

AN EVALUATION OF THE
ROLE OF GANGLIOSIDES
AS RECEPTORS FOR
FIBRONECTIN

By

Robert M. Perkins

A dissertation submitted to
the University of Leicester for
the degree of Doctor of Philosophy
October, 1984.

UMI Number: U350638

All rights reserved

INFORMATION TO ALL USERS

The quality of this reproduction is dependent upon the quality of the copy submitted.

In the unlikely event that the author did not send a complete manuscript and there are missing pages, these will be noted. Also, if material had to be removed, a note will indicate the deletion.



UMI U350638

Published by ProQuest LLC 2015. Copyright in the Dissertation held by the Author.
Microform Edition © ProQuest LLC.

All rights reserved. This work is protected against
unauthorized copying under Title 17, United States Code.



ProQuest LLC
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106-1346

Thesis



x750087934

To

Susanne

(i)

Acknowledgements

I would like to thank the following for assistance and useful discussion during this project; Dr. K.M. Jones, Dr. J. Sampson, Dr. S. Kellie, Dr. C.H. Streuli, Mr. P. Hissey and Miss S.L. Griffiths. I am indebted to Dr. R.A. Badley for assistance with interpretation of IRM studies, Dr. R.C. Hughes for helpful discussions on cell spreading on CT, Mr. B. Patel for excellent technical assistance and the MRC for supporting my studies. In particular, I would like to thank Dr. D.R. Critchley for his support and encouragement during the course of this study.

This dissertation was completed in November, 1984, and submitted in accordance with the requirements of the University of Leicester for the degree of Doctor of Philosophy. Experimental work was carried out during the period October 1979 to December 1982.

Abbreviations

BHK	Baby hamster kidney
BSA	Bovine serum albumin
CAPS	Cyclohexylamino propane sulphonic acid
°C	Degrees centigrade
C	Carbon
CHO	Chinese hamster ovary
CIG	Cold insoluble globulin
CO ₂	Carbon dioxide
ConA	Concanavalin A
CPM	Counts per minute
CSP	Cell surface protein
CT	Cholera toxin
DMEM	Dulbecco's modified Eagles medium
DMSO	Dimethylsulphoxide
DTT	Dithiothreitol
EDTA	Ethylene diamine tetracetic acid
FCA	Freund's complete adjuvant
FTIC	Fluorescein isothiocyanate
cFN	Cellular FN
pFN	Plasma FN
GAG	Glycosaminoglycan
GD, GT	Di- and Tri-sialogangliosides respectively
gms.	Grams (mg, milligrams etc.)

h	Hours
I	Iodine
IEF	Immunoelectrophoresis
Kd	Kilodaltons
L	Litre (ml, millilitre, etc.)
M	Molar (mM, millimolar, etc.)
MES	Morpholino ethane sulphonic acid
'	Minutes
NaN ₃	Sodium azide
NaOH	Sodium hydroxide
NH ₄ OH	Ammonium hydroxide
NP40	Nonidet P-40
OD _{280nm}	Optical density at a wavelength of 280 nm
PBS	Phosphate buffered saline (137mM sodium chloride, 2.79mM potassium chloride, 8mM di-sodium hydrogen phosphate, 1.53mM potassium di-hydrogen phosphate, pH 7.4
cPBS	Complete PBS as above with calcium chloride (1.34 mg/ml) and magnesium chloride (1 mg/ml) added
PBI	As PBS with sodium iodide replacing sodium chloride
PMSF	Phenylmethyl sulphonyl fluoride
RES	Reticuloendothelial system
RT ^o	Room temperature
S	Sulphur
SAM	Substrate attached material
<u>S. aureus</u>	Staphylococcus aureus

SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
TBS	Tris buffered saline
TCA	Trichloroacetic acid
TEMED	N, N, N', N' - tetramethylethylene diamine.

<u>CONTENTS</u>	<u>Page. No.</u>
Dedication	(i)
Acknowledgements	(ii)
Abbreviation	(iii)
Contents	(vi)

CHAPTER 1 - GENERAL INTRODUCTION

A. FIBRONECTIN - STRUCTURE AND FUNCTIONS	2
1. Discovery and Nomenclature	2
2. Distribution	5
3. Structure and Molecular Interactions	9
a) Chemical properties	9
b) Molecular interactions via structural domains	12
(i) Self association	15
(ii) Interaction with collagen/gelatin	15
(iii) Interaction with fibrin and plasma transglutaminase	17
(iv) Interaction with glycosaminoglycans	18
(v) Interaction with DNA	20
c) Plasma versus cellular FN	21
4. Fibronectin as an Adhesive Glycoprotein	24
a) Involvement in cell-substratum adhesion and spreading	24
b) Role in cell transformation and malignancy	24

	<u>Page No.</u>
c) Other functions of FN	31
(i) Wound healing and haemostasis	31
(ii) Opsonisation	32
(iii) Development and differentiation	33
d) The nature of the cell surface receptor for FN	34
 B. GANGLIOSIDES AS CELL SURFACE RECEPTORS	 38
1. Structure and Nomenclature	39
2. Membrane Organisation of Gangliosides	43
3. Ganglioside GM ₁ as the Cell Surface Receptor for Cholera Toxin	45
4. Gangliosides as the Receptor for FN.	47

CHAPTER 2 - GANGLIOSIDE INHIBITION
OF CELL SPREADING

Introduction	51
Materials and Methods	54
Cell culture	54
Ganglioside purification	54
CHO cell attachment and spreading assays	55
(i) Preparation of collagen-coated substrata	55
(ii) CHO cell attachment assay	56
(iii) CHO cell spreading assay	56
BHK cell spreading assay	57
Radioiodination procedures	58
(i) Fibronectin	58
(ii) Cholera toxin	58
(iii) Protein A	59
Binding of FN to plastic adsorbed gangliosides	62
Results	63
Ganglioside inhibition of FN-mediated CHO cell attachment and spreading	63
(i) Serum dependent CHO cell attachment	63
(ii) Effects of gangliosides	67
(iii) Ganglioside inhibition of CHO cell spreading	70
Ganglioside inhibition of FN-mediated BHK cell spreading	78
Binding of FN to plastic-adsorbed gangliosides	90

	<u>Page No.</u>
Ganglioside inhibition of ConA-mediated BHK cell spreading.	96
Discussion	106

CHAPTER 3 - A COMPARISON OF CELL SPREADING
ON SUBSTRATA COATED WITH FN, CT AND CONA

Introduction	118
Materials and Methods	124
Cell culture	124
Attachment and spreading on plastic substrata	124
Radioiodination of ligands	125
Quantitation of ligand binding to substrata	127
Derivitisation of glass coverslips	127
Effects of cytoskeletal disrupting agents on cell spreading	128
Immunofluorescent staining of F-actin	128
Visualisation of focal contacts	129
(A) Antibody exclusion technique	129
(B) Interference reflection microscopy	130
Scanning electron microscopy	131
Results	132
Cell attachment to ligand-coated substrata	132
Cell spreading on ligand-coated substrata	132
Specificity of ligand-cell interactions	139
SEM analysis of the spreading induced by FN, CT and ConA	144
Adsorption of ligands to microtitre wells	150
The effect of ligand density and receptor number on cell spreading	150

	<u>Page No.</u>
The effects of cytoskeletal disrupting agents on cell spreading	158
F-actin organisation of cells spread on FN-, CT- and ConA- coated substrata	162
Ability of each ligand to trigger focal contact formation	166
Discussion	181

CHAPTER 4 - THE RELATIONSHIP BETWEEN LEVELS OF
CELLULAR GANGLIOSIDES AND THE ABILITY TO INTERACT WITH FN

Introduction	192
Materials and Methods	197
Cell culture	197
Ganglioside analysis	197
Cell spreading on ligand-coated substrata	198
Incubation of cells with ganglioside-containing medium	199
Immunofluorescence	199
Interference reflection microscopy	200
Results and discussion	201
The ability of ganglioside-deficient variants of Balb/c 3T3 cells to interact with FN	201
The effect of exogenous gangliosides on the ability of cells to interact with FN	224
The effect of neuraminidase treatment of Balb/c 3T3 and BHK cells on their ability to spread on FN-coated substrata	234

	<u>Page No.</u>
<u>CHAPTER 5 - CONCLUDING COMMENTS</u>	246
 <u>APPENDIX I - PURIFICATION OF FN FROM HUMAN PLASMA</u>	
Protein estimation - Micro-Lowry	251
SDS - polyacrylamide gel electrophoresis	251
Cyanogen bromide activation of Sepharose 4B	253
Coupling of gelatin to cyanogen bromide-activated Sepharose 4B	253
Purification of human plasma FN.	254
 <u>APPENDIX II - PRODUCTION AND CHARACTERISATION OF ANTI-FN ANTISERUM</u>	
Preparation of FN for immunisation	259
Immunisation protocol	262
Affinity purification of anti-FN antiserum	262
Characterisation of rabbit anti-FN antiserum	264
(i) Ouchterlony double diffusion test	264
(ii) Immunofluorescence	264
(iii) Immunoelectrophoresis	267
(iv) Immune precipitation of FN from ³⁵ S-methionine labelled BHK cells	270
(v) Immunoblotting	275
 <u>BIBLIOGRAPHY</u>	279

CHAPTER 1

GENERAL

INTRODUCTION

A. FIBRONECTIN - STRUCTURE AND FUNCTIONS (see reviews by Pearlstein et al. 1980; Mosesson and Amrani, 1980; Ruoslahti; et al.)

1. Discovery and Nomenclature

Fibronectin (FN) was originally described by Morrison et al. (1948) as a fraction of human plasma isolated during the purification of fibrinogen and was referred to as cold insoluble globulin (CIG) due to its cryoprecipitability. Subsequently a number of investigators, working on a variety of research topics, have described proteins which are now known to be one of two closely related forms of FN, a soluble plasma form (pFN) and an insoluble cellular form (cFN) (see Table 1.1). Although not identical the two forms are very similar (Yamada and Kennedy, 1979) and in the following discussion the term fibronectin is used to include both the plasma and cellular forms. Only in cases where the two forms may differ significantly will the type be specified.

The discovery of FN associated with the surface of normal fibroblasts in cell culture (Hynes and Bye, 1974) and its loss following oncogenic transformation (Vaheri and Mosher, 1978) focused attention on this protein and it has subsequently been shown to possess a number of different functions (see Table 1.2). In general these involve the ability of FN to mediate the interaction of the extracellular matrix with the cell surface. Such interactions can have profound effects on cell adhesion and mobility and may also influence the differentiated state of a cell as a result of altered gene expression (Kleinman, Klebe and Martin, 1981).

Table 1.1 Fibronectin nomenclature

Name	Reference
1. Cold-Insoluble Globulin (CIG)	Morrison, Edsall and Miller, 1948.
2. Antigelatin Factor	Wolff, Timpl, Pecker and Steffen, 1967.
3. Opsonic Protein (α_2 Surface Binding Glycoprotein)	Allen, Saba and Molnar, 1973.
4. Cell Attachment Protein	Klebe, 1974.
5. Cell Spreading Factor	Grinnell, 1976.
6. Cell Adhesion Factor	Pearlstein and Gold, 1978.
7. Microfibrillar Protein	Muir, Bornstein and Ross, 1976.
8. Fibroblast Surface Antigen	Ruoslahti, Vaheri, Kuusela and Linder, 1973.
9. Galactoprotein A	Gahmberg, Kiehn and Hakomori, 1974.
10. Large External Transformation Sensitive Protein (LETS)	Hynes and Bye, 1974.
11. Cell Surface Protein (CSP)	Yamada and Weston, 1974.
12. Zeta-Protein	Jones and Hager, 1976.
13. Major Fibroblast Protein	Sear, Grant and Jackson, 1977.

Table 1.2 Functions of fibronectin

-
1. Cellular Adhesion.
 2. Regulation of Cellular Morphology, i.e. Spreading.
 3. Cell Motility and Chemotaxis.
 4. Organisation of the Microfilament System.
 5. Opsonisation and Phagocytosis.
 6. Haemostasis.
 7. Determinant in Development and Differentiation.
 8. Organisation of Loose Connective Tissue and the Extracellular Matrix.
-

2. Distribution

FN is a ubiquitous glycoprotein found in all vertebrates and at least three lower invertebrates; the sea urchin embryo, dissociated freshwater sponge cells and dissociated seawater sponge cells (Akiyama, Yamada and Hayashi, 1981).

In vivo FN is widely distributed throughout the body being a major component of connective tissue and extensively associated with basement membranes (Hedman, Vaheri and Wartiovaara, 1978) with the possible exception of glomerular basement membranes (Linder, Stenman, Lehto and Vaheri, 1978). It is also found in exocrine glands, mucous membranes, sweat, sebaceous, mammary and parotid glands, spleen, intestine, liver sinusoids and the sarcolemma of striated muscle (Pearlstein, Gold and Garcia-Pardo, 1980). It is characteristically visualised as thin fibrillar structures associated with other connective tissue components such as collagen and glycosaminoglycans (GAG's). As a soluble form FN is found in human blood plasma at a concentration of 200-300µg/ml (Mosher and Williams, 1978). The absolute levels have been shown to increase with age (Labat-Robert, Polazman, Deroutte and Robert, 1981) and are generally slightly higher in males than females (Erikson, Clemmensen, Hansen and Ibsen, 1982). Interestingly, the levels of pFN have been reported to be reduced in patients with advanced malignancy (Mosher and Williams, 1978) and following major surgery or trauma (Saba, Blumenstock, Scovill and Bernard, 1978). Although the reasons for these variations are unclear at present a connection with FN's opsonic function has been proposed (Saba and Jaffe, 1980). FN is also found in human amniotic

fluid (~170µg/ml), cerebrospinal fluid (1-3µg/ml), urine and synovial fluid (Ruoslahti, Engvall and Hayman, 1981). Serum levels of FN are approximately 35% less than plasma levels due to incorporation of FN into the fibrin clot. A small proportion (~0.5%) of the total blood FN in man is also found in the α granules of blood platelets and is released into the circulation during platelet stimulation by thrombin (Zucker, Modesson, Brockman and Kaplan, 1979).

In vitro cFN was first identified associated with the surface of fibroblast by cell surface labelling techniques (Hynes and Bye, 1974) and was later visualised by immunofluorescence microscopy as a complex fibrillar structure enmeshing most fibroblastic cell types (Yamada, 1978). It has also been shown to be produced by epithelial cells from the liver, kidney, gut, breast and amniotic membranes, together with myoblasts, Schwann cells, macrophages, undifferentiated chondrocytes, hepatocytes, endothelial cells and astroglial cells (see reviews by Ruoslahti et al., 1981). These in vitro findings further emphasise the widespread distribution of FN in vivo although some discrepancies between in vivo and in vitro studies do exist. For example, FN is apparently not associated with astroglial cells in vivo (Schachner, Schoonmaker and Hynes, 1978) but is clearly present when these cells are taken into tissue culture conditions (Vaheri, Ruoslahti, Westermarck and Ponten, 1976).

Cellular FN is commonly found as complex fibrillar structures in confluent fibroblast cultures although radially orientated striae or punctate patterns have been observed in other cell types. The distribution of FN in relation to cell polarity also varies between cell types. In

cultured endothelial and amniotic epithelial cells FN is primarily found associated with the ventral cell surface abutting the substratum (Birdwell, Gospodarowicz and Nicolson, 1978). In epithelial crypt cells it is found exclusively in areas of cell-cell contact (Quaroni, Isselbacher and Ruoslahti, 1978) and in fibroblastic cells it can be found in a pericellular distribution (Vaheri and Mosher, 1978). This suggests that the interaction of FN with the cell surface may be restricted to particular sites under certain conditions. This is further supported by the reported codistribution of FN with intracellular microfilament bundles, and its localisation to specialised areas of cell-substratum contact known as focal contacts (Hynes, Destree and Wagner, 1982).

The distribution of FN is dependent on the cell cycle and cell density (Gahmberg and Hakomori, 1974). In sparse fibroblast cultures it is found mainly on the underside of the cell and in regions of cell-cell contact, often forming small stitch-like structures. In more confluent cultures a complex FN matrix is formed pericellularly in association with other matrix components, e.g. collagen and proteoglycans. Much of this matrix FN is not in close association with the cell surface and is disulphide bonded into multimeric complexes (Hynes and Destree, 1977). The presence of FN in a matrix form makes studies on the nature of its interaction with the cell surface particularly difficult. Subcellular fractionation experiments show that FN is not normally present in plasma membrane fractions but copurifies with glycocalyx material (Pearlstein *et al.*, 1980). However, antibodies to FN are known to be toxic to FN-containing cells in the presence of complement, but not to cells lacking FN (Milhaud, Yamada

and Gottesman, 1980) suggesting that at least part of extracellular FN is in close proximity to the cell surface.

In relation to the cell cycle, cell surface FN is depleted during mitosis and slowly accumulates after division during the G₁ phase (Hynes and Bye, 1974). Levels of FN are also dependent on cellular differentiation since during myoblast and chondrocyte differentiation in vitro FN expression is switched off as it is during embryonic differentiation of mesenchymal cells into muscle, cartilage and renal tubule epithelium in vivo (Mosesson and Amrani, 1980). FN expression is apparently switched on during differentiation of teratocarcinoma stem cells into endodermal cells, which are known to have FN associated with them (Wartiovaara, Leivo, Virtanen, Vaheri and Graham, 1978).

Finally, FN is found as a soluble form in vitro in the culture medium of many cell types and may be analogous to pFN in vivo. The relationship between FN present in the culture medium and the matrix form is unclear although the two may be in equilibrium since pFN will incorporate into matrix material (Oh, Pierschbacher and Ruoslahti, 1981).

Although a great deal of data on the structure and function of FN has been accumulated it is perhaps important to note that the vast majority of this work has been carried out using FN from either plasma, conditioned medium or tissue culture cell surfaces with little data on the behaviour of FN present in tissue samples.

3. Structure and Molecular Interactions

a) Chemical properties

FN is a disulphide linked, dimeric glycoprotein with a subunit molecular weight of approximately 220Kd. In SDS-PAGE run under non-reducing conditions, pFN migrates as a single band of apparent molecular weight 450Kd and after reduction it migrates as a closely spaced doublet (approximately 220Kd) (Mosesson, Chen and Huseby, 1975). This is in close agreement with sedimentation analysis which gives pFN a molecular weight of 450 ± 25 Kd and an S value of 12-14 (Mosesson et al., 1975). Cellular FN migrates as a single band on reduced SDS-PAGE corresponding to the upper band of the pFN doublet (Hynes et al. 1978).

Peptide mapping in both 1 and 2 dimensions (Kurkinen, Vartio and Vaheri, 1980; Birdwell, Brasier and Taylor, 1980) suggests that the two polypeptides of pFN are very similar and a common amino-terminal sequence has been demonstrated which is blocked by a pyroglutaminic acid residue (Mosesson et al., 1975).

FN has been shown to contain both intra- and inter-chain disulphide bridges (Wagner and Hynes, 1979). The interchain bridges are located within 10Kd of the carboxyl terminus although the number of bridges is not clear. The intrachain disulphides are localised mainly in the amino terminal third of the molecule, as shown by S-cyanylated fragmentation (Fukuda and Hakomori, 1979), although some may also be present in the carboxyl terminal third.

There are apparently 1-2 free sulphydryl groups per FN monomer, one situated about 170Kd from the amino terminus and probably another one closer to the carboxyl end (Wagner and Hynes, 1980; Smith, Mosher, Johnson and Furcht, 1982). It has been suggested that these groups may be involved in the interaction of FN with the cell surface (Ali and Hynes, 1978) although it has been reported that reduced and alkylated FN is still capable of mediating cell adhesion (Gold and Pearlstein, 1979).

Both cFN and pFN contain approximately 5% carbohydrate consisting of 4 to 6 complex asparagine-linked units per monomer. Mannose, galactose, N-acetyl glucosamine and sialic acid are the common sugar residues in pFN while cFN contains fucose and much less sialic acid (Mosesson and Amrani, 1980). Glucose and xylose have been shown to be present in chick embryo fibroblast cFN (Yamada, Schlesinger, Kennedy and Pastan, 1977). Other variations in sugar content and linkages have been reported between cell and plasma FN and also between FN's from different sources. Amniotic fluid FN has an exceptionally high carbohydrate content of between 7-9.5% (Ruoslahti, Engvall, Hayman and Spiro, 1980). This may account for its higher apparent molecular weight as determined by SDS-PAGE since unglycosylated FN reportedly migrates faster than its normal counterpart (Olden, Pratt and Yamada, 1979). Thus, altered glycosylation may account for the variable electrophoretic mobilities of FN's from different sources. Most of the carbohydrate in FN is situated in a 40Kd sequence approximately 30Kd from the amino terminal end which also has the ability to bind to collagen (Sekiguchi and Hakomori, 1980). Glycosylation of FN is not required for its functional activity although it protects the molecule from proteolytic attack, the unglycosylated FN being 2-3 times more sensitive to

degradation than normal FN (Olden et al., 1979).

One of the key areas of interest in FN research is the elucidation of its complete amino acid sequence. Working on bovine pFN Peterson and co-workers have sequenced 27% of the molecule and have recently proposed three types of internal homology (Skorstengaard, Thøgersen, Vibe-Pederson, Peterson and Magnusson, 1982). The amino terminal end is believed to form a "5 fingered" arrangement due to looping out of the polypeptide backbone, each loop being held in position by disulphide linkages. A further 4 "fingers" are found adjacent to these and possibly also at the carboxyl terminal end. This repeating structure is referred to as type I homology and sequences corresponding to type II and type III homology are found in the central and carboxyl half of the molecule respectively (Peterson et al., 1983).

Using recombinant DNA technology it has been shown that the chicken FN gene is 48 kilobases long, consisting of at least 48 constantly sized exons (147 ± 37 base pairs) separated by 40 introns of widely differing size (Hirano et al., 1983). Interestingly, the exon size of 150 base pairs, or 50 amino acids, corresponds approximately to the size of one of the "fingers" in the type I homology regions described above. This might suggest that the gene for FN may have arisen by gene duplication of a primordial gene approximately 150 base pairs long. More recently cDNA clones for FN have been isolated from a rat liver library and used to establish the sequence of the C-terminal third of rat FN consisting of the cell-, heparin-, and fibrin-binding domains (Schwarzbauer, Tamkun, Lemischka and Hynes, 1983). It was clearly demonstrated that the cell- and

heparin-binding domains consist of homologous, repeating sequences perhaps indicating a polyvalent interaction of FN with the cell surface.

b) Molecular interactions via structural domains (see reviews by Hynes and Yamada, 1982; Akiyama, Yamada and Hayashi, 1981; Sekiguchi, Fukuda and Hakomori, 1981 and Fig. 1.1).

FN has been shown to interact with a large number of macromolecules, including some as yet undetermined components of the cell surface, and has a diversity of functional properties. During recent years it has become possible to dissect the FN molecule into functional domains by the use of specific proteolytic fragmentation together with affinity chromatography. Using such a strategy it is possible to allocate specific molecular interactions to particular parts of the polypeptide backbone, commonly referred to as "domains". The presence of distinct domain structures in FN had previously been suggested by physical studies which described FN as consisting of a number of globular regions connected by flexible link regions (Alexander, Colonna, Yamada, Pastan and Edelhoch, 1978; Alexander, Colonna and Edelhoch, 1979). Using differential scanning calorimetry of native FN Wallace et al. (1981) showed three distinct endothermal denaturatuon transitions suggesting the presence of at least three ordered "domain" regions. At the electron microscopic level native FN is seen as a V-shaped molecule with an angle of approximately 70° between the two polypeptide subunits. Although no globerular structures were apparent three hinge regions displaying considerable flexibility were present on each monomer and may connect more compact domain regions (Odermatt and Engel, 1982).

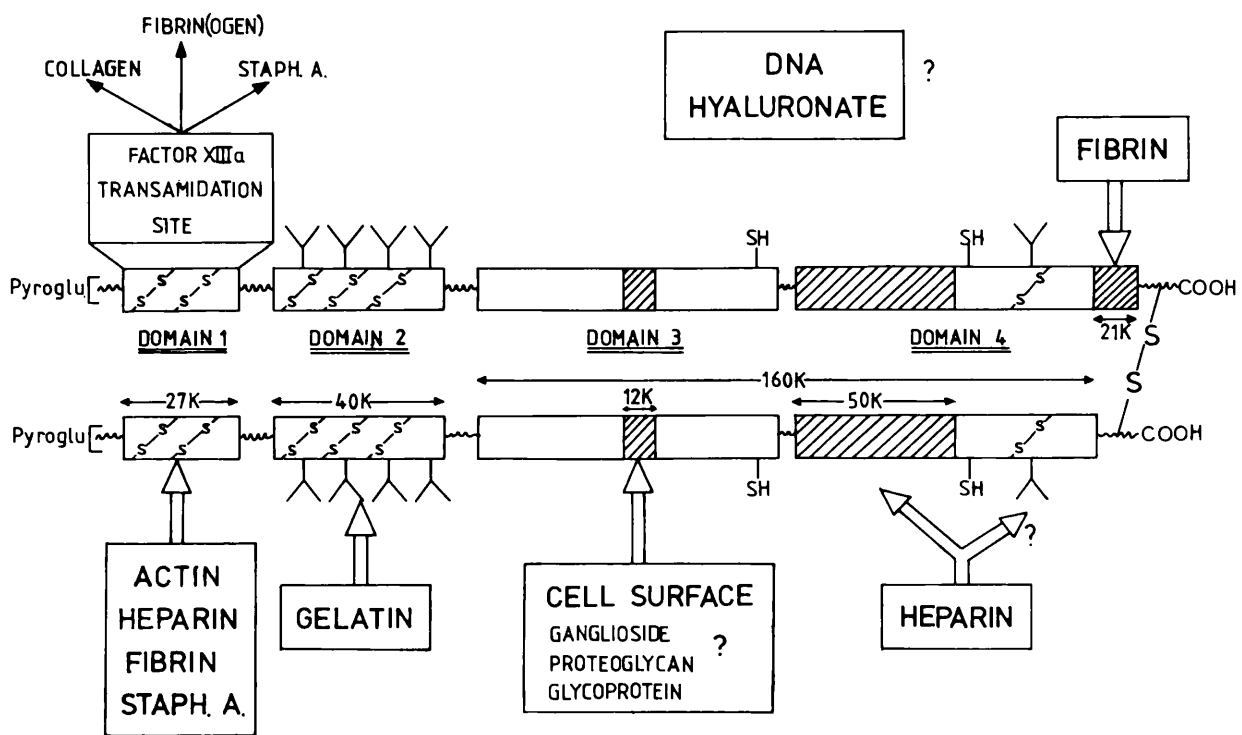
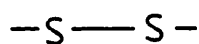


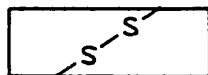
Fig. 1.1 Domain structure of FN



complex asparagine-linked oligosaccharide units



Interchain disulphide bonds



Intrachain disulphide bonds



Domain regions



Flexible hinge regions usually corresponding
to sites of proteolytic attack.

(see refs. Sekiguchi and Hakomori, 1980'; Sekiguchi et al. 1981';
Akiyama et al. 1981'; Hynes and Yamada, 1983'; Sekiguchi and Hakomori,
1983'; Yamada, 1983).

FN is capable of interacting with various macromolecules via specific domains within the molecule. Co-operativity between domains may be an important factor in some of these complex molecular interactions. These are outlined below.

i) Self Association - The ability of cFN to self-associate is implied by its presence as fibrillar structures in matrix material and at the cell surface and also by its ability to form fibre-like structure in concentrated solutions. Ehrishman et al. (1982) have recently proposed that horse serum FN contains two self-association sites at least 135Kd apart. One site is within an amino terminal 60Kd peptide and the other site is within 35Kd of the carboxyl terminus. It was suggested that this would allow a "head to tail" association of FN molecules and assembly into filamentous structures. This ability of FN to self associate may be important in the formation and organisation of the extracellular matrix.

ii) Interaction with collagen/gelatin - The interaction of FN with collagen was first identified by the requirement of a serum or culture medium factor to mediate cell attachment to collagenous substrata (Klebe, 1974; Pearlstein, 1976). This factor was later shown to be FN and its affinity for collagen demonstrated by direct binding assays (Engvall and Ruoslahti, 1977). The affinity of FN for different genetic types of collagen has been investigated by a number of workers with somewhat contrasting results. Gold et al. (1979) demonstrated equal binding to type I, II and III collagen, before and after denaturation in agreement with the results of Dessau et al. (1978). However, other workers have shown preferential binding to type III collagen and that denaturation of all collagens

substantially increases FN binding to levels higher than any native collagen type (Ruoslahti and Engvall, 1978).

The binding of FN to collagen type I has been shown to be predominantly to a cyanogen bromide fragment of the α -1(I) chain of collagen consisting of residues 757 to 791 (Kleinman, McGoodwin, Martin, Klebe, Fietzek and Wolley, 1978). This fragment lacks carbohydrate and has a hydrophobic nature suggesting that the high affinity binding of FN with collagen may be due to hydrophobic interactions. This is further supported by the fact that the interaction is stable to high salt concentrations (Gold et al., 1980). Low affinity binding to other sites on the collagen molecule have also been demonstrated (Dessau, Adelman, Timpl and Martin, 1971).

The collagen binding site on FN has been localised to a 40Kd fragment by enzymatic digestion and affinity chromatography (Hahn and Yamada, 1979). It is rich in intrachain disulphide bridges and contains most of FN's carbohydrate (Sekiguchi and Hakomori, 1980) although this is not required for collagen binding activity. The 40Kd fragment originates from a site approximately 30Kd from the amino terminus (Furie, Frey and Rifkin, 1980) and although still retaining gelatin binding properties it shows a lower affinity than the intact FN molecule. It has also been shown to possess transformation promoting activity although the significance of this is not clear at present (DePetro, Barlati, Vartio and Vaheri, 1981). The gelatin binding site has recently been further restricted to a 5-10Kd region within the 40Kd domain by the use of monoclonal antibodies (Smith and Furcht, 1982).

The biological significance of the interaction of FN with collagen is unclear although extensive codistribution of these two matrix components in vitro and in vivo together with their coordinated loss following viral transformation (Vaheri, Kurkinen, Lehto, Linder and Timpl, 1978) suggests that the interaction is important. It seems likely that collagen-FN interactions may play a key role in the development of the extracellular matrix. However the presence of FN matrices in the absence of collagen demonstrates that such interactions are not essential.

iii) Interaction with fibrin and plasma transglutaminase (Factor XIIIa) - FN has been shown to bind to both fibrin and fibrinogen either non-covalently or covalently following Factor XIIIa-mediated cross-linking. FN interacts rather poorly with fibrinogen although it has been shown to coprecipitate with fibrinogen in the cold and to bind to fibrinogen coupled to Sepharose beads at low temperatures (Stemberger and Hörmann, 1976). The interaction of FN with fibrinogen is apparently increased by the presence of heparin which has also been shown to bind to FN (Stathakis and Mosesson, 1977). In the presence of Factor XIIIa (thrombin-activated plasma transglutaminase) FN and fibrinogen can be covalently linked via the carboxyl terminal end of A α chain of fibrinogen and a glutamine residue three residues from the amino terminus of FN (Peterson et al., 1983). This glutamine residue is also the site at which Factor XIIIa crosslinks FN to itself, fibrin, collagen and S. aureus. It is present in a 27Kd amino-terminal domain involved in non-covalent interaction with actin, S. aureus, fibrin and heparin, but interestingly not collagen. Thus, the major site of collagen crosslinking to FN is at a different site from the 40Kd collagen binding domain. The

significance of this is not clear although the finding that physiological levels of polyamines inhibit cross-linking between FN and collagen but not between FN and fibrin suggests that the former interaction may not be physiologically important (Mosher, Schad and Kleinman, 1979).

The non-covalent interaction of FN with fibrin is apparently of higher affinity than that with fibrinogen since FN will bind to fibrin monomers coupled to Sepharose at room temperature but not fibrinogen (Iwanaga, Suzuki and Hashimoto, 1978). Taking advantage of this in conjunction with proteolytic fragmentation Sekiguchi et al. (1981; 1983) have demonstrated binding of fibrin to a 21Kd fragment as well as to the 27Kd domain mentioned above. This 21Kd domain is reported to be present on only one of the FN polypeptides, being located close to the carboxyl terminus, and is apparently not a substrate for Factor XIIIa (Sekiguchi et al., 1981). Richter et al. (1981) however have proposed the presence of a transamidation susceptible site, in addition to that present in the 27Kd domain, in a similar position to the 21Kd domain suggesting that under certain conditions it may be involved in transamidation. The interaction between FN, Factor XIIIa and fibrin may be an important factor in wound healing and opsonisation.

iv) Interaction with glycosaminoglycans (GAG's) - GAG's are polysaccharides that are found in animal tissues usually in covalent association with protein, forming proteoglycans (see review by Lindahl and Höök, 1978). The interaction between FN and GAG's appears to be of a complex nature with the binding to heparin being the best characterised (Hayashi and Yamada, 1982). Hyaluronic acid, heparin and heparan sulphate

GAG's cause the agglutination of gelatin-coated beads in the presence of FN, agglutination only occurring if FN and GAG's are present together (Ruoslahti et al., 1981). Interestingly, heparin reportedly stabilises the interaction between FN and collagen and also between FN and fibrinogen (Ruoslahti and Engvall, 1980). Thus, interactions at one domain in FN may promote or stabilise binding at a different domain.

The interaction of chick cFN with hyaluronic acid and heparin was investigated by Yamada et al. (1980) using a filter binding assay which relied on cFN being precipitated onto filters at neutral pH. They demonstrated high affinity binding ($K_D = 10^{-7}$ - 10^{-8} M) of both hyaluronic acid and heparin to cFN and suggested that binding was to separate sites on the basis of competition experiments. Extending this work it was shown that heparin binding to FN was via at least two distinct sites with varying binding requirements (Hayashi and Yamada, 1982). One binding site was identified as being within a 30Kd plasmin fragment which was eluted from a heparin-Sepharose column by 0.25M salt and is thought to be equivalent to the 27Kd amino terminal domain described above (Richter, Seidl and Hormann, 1981). Binding to this site was shown to be sensitive to physiological levels of calcium ions. A second heparin binding fragment (50Kd) has been located in the carboxyl terminal third of FN (Hayashi, Schlesinger, Kennedy and Yamada, 1980) and was less sensitive to salt and insensitive to divalent cations (Hayashi and Yamada, 1982). These two heparin binding sites have recently been identified independently by the use of monoclonal antibodies, one site being localised within the amino terminal 27Kd domain, the other being approximately 30Kd from the carboxyl terminus (Smith and Furcht, 1982). Heparin can also interact with the

carboxyl terminal third of FN by a divalent cation sensitive mechanism (Hayashi and Yamada, 1982).

The finding that GAG's and FN are the major constituents of substrate attached material (SAM) remaining following detachment of cells from their substratum with EDTA (Culp, Murray and Rollins, 1979) and their codistribution in tissue culture (Hedman, Johansson, Vartio, Kjellen, Vaheri and Hook, 1982) suggest that the interactions outlined above may be physiologically important. In addition FN has been shown to associate with proteoglycans at the cell surface using a crosslinking reagent (Perkins, Ji and Hynes, 1979).

As mentioned above FN binds with moderately high affinity to hyaluronic acid, however the position of this binding site(s) is unclear due mainly to the fact that FN will only bind effectively to hyaluronic acid when FN is in an aggregated form (Laterza and Culp, 1982).

v) Interaction with DNA - FN has been shown to bind to DNA coupled to Sepharose and bound to nitrocellulose membranes. A number of DNA-binding fragments were identified with molecular weights of 60Kd, 50Kd and 25Kd (Zardi, Siri, Carnemolla, Santi, Gardner and Hoch, 1979; McMaster and Zardi, 1982; Hoch, 1982). However, under physiological conditions the interaction between FN and DNA is of low affinity and is abolished by 10mM CaCl_2 or MgCl_2 , putting doubt on the importance of this interaction. Recent evidence showing sequence homology between a 25Kd DNA-binding fragment of FN and the α subunit of the DNA-dependent RNA polymerase from E. coli on the other hand, suggests that the interaction may be of biological significance (Pande and Shively, 1982).

vi) Interaction with the cell surface - The interaction of FN with the cell surface is perhaps the most important of its binding activities since it is thought to be involved in most, if not all, of the proposed functions of FN. The cell binding domain of FN has been shown to be separate from the gelatin- and heparin-binding domains and has recently been identified in a 12Kd fragment which is capable of mediating cell attachment and spreading (Pierschbacher, Hayman and Ruoslahti, 1981). The primary structure of this domain has now been determined and shown to consist of 108 amino acid residues and to contain no carbohydrate (Pierschbacher, Ruoslahti, Sundelin, Lind and Petersen, 1982). It has been positioned in a central region of FN between the gelatin-binding domain and the carboxyl heparin-binding domain 127-197Kd from the amino terminus. This is supported by Ehrishman et al. (1982) who localised a cell attachment site between 153 and 165Kd from the amino terminus of horse serum FN. The close proximity of the heparin domain and cell attachment domain was shown by the isolation of a 42Kd chymotryptic/pronase fragment from horse serum FN which promoted myoblast attachment and also bound to heparin-Sepharose (Ehrishmann et al., 1982). The two are clearly distinct however since other workers have identified fragments which promote cell attachment but do not bind to heparin (Hayashi et al., 1980; Pierschbacher et al., 1981).

c) Plasma versus cellular FN

The relationship between plasma and cellular FN is not clear at present although they appear to be structurally similar and to qualitatively display the same biological activities. As well as having related amino

acid compositions, peptide maps, spectroscopic and hydrodynamic properties, and being immunologically cross-reactive both forms are capable of binding to gelatin, heparin, actin, DNA, S. aureus, Factor XIIIa and the cell surface (Hayashi and Yamada, 1981). Differences between the two forms do however exist. Cellular FN is generally insoluble at neutral pH, exists as a multimeric form at the cell surface and migrates as a single band on reduced SDS-PAGE while pFN is a soluble dimer at neutral pH and migrates as a doublet on reduced SDS-PAGE (Alexander et al., 1978 and 1979; Hynes et al., 1978; Hynes and Destree, 1977). As mentioned earlier variations in carbohydrate content and sugar linkages occurs between the two forms with cFN containing fucose and less sialic acid than pFN which in turn lacks fucose. Quantitative differences in the biological activities of cellular and plasma FN have also been reported. Cellular FN is more active than the plasma form in restoring normal morphology to transformed cells (50x), haemagglutination of sheep red blood cells (150-200x), and mediating NIL/HSV cell attachment to plastic (2-3x) (Hynes et al., 1978; Yamada and Kennedy, 1979). The two forms are however equally active in promoting cell attachment and spreading (Yamada and Kennedy, 1979) and also in promoting uptake of particles by macrophages (Marquette, Molnar, Yamada, Schlesinger, Darby and Van Alten, 1981). Clearly pFN can bind to the cell surface receptor as well as cFN under certain assay conditions. Indeed molecular cloning and nucleotide sequencing of the cell attachment domains of human pFN and cFN from a fibrosarcoma cell line show the two to be identical (Oldberg, Linney and Ruoslahti, 1983). It may be that the assays in which the biological activity of the two forms differ requires FN in an aggregated or multimeric form and since pFN shows a reduced tendency to aggregates than cFN it would be less active in these

assays. This requirement for aggregation may be alleviated by adsorption to a solid substratum. Ehrishmann et al. (1982) proposed that the reduced tendency of pFN to agglutinate, or self-associate, is due to it lacking a self-association site on one of its polypeptides resulting in a heterodimeric structure incapable of the head-to-tail association displayed by the homodimeric cellular form.

Until recently the structural basis for these variations in biological activity has remained unclear although it appears not to be due to differences in glycosylation. However, it is now becoming clear that differences in the polypeptide backbone of cell and plasma FN do exist since homologous fragments from the two forms differ in molecular weight and a monoclonal antibody which preferentially recognises cFN has recently been produced (Hayashi and Yamada, 1981; Atherton and Hynes, 1981). From fragmentation studies on chicken plasma and cellular FN three difference regions have been identified. A 44.5Kd thermolysin fragment of pFN, containing the gelatin-binding domain, is 1.3Kd larger than its cellular equivalent and a 132Kd thermolysin fragment of pFN is apparently 10Kd smaller than in cFN and is located in the carboxyl half of the molecule. Further digestion of this fragment shows that in fact, two difference regions are present, pFN being 11Kd smaller at one position and 1Kd larger at a site towards the carboxyl terminal end (Hayashi and Yamada, 1981). How these differences are related to specific differences in biological activity has yet to be determined and until the complete amino acid sequences have been determined the full extent of these differences will remain unresolved. However, the finding of difference regions at internal positions on the polypeptide backbone shows that pFN

is not derived from cFN by post-translational proteolytic modification and suggest that the two forms are derived from different genes or are produced by different RNA splicing mechanisms. Interestingly, it has recently been shown that three different FN mRNA species could be detected in rat liver. These appear to arise by alternative splicing of a single FN gene transcript Schwarzbauer et al., 1983) although their relationship to the different FN polypeptides is unclear. As a result of these differential splicing events mRNA's are generated which code for polypeptides containing internal difference regions of 95 to 120 amino acids. This is of the appropriate size and position to account for the molecular weight differences between the two polypeptides of pFN and also the difference between cFN and the smaller polypeptides of pFN.

4. Fibronectin as an Adhesive Glycoprotein

FN, through its ability to interact with a number of different components has been shown to be involved in a diversity of functional roles each important in their own right and extensively reviewed elsewhere. For these reasons I do not intend to talk at length about all of these topics but instead will concentrate on those aspects of FN function which are related to its interaction with its cell surface receptor, in particular its role in cellular adhesion and spreading.

a) Involvement in cell-substratum adhesion and spreading

Cell attachment and spreading on a substratum is an extremely complex process which essentially involves the interaction of cell surface

components with the substratum or molecules adsorbed onto it. This is followed by a series of ill-defined events which result in a general flattening of the cell, a process referred to as cell spreading (see review by Grinnell, 1978). Cell spreading is an active process which is sensitive to agents which specifically disrupt actin-containing microfilaments and requires the presence of divalent cations. It can occur on substrata coated with various ligands with an affinity for the cell surface such as lectins, glycosidases and antibodies directed against the cell surface, as well as FN. However, the degree and mechanism of cell attachment and spreading on these substrata may differ (Grinnell and Hays, 1978; Rauvala, Carter and Hakomori, 1981; Carter, Rauvala and Hakomori, 1981).

When considering cell attachment it is important to distinguish between passive and active adsorption of cells to the substratum. In the absence of serum cells will adsorb to some substrata almost instantly in a temperature and pH independent manner not requiring the cells to be metabolically active or even viable. This non-specific or passive attachment of cells probably represents direct adsorption to sites on the substratum to which serum proteins would normally bind. Passive adsorption is thus blocked in the presence of serum or other concentrated protein solutions. Following passive adsorption some cells are capable of going on to spread by the secretion of endogenous spreading factors such as fibronectin. Many cells however, e.g. BHK, HeLa, L and CHO cells show little tendency to spread under these conditions.

A role for FN in cell attachment and spreading originally developed from the finding that serum factors were capable of promoting the adhesion of tissue culture cells to plastic or collagen substrata by a mechanism involving activation of the substratum rather than a direct effect on the cell. Fractionation of serum has shown it to contain at least two different factors capable of mediating cell attachment and spreading, FN and a recently identified 70Kd protein (Whateley and Knox, 1980; Hayman et al., 1982; Barnes, Silnutzer, See and Shaffer, 1983). It has subsequently been shown that many cells require FN for efficient attachment and growth in vitro Orly et al. (1979) demonstrated that FN could mediate cytokinesis and growth of rat follicle cells in serum-free medium. Some cells however do not require an exogenous supply of FN for efficient attachment. These either produce sufficient levels of endogenous FN or utilise other attachment factors, e.g. chondronectin, laminin or vitronectin (Hewitt, Varner, Silver, Dessau, Wilkes and Martin, 1982; Hayman, Pierschbacher, Ögren and Ruoslahti, 1983).

FN's ability to mediate cell attachment and spreading requires it to be bound to a solid substrate as in solution it has only a low affinity for the surface of cells in suspension. To explain this phenomena it has been postulated that FN undergoes some activation process as a result of adsorption to the substratum causing an increased affinity for the cell surface. This activation process may be due to a conformation change in FN or it may be that, as a result of substrate adsorption FN's cell binding activity is increased in a cooperative manner since it effectively becomes a single molecule with multiple cell binding sites. As a result of FN's poor affinity for the cell surface when in solution most studies

on the interaction of FN with the cell surface have used substratum-bound FN thus involving cell attachment and spreading experiments. An alternative approach has been to study the uptake of FN-coated particles by cells, a process which may be analogous to cell spreading.

As mentioned above the interaction of a cell with substratum bound FN triggers an energy dependent cytoskeletal reorganisation resulting in cell spreading. It is thus apparent that FN is capable of interacting, presumably through its receptor, with the intracellular cytoskeleton, in particular the microfilament system (Hynes, 1981; Hynes, Destree and Wagner, 1982). The transmembrane association of FN and actin-containing microfilaments is supported by the following data:-

i) Treatment of cells with a microfilament-disrupting agent (cytochalasin B) results in a co-ordinated loss of cell surface FN (Kurkinen, Wartiovaara and Vaheri, 1978).

ii) Addition of FN to transformed cells (which normally lack FN and have a disorganised microfilament system) results in formation of surface FN fibrils and reorganisation of intracellular microfilaments (Ali, Mautner, Lanza and Hynes, 1977).

iii) External FN fibrils show extensive codistribution with intracellular microfilaments on the ventral surface of well spread cells (Hynes and Destree, 1978a). At higher resolution the two systems were shown to form regions of very close, transmembrane association termed the fibronexus (Singer, 1979).

iv) FN has been shown to be localised to specialised areas of cell-substratum adhesion, called focal contacts in stationary hamster fibroblasts (Hynes, 1981). These correspond to the sites at which microfilament bundles connect with the plasma membrane. Vinculin, a 130Kd actin-binding protein, has also been shown to codistribute with FN at focal contacts (Burridge and Feramisco, 1980; Singer and Paradiso, 1981). The localisation of FN at focal contacts is at present controversial with many workers suggesting that FN is not present at these sites and under some circumstances appears to be actively removed (Birchmeier et al., 1980; Chen and Singer, 1980; Aznur and Geiger, 1981). However, the finding that FN and actin are both found in material remaining attached to the substratum following removal of cells with EDTA or detergent (substrate attached material = SAM) and believed to represent sites of focal contacts, supports the idea that these two components are associated with specialised areas of the cell surface involved in cell-substratum adhesion (Culp et al., 1979).

There is thus good evidence that FN is capable of a transmembrane association with the actin-containing microfilament system and this capability may be a key factor in cell spreading. The findings of Folkman and Moscona (1978) demonstrating a strict correlation between the degree of cell spreading and DNA synthesis raised the possibility that FN, through its ability to regulate cell shape, may be an important factor in the regulation of cell growth in normal cells, although this does not appear to be the case for transformed cells.

b) Role in cell transformation and malignancy

Much of the recent interest in FN has stemmed from the finding that it is commonly lost from the surface of transformed cells (Hynes, 1976). In vitro transformation of a variety of cell lines by tumour viruses, chemical carcinogens and spontaneous transformation has been reported to result in reduced levels of surface FN. Similarly, reduced levels of FN have been shown to correlate with the tumourigenic potential of a series of adenovirus transformants (Chen, Gallimore and McDougall, 1976). However, a ten-fold reduction in the levels of surface FN accompanying the establishment of several rodent and chicken cell lines did not result in the acquisition of tumourigenicity (Chen, Maitland, Gallimore and McDougall, 1977). A lack of correlation between decreased expression of FN and tumourigenicity has also been demonstrated in human cell hybrids (Der and Stanbridge, 1978). Other studies suggest that a better correlation exists between FN levels and the metastatic potential of tumour cells than their tumourigenicity per se. Using human epithelial cell lines derived from carcinomatous and nonmalignant tissue, Smith et al. (1979) demonstrated the presence of matrix FN in nonmalignant tissue, and primary carcinomas. In contrast, cell lines derived from metastatic carcinomas displayed very little or no FN. However, a recent study in this laboratory failed to detect any significant correlation between the levels of FN in various human colorectal tumours and their metastatic potential (Niemczuk, Perkins, Talbot and Critchley, 1982). The relationship between FN and malignancy is thus complex and at present incompletely understood.

The mechanism of FN loss from transformed cells and its significance to the transformed phenotype has been extensively investigated. In transformed chick embryo fibroblasts the primary cause of reduced levels of cell surface FN is a reduction in the levels of FN-specific messenger RNA (Fagan, Sobel, Yamada, de Crombrughe and Pastan, 1981). Many transformed cells do however synthesise FN. For example, transformed rat kidney cells actually synthesise increased levels of FN following transformation but are unable to retain it at the cell surface (Hayman, Engvall and Ruoslahti, 1980). It does not appear that the FN produced by transformed cells differs significantly from that produced by normal cells in terms of biological activity and chemical structure (Wagner, Ivatt, Destree and Hynes, 1981). However, it has also been demonstrated that functional cell surface receptors for FN are still present on some transformed cells as they are able to attach and spread on FN-coated substrates (Hayman et al., 1980). Alternatively the loss of FN from transformed cells may reflect a more general inability of these cells to organised extracellular matrices. This is supported by the finding that collagen and some glycosaminoglycans are also lost or reduced following cell transformation (Adams et al., 1977; Satoh, Duff, Rapp and Davidson, 1973). Other explanations include:-

- i) the increased levels of cell surface proteases associated with transformed cells may release cell-associated FN,
- or ii) the disruption of the cytoskeleton following transformation may impair the cells ability to retain FN.

It is clear therefore that no simple explanation can be given for the reduction of FN at the cell surface following transformation.

The loss of FN from transformed cells appears to be responsible for their rounded morphology, reduced adhesiveness, disorganised microfilament system and the inability to undergo directed movement, since addition of exogenous FN to cultures of transformed cells reverses these characteristics to those displayed by their normal counterparts. However, there is no effect on growth control following such treatment (Yamada, Yamada and Pastan, 1976).

c) Other functions of FN

FN has been shown to play a part in a number of other biological activities which involve its interaction with the cell surface. These are briefly mentioned below.

i) Wound healing and haemostasis - As described above FN binds to fibrin and collagen and can be cross-linked to these components by Factor XIIIa. It thus becomes incorporated into the wound area at sites of tissue damage where it may serve as an organisational centre for the formation of new matrix structure. Additionally, it might increase the adhesive properties of the wound, promoting migration of fibroblasts into the damaged area and so promoting tissue repair. In vitro Grinnell et al. (1980a) have shown that cross-linking of FN to fibrin-coated substrata by Factor XIIIa markedly increased fibroblast attachment. Smaller increases in attachment are seen if cross-linking is omitted. Interestingly,

FN has been shown to be a potent chemo-attractant for human skin fibroblasts, a property which is retained in a 160Kd cell-binding fragment (Seppä, Yamada, Seppä, Silver, Kleinman and Schiffmann, 1981). Again this may promote migration into wound areas so increasing repair.

The function of platelet FN is at present unclear. Following platelet activation, FN is released from the α granules and become associated with the platelet surface (Zucker et al., 1979; Ginsberg, Pointer, Forsyth, Birdwell and Plow, 1980). It may thus play a part in platelet aggregation and it has been shown that FN can mediate the attachment and spreading of platelets, presumably via a component of the platelet surface (Grinnell, Feld and Snell, 1979). Using a chemical cross-linking reagent this component has recently been suggested to be thrombospondin (Lahav, Schwartz and Hynes, 1982).

ii) Opsonisation - FN has been shown to be a major plasma opsonic protein and is identical to the α_2 -surface binding glycoprotein which promotes uptake of gelatin-coated particles by liver slices (Blumenstock, Saba, Weber and Laffin, 1978). It was thus suggested that pFN could act as a non-specific opsonin for the reticuloendothelial system (RES). In support of this proposal is the fact that FN can bind to S. aureus, the Clq component of complement, denatured actin and fibrin suggesting that pFN may help to clear cellular debris, bacteria and fibrin from the circulation (Mosher and Proctor, 1980; Keski-Oja and Yamada, 1981; Bing, Almeda, Isliker, Lahav and Hynes, 1982).

Saba et al. (1978 and 1980) have reported extensively on the opsonic properties of FN and have demonstrated a general depression in the levels of pFN following major surgery, trauma and burns. They suggest this to be due to uptake of pFN by the RES during clearance of plasma debris and results in a number of clinical conditions which can apparently be alleviated by the administration of cryoprecipitate, a rich source of pFN.

The opsonic role of FN presumably involves it binding to cells of the RES via a specific receptor before its uptake and degradation.

iii) Development and differentiation - During embryonic differentiation of mesenchymal cells into muscle, cartilage and renal tubular epithelia the expression of FN is apparently switched off indicating that it is linked with the differentiated state of a cell in vivo (Linder, Vaheri, Ruoslahti and Wartiovaara, 1975; Wartiovaara, Stenman and Vaheri, 1976). This is supported by in vitro work on chondrocyte and myoblast differentiation. Dense culture of myoblasts, containing surface FN, loose it prior to their differentiation into myotubes (Chen et al., 1977), a process which is inhibited by the addition of exogenous FN and promoted by antibodies to FN. In vitro chondrogenesis is also accompanied by loss of surface FN. However, differentiated chondrocytes do produce FN suggesting that following differentiation chondrocytes are unable to retain FN at the cell surface. Treatment of chondrocyte cultures with vitamin A, which is known to inhibit in vitro chondrogenesis, results in increased accumulation of surface FN and addition of FN to chondrocytes alters the phenotypic expression of these cells from cartilage specific components to more fibroblastic components (Hassell, Pennypacker, Kleinman,

Pratt and Yamada, 1979). This is accompanied by reversion to a fibroblastic morphology. The presence of FN may thus influence the differentiated state of a cell as a result of its interaction with the cell surface.

FN has also been implicated as an agent responsible for directing morphogenetic movement during development, e.g. *Xenopus larvis* primordial germ cell migration and migration of embryonic tissue in early chick embryo development (Heasman, Hynes, Swan, Thomas and Wylie, 1981; Critchley, England, Wakely and Hynes, 1979).

d) The nature of the cell surface receptor for FN

From the above it is apparent that many of the functional activities of FN require its interaction with a cell surface receptor. The nature of this receptor is thus a key issue in these processes.

The characterisation of the FN receptor has been hampered due mainly to the following reasons. Firstly, FN can bind to many cellular components not necessarily involved in the initial interaction with the cell surface, e.g. heparin. It is thus necessary to separate these non-receptor activities from the true receptor-binding activity. The isolation of the cell-binding domain of FN may remedy these problems. Secondly and perhaps more importantly, FN displays a low affinity for the cell surface unless it is immobilised on a solid substratum. This precludes the use of classical ligand-binding experiments in measuring receptor activity. Instead many investigators have used the ability of substratum-adsorbed FN to promote cell adhesion as a measure of FN-receptor interactions.

An alternative, although essentially similar assay is the binding of FN-coated latex beads to the cell surface. In contrast to fibroblastic cells, specific and saturable binding of ^{125}I -labelled FN to thrombin-stimulated human platelets has been demonstrated (Plow and Grinsberg, 1981). Binding was divalent cation dependent and was inhibited by unlabelled FN and normal serum but not by FN-depleted serum or a variety of proteins or glycoproteins. Scatchard analysis suggested a single class of binding sites with an apparent K_D of $3 \times 10^{-7}\text{M}$, approximately 1.2×10^5 molecules of FN binding per platelet. It has recently been suggested that the interaction between FN and platelets may be mediated by thrombospondin since these two components can be cross-linked following platelet adhesion to FN-coated substrata (Lahav *et al.*, 1982). It thus seems likely that the interaction of FN with platelets is via a different mechanism to that involved in FN binding to fibroblastic cell types.

A number of lines of evidence suggest that carbohydrate moieties at the cell surface are involved in FN binding. Firstly, the use of tunicamycin, a drug which specifically inhibits N-linked glycosylation of proteins, showed that cells lacking surface carbohydrate display a reduced ability to spread on FN-coated substrata (Butters, Devalia, Aplin and Hughes, 1980). Secondly, mutant cell lines with defective glycosylation activities also have a reduced adhesive capacity on various substrata (Pouyssegur and Pastan, 1976; Aplin and Hughes, 1981a). BHK cells selected for resistance to the lectin ricin, display reduced ricin binding together with a reduced level of surface FN and impaired ability to spread on FN-coated substrata (Meager, Ungkitchanukit, Nairn and Hughes, 1975; Edwards, Dysart and Hughes, 1976; Pena and Hughes, 1978).

To which plasma membrane component the carbohydrate sequences involved in FN binding are attached remains unclear. Using FN coupled to a photoactivatable cross-linking reagent a 47Kd glycoprotein has been shown to come into close proximity to substrate-adsorbed FN following BHK cell spreading (Aplin, Hughes, Jaffe and Sharon, 1981c). The same glycoprotein is detected following cell spreading on the lectins ricin, soybean agglutinin and concanavalin A. Perkins et al. (1979), also using a cross-linking derivative of FN, demonstrated that following binding of FN to hamster cell monolayers it became coupled mainly to proteoglycans. However, it seems likely that this interaction represents the binding of FN to extracellular matrix material rather than to its cell surface receptor. Other evidence however also suggests that proteoglycans and GAG's may play a role in FN-mediated cell adhesion. For example, substrate-attached material (SAM), remaining following EDTA detachment of cells from the substratum, is enriched for FN and GAG's, in particular heparan sulphate (Culp, Murray and Rollins, 1979; Murray, Ansbacher and Culp, 1980) and highly sulphated heparan sulphate is also capable of binding to FN-Sepharose although undersulphated heparan sulphate, hyaluronate or chondroitin species of GAG's do not (Laterra, Ansbacher and Culp, 1980). The demonstration of membrane-associated heparan sulphate (Kjellen, Pettersson and Hook, 1981) and the ability of such material to mediate some of the adhesive responses seen following FN-mediated cell adhesion (Laterra, Silbert and Culp, 1983b) therefore make these GAG's candidate receptors for FN. Arguing against such a role however is the finding that the cell attachment domain of FN does not display heparin-binding activity (Pierschbacher et al., 1981).

A number of groups have raised broad spectrum antisera against whole cell or cell surface material in an attempt to identify components involved in FN binding (Wyllie, Damsky and Buck, 1979; Hsieh and Sueoka, 1980; Knudsen, Rao, Damsky and Buck, 1981; Hughes, Butters and Aplin, 1981). Such antisera generally cause cell rounding and detachment from the substratum as a result of binding to a restricted group of glycoproteins. In particular, a 140Kd glycoprotein is commonly recognised although others with molecular weights of 50-, 80- and 120-Kd have also been identified. Interestingly, a 140Kd cell surface glycoprotein has been identified as a major component of the detergent insoluble cytoskeleton together with FN, 250Kd and 170Kd glycoproteins (Lehto, Vartio and Virtanen, 1980; Carter and Hakomori, 1981; Carter, 1982). This glycoprotein may thus play an important role in connecting the FN matrix with the cytoskeleton although direct evidence of it acting as a receptor for FN is not apparent. In fact the 140Kd glycoprotein is apparently trypsin-resistant although binding of FN-coated latex beads to BHK cells is sensitive to mild trypsinisation conditions and monocyte binding to FN-coated substrata is also abolished by trypsin (Grinnell, 1980; Bevilacqua, Amrani, Mosesson and Bianco, 1981)¹. Also using proteases to investigate the nature of the FN receptor Tarone et al. (1982) demonstrated that pronase treatment of BHK cells completely inhibited their adhesion to FN-coated substrata although prolonged trypsin treatment had no effect. Protein synthesis was required to restore the adhesive capacity of the cells supporting a protein nature for the FN receptor. On the basis of their protease-resistance under conditions where cell adhesion was unaffected two glycoproteins of molecular weight 120- and 80-Kd were implicated in FN-mediated cell adhesion.

¹

These 140 kd glycoproteins are clearly different however and the Gp140 described by Carter et al has been shown to be the subunit of type VI collagen (Heller Harrison R.A. and Carter W.G. J. Biol. Chem.259,6858.)

Whilst most studies have emphasised the potential role of glycosylated membrane proteins of various types in the interaction of cells with FN, indirect evidence that the FN receptor is a cell surface sialoglycosphingolipid (ganglioside) has also been put forward (Kleinman, Martin and Fishman, 1979a; Kleinman, Hewitt, Murray, Liotta, Rennard, Pennypacker, McGoodwin, Martin and Fishman, 1979b). Gangliosides were able to inhibit the FN-dependent attachment of CHO cells to collagen and the inhibitory activity was partially retained in the oligosaccharide moiety. Gangliosides have been implicated as receptors for a large number of molecules and in the following I will briefly outline our current understanding of these molecules and their role as cell surface receptors.

B. GANGLIOSIDES AS CELL SURFACE RECEPTORS (see reviews by Critchley and Vicker, 1977; Hakomori, 1981; Svennerholm, Mandel, Dreyfus and Urban, 1980; Wiegandt, 1982).

Gangliosides are sialic acid containing glycolipids, or more correctly glycosphingolipids, that are found in high concentrations in neural tissue, in particular the brain, where they were first discovered by Klenk in 1937. This localisation led to the belief that ganglioside might play a specific role in the functioning of the central nervous system and was supported by the finding that ganglioside accumulation is one of the symptoms of the mental disorder, Tay Sachs' disease. More recently it has become evident that gangliosides are ubiquitous membrane components being found in extra neural tissue and most animal cells in culture. The use of modern analytical techniques, such as HPLC, mass spectroscopy and NMR,

have shown that a surprising diversity of ganglioside species exist and coupled with their localisation in the plasma membrane they are uniquely qualified for specific recognition functions. Gangliosides have been implicated in cell growth and differentiation, malignant transformation, viral infection and as receptors for the glycoprotein hormones, interferon and many bacterial toxins (see Table 1.3).

1. Structure and Nomenclature (see Fig. 1.2 and Table 1.4)

Gangliosides consist of a membrane embedded lipid moiety linked to an externally exposed carbohydrate moiety. The lipid component, termed ceramide, consists of a sphingosine base linked to a fatty acid chain via an amide bond. The carbohydrate component necessarily contains one or more sialic acid residues (either N-acetyl or N-glycoyl neuraminic acid) usually at terminal positions. This combination of lipophilic ceramide and hydrophilic carbohydrate makes gangliosides highly polar lipids with complex physicochemical properties such as the tendency to form micellar structures at concentrations as low as 10^{-8} - 10^{-10} M (Formisano, Johnson, Lee, Aloj and Edelhoch, 1979).

The specificity of gangliosides as membrane receptors has been shown to reside in their oligosaccharide component. Synthesis of these structures is believed to occur in the Golgi apparatus by sequential addition of nucleotide-activated monosaccharides to the ceramide backbone. These reactions are catalysed by a series of highly specific membrane-bound glycosyl transferases which have been postulated to exist as an ordered multienzyme complex to allow regulated sequential transfer of sugar residues (Fishman and Brady, 1976; Keenan, Morre and Basu, 1974).

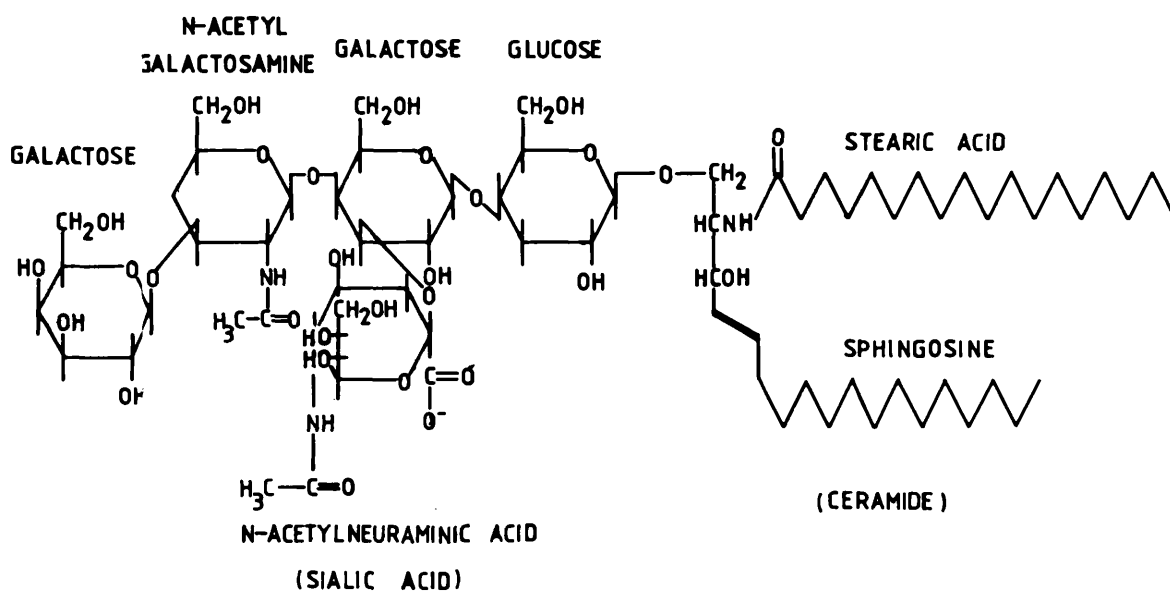


Fig. 1.2 - Structure of ganglioside GM₁ (Opposite)

Table 1.4 Ganglioside structure and nomenclature (Svennerholme, 1970)

GM ₃	Cer - Glc - Gal - AcNeu
GM ₂	Cer - Glc - Gal (AcNeu) - GalNac
GM ₁	Cer - Glc - Gal (AcNeu) - GalNac - Gal
GD _{1a}	Cer - Glc - Gal (AcNeu) - GalNac - Gal - AcNeu
GD _{1b}	Cer - Glc - Gal (AcNeu) ₂ - GalNac - Gal
GT _{1a}	Cer - Glc - Gal (AcNeu) - GalNac - Gal (AcNeu) ₂
GT _{1b}	Cer - Glc - Gal (AcNeu) ₂ - GalNac - Gal - AcNeu
GQ _{1a}	Cer - Glc - Gal (AcNeu) - GalNac - Gal (AcNeu) ₃
GQ _{1b}	Cer - Glc - Gal (AcNeu) ₂ - GalNac - Gal (AcNeu) ₂

Cer., ceramide; Glc., glucose; Gal., galactose;

GalNac., N-acetylgalactosamine; AcNeu., N-acetylneuraminic acid (sialic acid).

Table 1.3 Role of gangliosides as membrane receptors

<u>Ligand</u>	<u>Ganglioside specificity</u>
1. Bacterial toxins	
Cholera toxin	GM ₁
E.coli toxin	GM ₁
Botulinus toxin	GT _{1b}
Staphylococcus α toxin	sialylparagloboside
2. Glycoprotein hormones ¹	
Thyrotropin (TSH)	GD _{1b}
Chorionic gonadotropin	GT ₁
Luteinising hormone	GT ₁
3. Migratory inhibitory factor (MIF)	fucose containing ganglioside
4. Sendai virus	GT _{1a} GQ _{1b}
5. Interferon	GM ₂ GT ₁
6. Fibronectin	GT _{1b} ?

see Hakomori (1981) and Wiegandt (1982) for references.

¹Recent evidence now seriously questions the role of gangliosides as receptors for the Glycoprotein hormones (Pacuszka et al Proc. Natl. Acad. Sci. USA 75, 764(1978)).

2. Membrane Organisation of Gangliosides.

Gangliosides are present in membranes from most mammalian cells and, being incapable of spanning the lipid bilayer, they are generally considered to be localised to the outer leaflet of the plasma membrane with the carbohydrate component exposed to the exterior.

The levels of membrane glycolipids labelled by galactose oxidase/borotritiide have been shown to vary during the cell cycle, maximum labelling occurring at G1. However, the chemical levels of glycolipids appear to remain constant throughout the cell cycle suggesting that glycolipids and thus gangliosides may become masked or cryptic under certain conditions (Gahmberg and Hakomori, 1974).

Various studies have been carried out to investigate the organisation of gangliosides in membranes using model systems. It is thought that in the presence of physiological levels of calcium or magnesium ions gangliosides are aggregated into lipid domains due to complexing of sialic acid residues by these divalent cations (Sharom and Grant, 1978). Gangliosides may also become partially immobilised by charge interactions with the carbohydrate function of integral membrane glycoproteins (Sharom and Grant, 1978).

The interaction of gangliosides with specific membrane proteins has been considered for some time with the proposal that a ganglioside domain around a particular protein may provide an optimal local environment for the functional configuration of a particular protein. As such, gangliosides

have been shown to greatly enhance the activity of the Mg^{2+} -dependent ATPase enzyme from brain microsomes (Capulto, Maccioni and Capulto, 1977) and can be cross-linked to specific "gangliophilic" proteins in the plasma membrane (Lingwood and Hakomori, 1980). A low molecular weight protein was isolated from bovine erythrocyte membranes with specific affinity for GM_3 (Watanabe, Hakomori, Powell and Yokota, 1980). As a consequence of their localisation in the outer leaflet of the plasma membrane gangliosides are incapable of a direct interaction with intracellular components. This raises the question of how, if at all, such interactions can occur. The demonstration that gangliosides can be induced to cap on the surface of lymphocytes by the addition of cholera toxin (a GM_1 -specific ligand) (Révész and Greaves, 1975) and the finding that antibodies directed against GM_1 cause thymocyte stimulation (Sela, Raz and Geiger, 1978) suggests that gangliosides are capable of such transmembrane interactions. This is supported by the recent evidence showing that gangliosides are associated with detergent-insoluble cytoskeletons (Streuli, Patel and Critchley, 1981).

A property of gangliosides which has often been exploited in receptor studies is their ability to become incorporated into membranes when added exogenously (Callis, Schwarzmann, Radsak, Siegert and Wiegandt, 1977; Radsak, Schwarzmann and Wiegandt, 1982). Using such an approach it is possible to reconstitute receptor-deficient membranes with specific gangliosides and identify which species functionally reconstitutes the membrane. This interaction between membranes and exogenous gangliosides appears to be a complex phenomena although it has been demonstrated in a number of systems that at least part of the membrane-associated ganglioside is functionally active (Moss, Fishman, Manganiello, Vaughan and Brady, 1976).

3. Ganglioside GM₁ as the Cell Surface Receptor for Cholera Toxin (CT).

As mentioned above gangliosides have been suggested to act as receptors for a number of biologically active factors and many also play a role in regulation of receptor function. However, apart from the interaction between CT and ganglioside GM₁ most of this data is of an indirect nature and in some cases disputed. I would thus like to outline the data indicating a role for GM₁ as the receptor for CT as this represents a well characterised system for studying ligand-ganglioside interactions. Also, this system is frequently used in this study as a comparison with FN-ganglioside interactions.

The interaction of CT with gangliosides has been extensively investigated and reviewed (Van Heyningen, 1982). It binds with high affinity ($K_D = 10^{-9}$ - 10^{-10} M) and specificity to ganglioside GM₁ at the surface of many cells in culture and isolated intestinal brush borders (Cuatrecasas, 1973; Critchley, Ansell, Perkins, Dilks and Ingram, 1979; Critchley, Magnani and Fishman, 1981). This binding results in an irreversible activation of adenylate cyclase at the cytoplasmic face of the plasma membrane causing an increase in intracellular cyclic AMP and the associated pathological effects of CT (Van Heyningen, 1982). Initial evidence that GM₁ was the CT receptor came from studies involving inhibition of CT binding to membrane fractions. It was demonstrated that the GM₁ was the most potent inhibitor of toxin binding to target cells or membranes (Cuatrecasas, 1973). Numerous studies have correlated the levels of GM₁ with the ability to bind and react with CT. NCTC 2071 cells lack higher gangliosides, including GM₁, and

are unresponsive to CT. Preincubation of these cells with GM₁ however causes them to become sensitised to CT (Moss et al., 1979).

Specific binding of CT to GM₁ has also been demonstrated using gangliosides adsorbed to plastic tubes and separated on thin layer chromatograms (Holmgren, Elwing, Fredman and Svennerholm, 1980; Magnani, Smith and Ginsberg, 1980). Specific binding could only be seen corresponding to GM₁. To demonstrate the absence of any glycoprotein component in CT binding, intestinal brush border glycoproteins were separated on SDS-PAGE and the gel overlaid with radiolabelled CT. Binding could only be demonstrated at the dye front corresponding to the position lipids run in this system. Binding of CT to the cell membranes specifically protected GM₁ from galactose oxidase/borotritiide labelling while glycoproteins showed no such protection (Critchley et al., 1981).

Direct evidence for CT binding to GM₁ at the cell surface has been demonstrated by Critchley et al. (1979). CT-receptor complexes were isolated from galactose oxidase/borotritiide labelled cells by immune precipitation and shown to contain GM₁ as the only labelled component specifically recovered. There is thus a wealth of information showing that CT binds specifically to ganglioside GM₁ at the cell surface and that this binding is functionally involved in the physiological effects of CT.

4. Gangliosides as the Receptor for FN.

Speculation about the involvement of glycolipids or gangliosides as potential receptors for FN probably originated from the finding that both complex gangliosides and FN were reduced following cell transformation. It was also known that the levels of certain gangliosides, the so called density-dependent glycolipids, increased as cells approached confluence, a similar stage at which FN begins to accumulate at the cell surface (Critchley and Vicker, 1977; Hynes and Bye, 1974). It was thus tempting to suggest that gangliosides may be important in the interaction of FN with the cell surface. Further circumstantial evidence linking these two components came from evidence demonstrating a correlation between reduced levels of gangliosides and metastatic potential (Skipski *et al.*, 1980) since the levels of FN are also believed to correlate with a cell's metastatic potential. More recently Yogeeswaran (1981) demonstrated that incorporation of mixed ganglioside into transformed Balb/c 3T3 cells caused a moderate increase in in vitro substrate adhesion and a substantial increase in tumour cell take in vivo. It would thus appear that gangliosides can influence the adhesive capacity of these cells and may thus be involved in the interaction with adhesive molecules such as FN.

In 1979 Kleinman *et al.* provided indirect evidence that gangliosides, in particular GD1a and GT₁, could bind to FN and thereby block its subsequent interaction with the cell surface. This was based on the ability of these gangliosides to inhibit FN-mediated adhesion of Chinese hamster ovary (CHO) cells to collagen-coated substrata. The inhibitory activity was partially retained in the oligosaccharide moiety of the

gangliosides and it was thus proposed that gangliosides, or glycoproteins bearing similar carbohydrate moieties (Rauvala and Finne, 1979) could act as cell surface receptors for FN.

I have attempted to make a critical evaluation of the role of gangliosides as receptors for FN using the following approaches.

- i) Extending Kleinman's original data to examine the ability of gangliosides to inhibit BHK cell spreading on FN. In addition, the specificity of this effect for FN induced cell adhesion was investigated by examining the ability of gangliosides to inhibit cell spreading on concanavalin A-coated substrata. Concanavalin A is believed to induce cell adhesion by interaction with cell surface glycoproteins and not involving gangliosides.
- ii) Investigating the ability of FN to bind to gangliosides immobilised on plastic surfaces using a sensitive radio-immune detection system.
- iii) In an attempt to determine whether a cell surface ganglioside is capable of mediating cell attachment and spreading I have investigated the ability of a ganglioside-reactive substratum to promote Balb/c 3T3 cell adhesion. In addition, by comparison of the adhesive response of these cells to ganglioside- and glycoprotein-reactive substrata with that to FN-coated substrata, it was hoped to be able to assess the relative importance of these cell surface components in mediating FN-induced cell adhesion.

iv) Finally I have analysed the relationship between levels of cellular gangliosides and their ability to interact with FN. To do this I have used a series of ganglioside-deficient variants of Balb/c 3T3 cells previously isolated in this laboratory. The effect of altering the levels of cell surface gangliosides by neuraminidase-treatment and ganglioside add-back experiments is also examined.

CHAPTER 2

GANGLIOSIDE INHIBITION **OF CELL SPREADING**

INTRODUCTION

Characterisation of the cell surface receptor for FN has met with a number of problems. It has proved difficult to carry out classical ligand binding studies due to poor binding of FN to cells in suspension (Rennard, Wind, Hewitt and Kleinman, 1981). Although binding of FN to cell monolayers has been shown (Rennard et al., 1981; Perkins et al., 1979), this probably represents binding to other matrix components, such as collagen, rather than its cell surface receptor. This is supported by data suggesting that receptor sites for FN become depleted from the dorsal surface of the cell following attachment and spreading (Grinnell, 1980). To demonstrate binding of FN to the cell surface it is necessary to activate the FN by adsorption to a suitable substrate, usually plastic (Klebe, Bentley and Schoen, 1981). This activation step is not understood although a number of explanations have been put forward. Following surface adsorption FN may undergo a conformational change such that a previously cryptic cell binding site becomes exposed. A similar such mechanism has been shown to occur during surface activation of blood clotting factor XII (the Hageman factor) by glass and other non-physiological substrata (Griffin, 1978). Alternatively, adsorption may allow cooperative binding of FN to the cell surface thereby increasing the overall affinity of the interaction. Due to the above limitations many studies of FN-cell interactions have involved the use of assays based on the ability of FN to promote cell-substrate attachment and spreading. This is thought to be due to the binding of substrate-adsorbed FN to a specific cell surface receptor. Klebe (1974)

demonstrated that SV-3T3 cell attachment to collagen-coated dishes was mediated by a high molecular weight (>200Kd) serum component which was also capable of inducing cell spreading. Pearlstein (1976) later demonstrated FN-dependent attachment of PyBHK cells to collagen. Similar such assays have subsequently been developed by a number of other workers also using collagen-coated substrata (Grinnell and Minter, 1978; Kleinman et al., 1979c). However, there is no absolute requirement for collagen since FN can adsorb to a number of substrata and promote cell attachment and spreading (Klebe, Bentley and Schoen, 1981; Hughes, Pena, Clark and Dourmashkin, 1979; Grinnell, Hays and Minter, 1977).

Using a CHO cell attachment assay Kleinman et al. (1979a) demonstrated that gangliosides, in particular di- and tri-sialogangliosides (GD1a and GT₁), could inhibit FN-mediated cell attachment to collagen-coated substrata. CHO cell attachment to collagen was shown to be a consequence of FN binding to the collagenous substrata and subsequent cell attachment to the collagen-FN complex. Preincubation of the collagen-FN complex with gangliosides reduced the adhesive capacity of the substratum to the low levels seen on collagen alone. This inhibitory capacity was partially retained in the oligosaccharide moiety of the ganglioside and was not attributable to an effect on collagen-FN interactions. Using periodate modifications of the gangliosides it was demonstrated that sialic acid formed an essential component of the inhibitory activity, although sialic acid alone was not active. From this data it was proposed that gangliosides or other sialic acid-containing glycoconjugates with a similar carbohydrate sequence, may act as cell surface receptors for FN.

In this study I have extended Kleinman's original observations by examining the ability of gangliosides to inhibit CHO and BHK cell spreading on FN-coated substrata. In addition, I have attempted to obtain further evidence of FN-ganglioside interaction by investigating the binding of FN to gangliosides bound to plastic. A similar such approach has been used to study the binding of cholera toxin and tetanus toxin to gangliosides (Holmgren, Elwing, Fredman and Svennerholm, 1980). Gangliosides adsorb to plastic substrata via a strong hydrophobic interaction between their ceramide moiety and the plastic substrata leaving the oligosaccharide portion free to react with specific ligands. Since it is believed to be the carbohydrate component of ganglioside to which FN binds, this system is ideally suited to studying the interaction of FN with immobilised gangliosides. In order to establish the methodology for this technique I have used the binding of radioiodinated cholera toxin (CT) to plastic coated with ganglioside GM₁, its endogenous cell surface receptor. Finally, I have asked if ganglioside inhibition of cell spreading is specific for the FN-mediated process or whether it represents a more general inhibition of the cell spreading process. To do this I have examined the effect of gangliosides on ConA-mediated BHK cell spreading.

MATERIALS AND METHODS

Cell culture

Chinese hamster ovary (CHO) and baby hamster kidney (BHK) cells were all grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat inactivated newborn calf serum, penicillin (100 units/ml), streptomycin (100µg/ml) and amphotericin B (0.235mg/ml) in 9cm tissue culture dishes (Nunc) in a 5% CO₂ incubator with a humidified atmosphere. Cells were routinely subcultured twice weekly using 0.25% trypsin. All tissue culture reagents were purchased from Gibco Bio-cult, Paisley, Scotland.

Cells were stored by slow freezing down to -70°C in 7.5% dimethyl sulphoxide/20% foetal calf serum before transfer to liquid nitrogen.

Ganglioside purification and analysis

Bovine brain mixed gangliosides were purchased from Sigma Chemical Company Ltd. and purified individual gangliosides from Supelco (Bellefonte Pa., U.S.A.). In addition a number of gangliosides were isolated from human brain tissue by Mr. B. Patel in this laboratory using previously published methodology (Svennerholm and Fredman, 1980; Fredman, Nilsson, Tayot and Svennerholm, 1980). This essentially consisted of extracting lipid material from human cerebral tissue with chloroform:methanol:water (4:8:3 v/v) and partitioning the lipid extract against water to isolate mixed gangliosides in the aqueous phase.

Mixed gangliosides were then separated into mono-, di-, and tri-sialogangliosides using a DEAE-Sephadex CL-6B column (acetate form) eluted stepwise with potassium acetate in methanol (0.005M-0.02M). Fractions were analysed by thin layer chromatography on silica gel G plates (Merck) using the solvent system chloroform: methanol:water (60:35:8). Separated gangliosides were visualised by resorcinol spray as follows:-

2.5ml of 2% aqueous resorcinol (BDH labs.) was mixed with 20ml of concentrated hydrochloric acid plus 62µl of 0.1M copper sulphate and made up to 25ml with water. The reagent was then left 4h before use. Plates were sprayed with the resorcinol reagent and baked at 110-120°C for 10' to visualise ganglioside bands.

The yield of gangliosides was determined by assaying the lipid-bound sialic acid with the resorcinol method (Svennerholm, 1957).

Chinese hamster ovary cell attachment and spreading assays

The attachment of CHO cells to collagenous substrata has been shown to be mediated by FN in a concentration dependent manner (Klebe, 1974). This system provides a sensitive assay for FN-cell interactions.

i) Preparation of collagen-coated substrata (Klebe, 1974). 10µg of acid soluble collagen (Sigma type III) was applied to 35mm tissue culture dishes (Falcon) in 2ml of 0.1% acetic acid containing 0.002% phenol red. Dishes were placed in a sealed container with a small beaker of NH₄OH and rocked gently until the phenol red indicator had changed from yellow

to a metallic red colour (5', RT⁰). Unpolymerised material was then decanted off, dishes washed three times with distilled water and air dried overnight. Collagen coated dishes were prepared freshly for each experiment.

ii) CHO cell attachment assay. Essentially as described by Kleinman et al. (1979a). Collagen-coated dishes were incubated with 1ml of DMEM containing 0.02% BSA and varying amounts of serum or FN for 1h, 37⁰C (for purification of FN see Appendix I). 4×10^5 freshly trypsinised CHO cells were added to each dish in 100 μ l of DMEM and allowed to spread for 1.5h, 37⁰C in a humidified CO₂ incubator. Unattached cells were removed by gently washing (x2) with 0.9% sodium chloride. Attached cells were then released with 0.25% trypsin/0.1% EDTA and counted on a Coulter counter. In ganglioside inhibition experiments varying amounts of stock ganglioside solution (10mg/ml, bovine brain mixed ganglioside in DMEM) were added to FN-treated dishes and incubated for 1h, 37⁰C. Cells were then added directly to the ganglioside-containing medium.

iii) CHO cell spreading assay. As described above except that attached cells were fixed with 3.8% formaldehyde in PBS for 40', RT⁰ and stained with crystal violet stain (0.02% in water) rather than counted. After preliminary experiments it was found that collagen treatment of the plastic dishes had little effect on FN-dependent cell spreading and the assay was modified as follows. Duplicate linbro wells (Flow labs.) were incubated with varying concentrations of FN in PBS for 1h, 37⁰C (250 μ l/well) allowing non-specific adsorption of the

protein to the plastic surface. Unbound material was decanted off and 250 μ l of DMEM added. 5×10^4 freshly trypsinised and washed (PBS containing 1mM phenylmethylsulphonyl fluoride, PMSF) CHO cells were then added to each well in 100 μ l of DMEM and allowed to spread for 1h, 37°C in a humidified CO₂ incubator. Cell spreading was quantitated using fixed and stained cells by microscopically counting the number of spread cells in 3-5 fields of view and expressing this as a percentage of the total cell number per field of view. A cell was scored as being spread if sufficient cell flattening had occurred to allow visualisation of the nucleus in crystal violet stained cells. This usually involved the cells adopting a tripolar morphology with bipolar cells not being sufficiently well spread to be scored as positive. Where appropriate, gangliosides (1mg/ml stock solution in DMEM) were added to FN-coated wells and incubated 1h, 37°C before direct addition of cells. Stained cells were photographed on a Leitz diavert microscope with the phase rings removed using Ilford Pan F film (50ASA).

Baby hamster kidney cell spreading assay

Essentially as described for CHO cell spreading. FN-coated linbro wells were incubated with 5×10^4 BHK cells for 1h, 37°C in a humidified CO₂ incubator. After fixing and staining, cell spreading was quantitated as described above. For concanavalin A (ConA)-mediated cell spreading, linbro wells were coated with ConA (Miles-Yeda) in PBS for 1h, 37°C as for FN-coating. It was found necessary to wash off unbound ConA prior to the addition of cells as free ConA tended to block cell binding to substrate-adsorbed ConA.

Radioiodination procedures

i) Fibronectin - FN was iodinated using enzymobeads (Biorad Labs.) according to the manufacturers instructions. Beads were rehydrated in 0.5ml of distilled water for 2h, 4°C and 25µl added to a 1.5ml microfuge tube. 50µl of PBS pH7.2, 8µg FN (20ul in PBS), 10µl Na¹²⁵I (1mCi, Radiochemical Centre, Amersham) and 25µl of 1% beta-D-glucose were then added and incubated for 25', RT^O. The beads were spun out in a Beckman microfuge and the supernatant transferred into 100µl 0.1M sodium iodide/PBS/0.05% BSA. After removing 5µl of the reaction mix into 2ml of PBI (as PBS with sodium iodide replacing sodium chloride) containing 0.05% BSA the remainder was loaded onto an 8ml Sephadex G25 (medium) column, equilibrated with 5 bed volumes of PBS/0.05% BSA, to separate free iodide from labelled protein. Labelled FN was then loaded onto a 1ml gelatin-Sepharose-4B column equilibrated with PBS/0.05% BSA and eluted with 4M urea/CAPS pH11.0. Between 60-70% of the counts loaded onto the column were retained and could be eluted with urea. Analysis of the affinity purified material by SDS-PAGE showed that the majority of the radio-activity was associated with a single band which co-migrated with unlabelled FN (Fig. 2.1). The specific activity of the labelled material, as determined by the percentage of TCA precipitable counts in the reaction mix, was 8µCi/µg of protein.

ii) Cholera toxin - CT was iodinated by a chloramine T method (Streuli et al., 1981). 10µg of CT (Schwarz-Mann) was labelled at RT^O with 500µCi of Na¹²⁵I for 1' using 25µg of aqueous chloramine T (Sigma, 10mg/ml) as an oxidising agent. The reaction was terminated with 50µg

of sodium metabisulphite (10mg/ml in PBS) followed by 50 μ l 0.1M sodium iodide in 0.1M Tris pH7.5 containing 0.2% (w/v) gelatin. 2.5 μ l of the reaction mix was taken into 2ml of PBI containing 0.1% BSA to calculate the efficiency of incorporation and the remainder loaded onto a Sephadex G25 column equilibrated with 0.1M Tris pH7.4/0.2% (w/v) gelatin to remove free iodide. Specific activities of 10-20 μ Ci/ μ g protein were routinely obtained with between 40-70% biological activity (as determined in cell binding assays).

iii) Protein A - protein A (Pharmacia) was radioiodinated using a modification of the chloramine T procedure. 5 μ l of 1mM chloramine T was incubated with 20 μ l of 0.1M sulphuric acid for 1' and for a further 2' after the addition of 5 μ l Na¹²⁵I (500 μ Ci). 30 μ l of potassium phosphate buffer pH7.0 was added followed by 20 μ l of protein A (1mg/ml) and incubated for 10', RT^O with mixing. The reaction was then terminated by the addition of 30 μ l of saturated tyrosine solution followed by 100 μ l of NET/gelatin buffer (150mM sodium chloride, 5mM EDTA, 50mM Tris pH7.4, 0.05% Nonidet-P40, 0.25% gelatin) plus 20 μ l of beta-mercaptoethanol. Labelled protein was separated from free iodide using a Sephadex G25 column equilibrated with NET/gelatin buffer. The specific activity of the labelled protein was 12 μ Ci/ μ g and greater than 65% was able to bind to rabbit IgG as determined in a microwell binding assay.

All labelled proteins were stored at 4^OC.

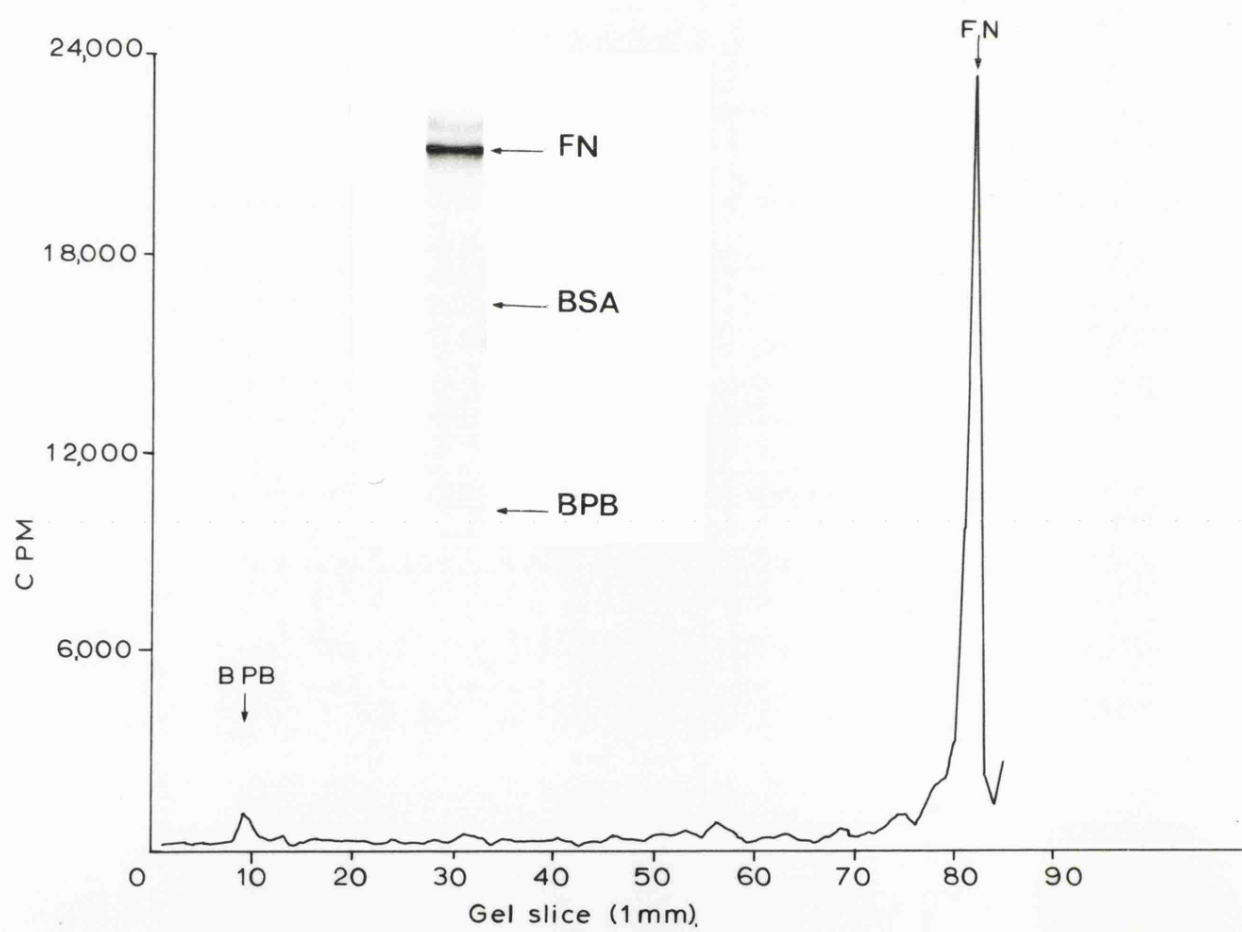


Fig. 2.1 Iodination of FN using Enzymobeads - SDS-PAGE
profile of iodinated materials

FN was iodinated using Enzymobeads according to the manufacturer's recommendations (see Bio-Rad product information bulletin 1060E) and as described in Materials and Methods. 8µg of human plasma FN was reacted with 1mCi Na¹²⁵I in the presence of enzymobeads and 1% beta-D-glucose for 25', RT⁰. Iodinated material was separated from free iodide by Sephadex G25 chromatography and biologically active FN then recovered by gelatin-Sepharose affinity chromatography. A sample of 4M urea eluted material was then analysed on a 7.5% SDS-PAGE by gel slicing or autoradiography (insert). BSA = bovine serum albumin marker, BPB = bromophenol blue marker.

Binding of FN to plastic-adsorbed gangliosides (Holmgren et al., 1980)

Gangliosides dissolved in PBS were adsorbed to 10 x 55mm polystyrene tubes (AB Heger-Plast. Stallarholmen, Sweden) for 18h, RT^O. Unbound ganglioside was removed and the tubes washed three times with PBS before incubation with 0.5ml of 10mg/ml BSA in PBS (1h, 37^OC) to prevent non-specific adsorption of ligands to the plastic. ¹²⁵I-labelled CT or FN was then added (100-200 x 10³ CPM/tube) in 200μl of PBS containing 5mg/ml BSA and the ligands allowed to bind for 2h, RT^O. Unbound material was aspirated and tubes washed three times with 0.05% Tween 20 in PBS before ¹²⁵I counting on a Beckman 5500 gamma counter.

Binding of FN or CT to ganglioside-coated tubes was also determined using ligand-specific antisera and ¹²⁵I-labelled protein A (see Appendix II for production and characterisation of anti-FN antiserum). Ganglioside-coated tubes were incubated with 100μl of CT or FN in PBS containing 5mg/ml BSA for 2h, RT^O and after removal of unbound ligand tubes were further incubated for 1h, 37^OC with 100μl of rabbit anti-ligand antiserum diluted 1:30 with PBS/BSA. Unbound antibody was removed by washing with PBS and tubes incubated 1h, 37^OC with ¹²⁵I-labelled protein A in PBS containing 5mg/ml BSA. Tubes were washed three times with PBS/0.05% Tween 20 and counted as above.

RESULTS

Ganglioside inhibition of FN-mediated CHO cell attachment and spreading

i) Serum-dependent CHO cell attachment. Collagenous substrata were prepared as described previously (Klebe, 1974; Pearlstein, 1976; Kleinman, McGoodwin, Rennard and Martin, 1979c). 35mm tissue culture dishes were coated with 10µg of collagen per dish by ammonia gelling and CHO cell attachment investigated in the presence of varying concentrations of bovine serum (Fig. 2.2). Maximal cell attachment occurred at between 0.25 and 1.0% serum although this rarely exceeded 50% of the total cells applied to the dish. In the absence of serum the substrata had a reduced capacity to mediate cell attachment although considerable variation was observed. In some instances maximal cell attachment was only 3-fold higher than that seen in the absence of serum due to high "non-specific" attachment. Pretreatment of the collagen-coated dishes with 8M urea as described by Klebe (1974) and Pearlstein (1976) did not significantly reduce attachment in the absence of serum (Table 2.1). Using phase microscopy it was noted that in the absence of serum or FN most of the attached cells were rounded and translucent in appearance with only a few opaque, rounded cells being seen. Following addition of serum or FN the attached cells underwent cell spreading, i.e. flattening of the cell body with cytoplasmic extension away from the nucleus.

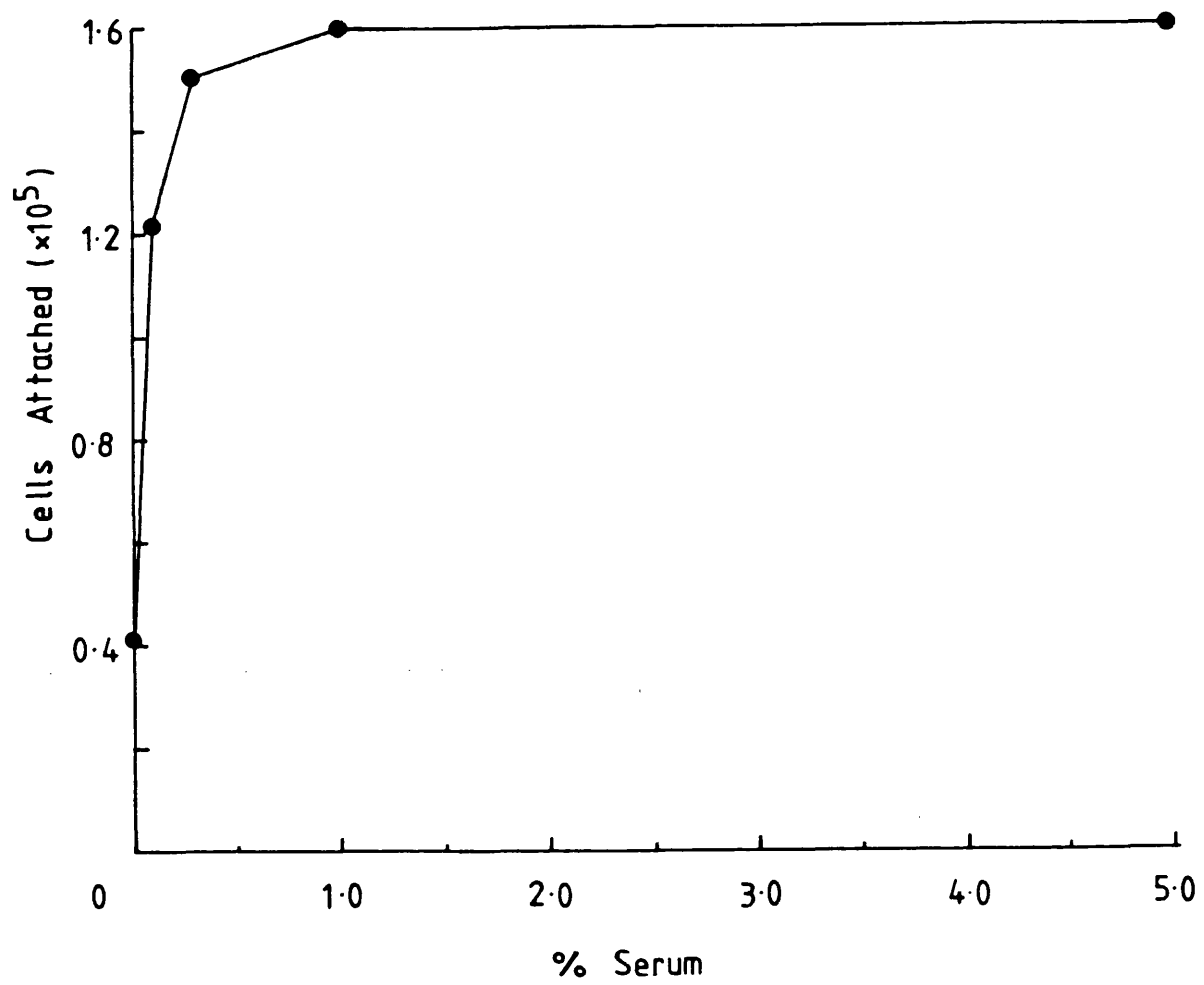


Fig. 2.2 Serum dependent CHO cell attachment to collagen-coated
substrata

35mm tissue culture dishes were coated with 10µg of collagen (type III, Sigma) by ammonia gelling. Each dish was then incubated with 1ml of DMEM/0.02% BSA and varying concentrations of bovine serum for 1h, 37°C. 4×10^5 freshly trypsinised CHO cells were then added in 0,1ml of DMEM/0.02% BSA and incubated for a further 1.5h, 37°C. After removal of unattached cells, adherent cells were released by trypsinisation and counted electronically.

Table 2.1 Effects of 8M urea treatment of collagen substrata
on CHO attachment

% Serum	Cell attachment ($\times 10^5$)	
	+ 8M urea treatment	- 8M urea treatment
0	0.24	0.17
0.1	0.82	0.78
0.5	0.90	0.93
1.0	0.84	0.89
5.0	1.0	0.82

Protocol essentially as described in Fig. 2.2. Where appropriate collagen-coated dishes were washed with aqueous 8M urea for 20', RT^O then three times for 5' with water and allowed to air-dry. Freshly trypsinised CHO cells were then added and allowed to attach for 1.5h, 37^OC.

ii) Effect of gangliosides. When collagen-coated dishes were incubated with 0.2% serum, a concentration capable of inducing approximately 90% of maximal cell attachment, and subsequently with various concentrations of bovine brain mixed gangliosides it was found that cell attachment decreased with increasing ganglioside concentrations (Fig. 2.3). However, the reduction in cell attachment demonstrated here was far less than that demonstrated by Kleinman et al. (1979a) under similar conditions. Examination of the attached cells using microscopy demonstrated that cell spreading was far more sensitive to gangliosides than cell attachment (Fig. 2.3). A ganglioside concentration of 0.67mM virtually abolished cell spreading while cell attachment was only inhibited by approximately 20%. 50% inhibition of cell spreading was seen with a ganglioside concentration of approximately 0.2mM. The remaining attached cells were translucent in appearance thus resembling cells attached in the absence of serum.

Due to the above difficulties in setting up a reliable CHO cell attachment assay it was decided to modify it by monitoring cell spreading, rather than attachment, since cell spreading displayed an absolute requirement for FN.

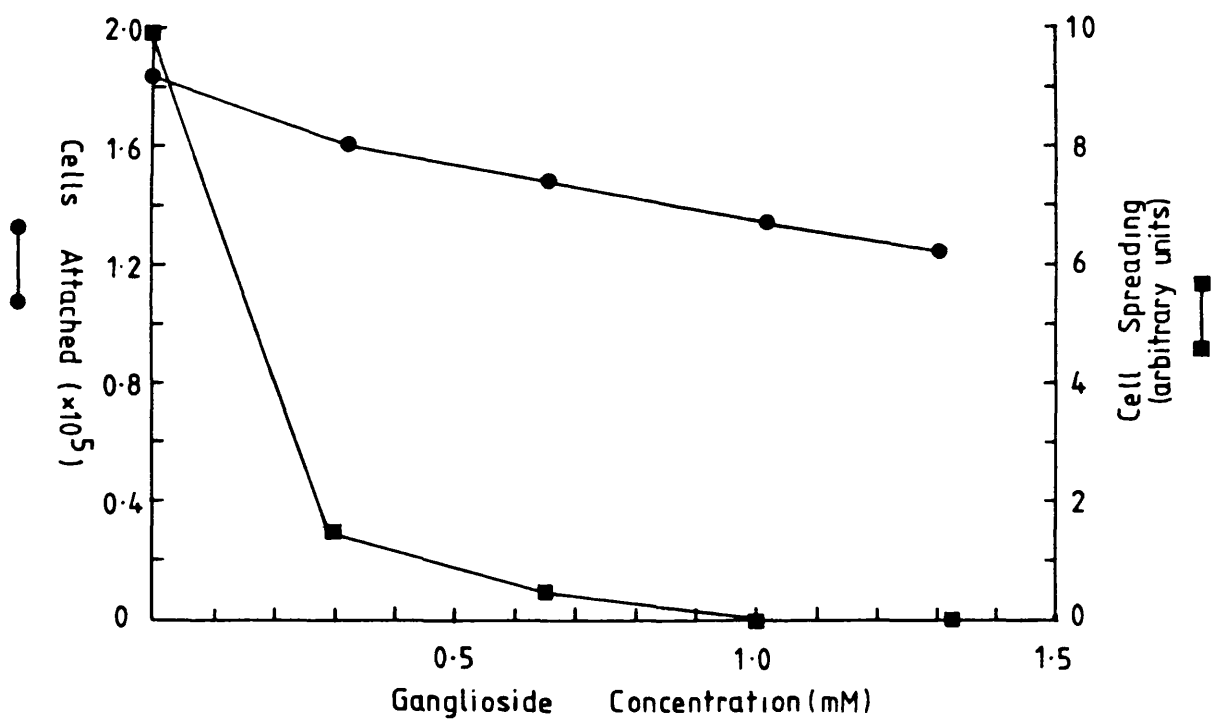


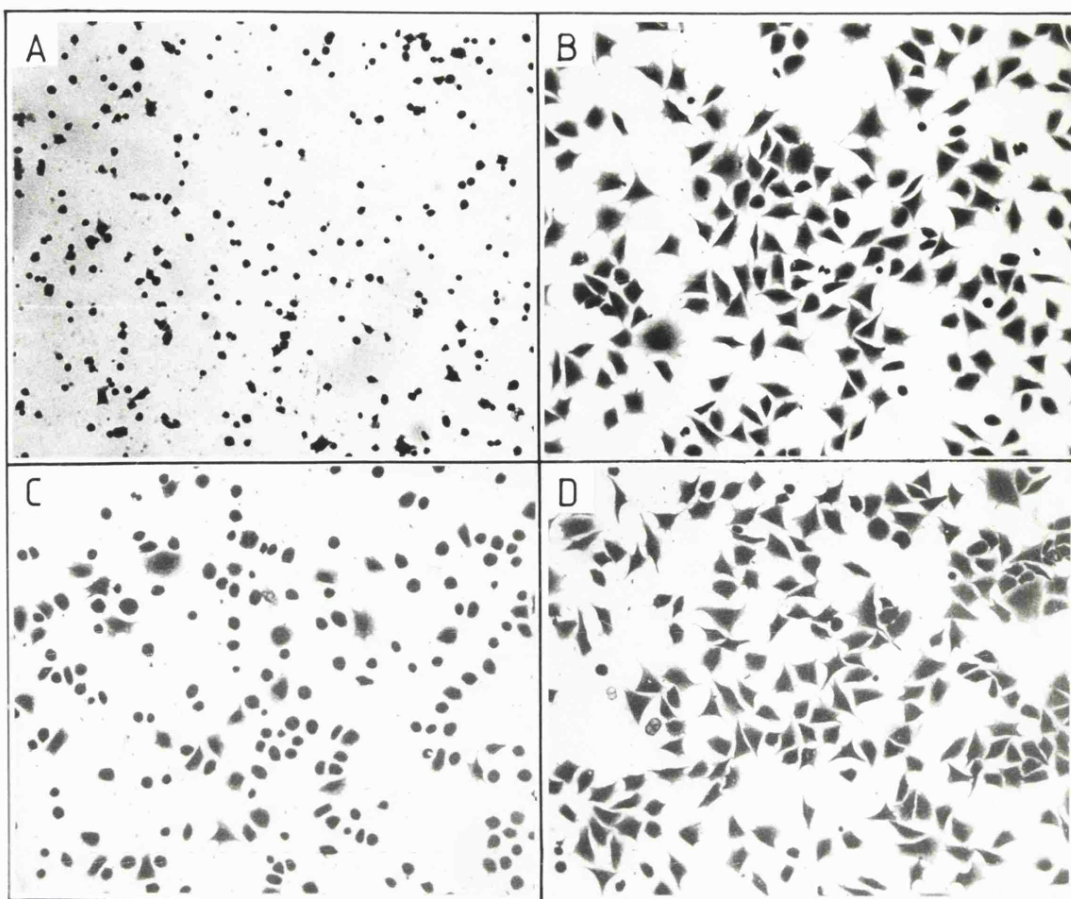
Fig. 2.3 Ganglioside inhibition of CHO cell attachment and spreading on collagen-coated substrata

35mm tissue culture dishes were coated with 10µg of collagen (type III, Sigma) by ammonia gelling. Dishes were then incubated with 1ml of DMEM/0.02% BSA plus 0.2% bovine serum and varying concentrations of mixed ganglioside for 1h, 37°C. Freshly trypsinised CHO cells (4×10^5) were then added and incubated for a further 1.5h, 37°C. Following removal of unattached cells, attached cells were released by trypsinisation and counted electronically. Cell spreading was scored semi-quantitatively on a 0-10 basis, 0 indicating a rounded morphology and 1-10 increasing numbers of well spread cells.

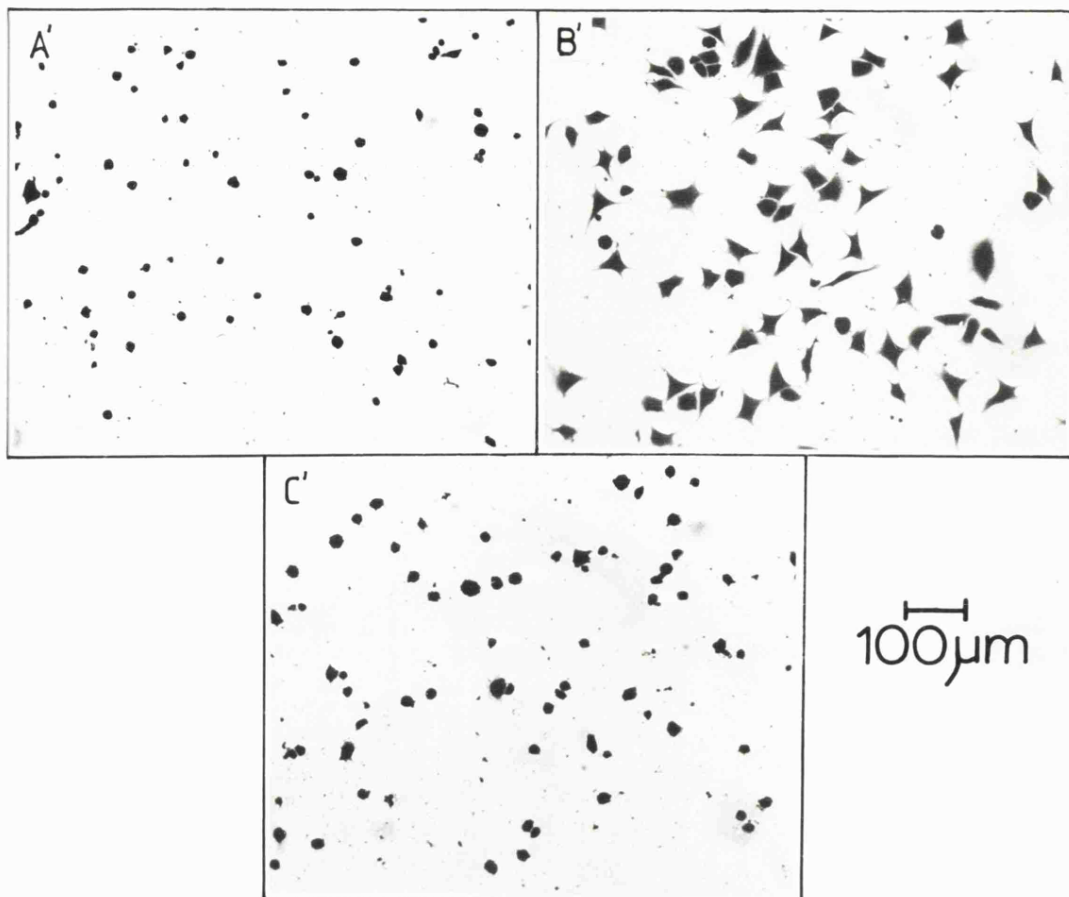
iii) Ganglioside inhibition of CHO cell spreading. The problems of the CHO cell attachment assay are clearly demonstrated in Fig. 2.4,A where a high level of cell attachment to collagen-coated dishes is evident in the absence of serum or FN. All the attached cells however remained rounded. Addition of 0.5% bovine serum to the dish caused a dramatic change in the shape of the attached cells to a flattened well spread morphology (Fig. 2.4,B). This ability of serum to promote cell spreading can be partially blocked by carrying out the assay in the presence of 500µg/ml bovine brain mixed gangliosides. This inhibitory effect can be overcome by increasing the level of serum to 5.0% (Fig. 2.4, C,D) suggesting that the inhibitory activity is not due to a cytotoxic effect on the cells.

The presence of collagen in Kleinman's attachment assay probably reduced non-specific attachment of the cells to plastic although in our case this was ineffective. Since we were now monitoring cell spreading it was decided to investigate whether or not CHO cells were capable of spreading on FN-coated linbro wells in the absence of collagen. Over the time course of the assay (1.5h) little or no cell spreading was evident in the absence of FN (Fig. 2.4,A'). Treatment of the wells with 3µg/ml FN in PBS for 1h, 37°C induced greater than 80% of the applied cells to spread (Fig. 2.4,B'). The spreading activity was completely abolished by the presence of 500µg/ml mixed gangliosides (Fig. 2.4,C'). Thus, CHO cell spreading on plastic could be induced by FN-coating in the absence of collagen and forms a rapid, sensitive assay for FN activity. This activity is sensitive to mixed gangliosides although cell attachment is not greatly affected.

(a)



(b)



100 μm

Fig. 2.4 Ganglioside inhibition of CHO cell spreading

a) 35mm tissue culture dishes were coated with 10 μ g of collagen as described in Materials and Methods. They were then incubated for 1h, 37 $^{\circ}$ C with 1ml DMEM plus serum and mixed ganglioside as indicated. 4 x 10⁵ freshly trypsinised CHO cells were then added in 100 μ l of DMEM and allowed to spread for 1.5h, 37 $^{\circ}$ C. Medium was then decanted off and cells fixed and stained.

- A - No serum; no ganglioside
- B - 0.5% serum; no ganglioside
- C - 0.5% serum; 500 μ g/ml mixed ganglioside
- D - 5% serum; 500 μ g/ml mixed ganglioside

b) Linbro wells were coated with FN in PBS for 1h, 37 $^{\circ}$ C followed by either DMEM or DMEM plus mixed ganglioside for a further 1h, 37 $^{\circ}$ C. Freshly trypsinised CHO cells (5 x 10⁴) were then added to each well in 100 μ l of DMEM and allowed to spread for 1.5h, 37 $^{\circ}$ C. Cell spreading was visualised as described above.

- A' - No FN; no ganglioside
- B' - 3 μ g/ml FN; no ganglioside
- C' - 3 μ g/ml FN; 500 μ g/ml ganglioside

Quantitative data for FN-dependent CHO cell spreading is presented in Fig. 2.5. In the absence of FN, linbro wells were unable to support CHO cell spreading although cell attachment clearly occurred. Coating of the wells with increasing concentrations of FN over the range 0.25-1.0 μ g/ml caused a linear increase in the number of cells spread. Maximal cell spreading was achieved at a coating concentration of 2.0 μ g/ml FN. Cell spreading in the absence of FN treatment was consistently less than 10% of the total number of attached cells and frequently as low as 5%. Slight variation in the amounts of FN required for maximal cell spreading were noticed between different batches of FN (between 1.5-2.5 μ g/ml FN). Repeated freeze thawing of FN stocks also reduced its ability to promote cell spreading and was thus avoided.

As previously shown (Fig. 2.4,b) mixed gangliosides inhibit FN-mediated CHO cell spreading. The concentration dependence of this phenomena is shown in Fig. 2.6. Linbro wells were coated with 3 μ g/ml FN followed by increasing concentrations of bovine brain mixed gangliosides in DMEM. Freshly trypsinised CHO cells were then added directly to the ganglioside containing medium. In the absence of gangliosides greater than 90% cell spreading was achieved. In the presence of gangliosides there was a steady reduction in the number of spread cells over the range 0-200 μ M mixed gangliosides. A 50% reduction in cell spreading was achieved at approximately 100 μ M mixed gangliosides. To determine the ganglioside specificity of this inhibition, 200 μ M purified gangliosides (Supelco) were added individually to the assay and cell spreading monitored (Table 2.2A). The di- and tri-sialogangliosides GD1_a and GT) were consistently better inhibitors of cell spreading than the monosialoganglioside, GM₁. This is in agreement with data for

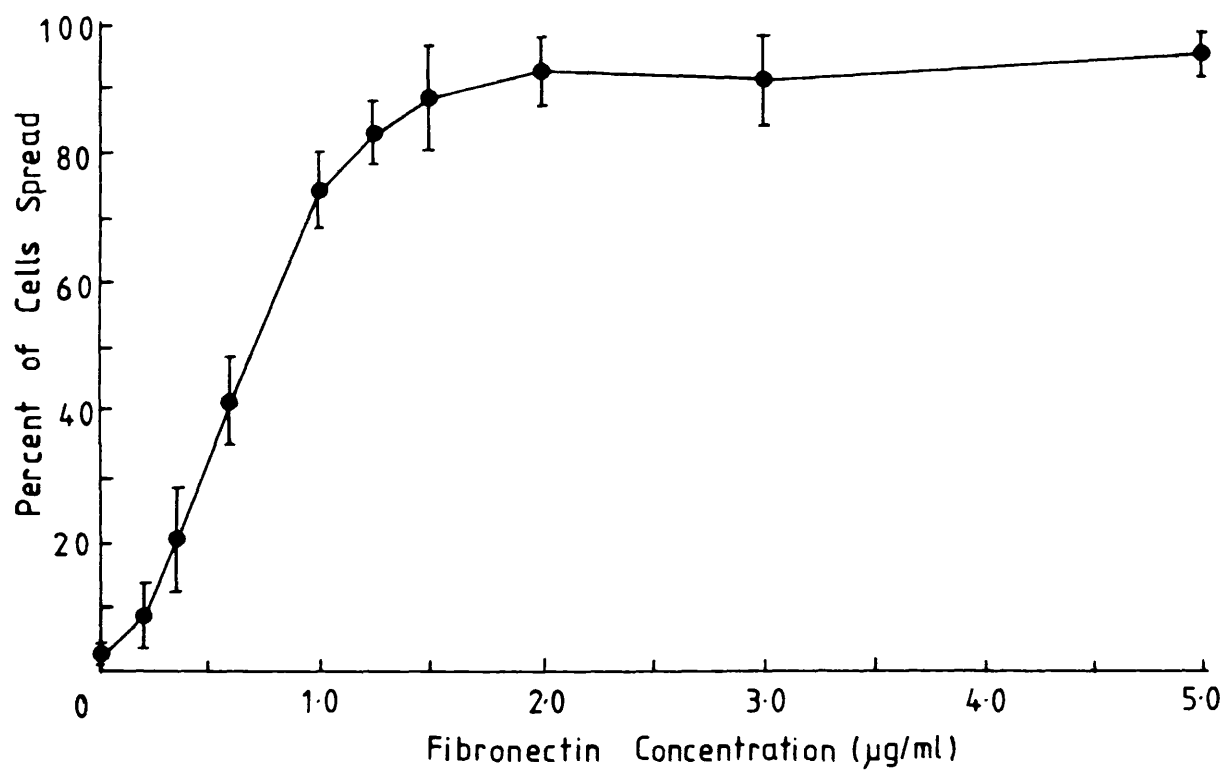


Fig. 2.5 FN-dependent spreading of CHO cells on plastic
substrata; concentration dependence

Linbro wells were incubated with varying concentrations of FN in PBS for 1h, 37°C (250µl/well). Unbound FN was then decanted off and 250µl DMEM added. Freshly trypsinised CHO cells (5×10^4 in 100µl DMEM) were added and allowed to spread for 1h, 37°C in a 5% CO₂ incubator. Cells were then fixed and stained and cell spreading quantitated as described in Materials and Methods. Error bars represent standard deviation ($n-1$).

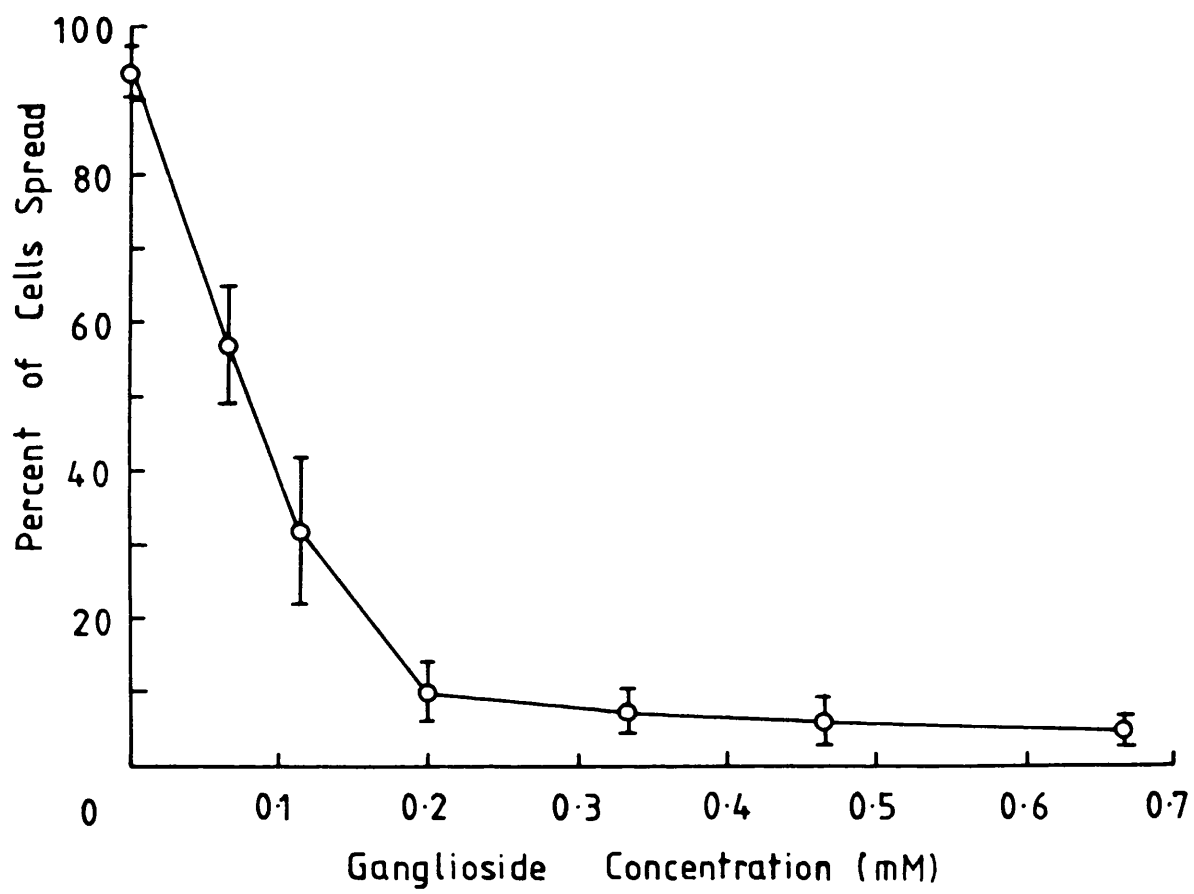


Fig. 2.6 Ganglioside inhibition of CHO cell spreading;
concentration dependence

Linbro wells were coated with 3µg/ml FN in PBS (250µl/well) for 1h, 37°C. Unbound FN was then decanted off and replaced by 250µl of DMEM containing varying concentrations of bovine mixed gangliosides. After incubating for 1h, 37°C. 5×10^4 freshly trypsinised CHO cells were added to each well in 100µl of DMEM and allowed to spread for 1h, 37°C. Medium was then gently decanted off and cells were fixed and stained and cell spreading quantitated as described in Materials and Methods.

ganglioside inhibition of CHO cell attachment (Kleinman et al., 1979a).

Because cells were added directly to ganglioside-containing medium in the above experiments the basis for the inhibitory activity of gangliosides on FN-dependent cell spreading is unclear. In an attempt to establish whether the inhibition was due to ganglioside binding to substrate-bound FN or to a direct effect on the cell we carried out the following experiments. Preincubation of FN-coated plastic with mixed gangliosides followed by removal of unbound gangliosides prior to the addition of cells only partially relieved the inhibition (Table 2.2,B). In addition, preincubation of the plastic substratum with gangliosides prior to FN had no effect on subsequent cell spreading demonstrating that ganglioside binding to the plastic substratum was not responsible for the inhibitory effect.

The above data is thus consistent with that presented by Kleinman et al. (1979a) suggesting that gangliosides, in particular GD1_a and GT, can bind to substratum adsorbed FN thereby preventing its subsequent interaction with the cell surface.

Ganglioside inhibition of FN-mediated BHK cell spreading

An important extension of the above data is to examine whether gangliosides are inhibitory in other assays commonly used to monitor FN-cell interaction. We therefore investigated the ability of gangliosides to inhibit FN-mediated BHK cell spreading, an established bioassay for FN (Pena and Hughes, 1978). It was demonstrated that BHK

(A)

Ganglioside (200 μ M)	% Cell spreading	% Inhibition
No addition	90.2 \pm 3.3	-
GM ₁	82.2 \pm 2.2	8.9
GD	7.3 \pm 4.7	91.9
GT	0	100
Mixed	10.6 \pm 3.3	88.2

(B)

Incubations	% Cell spreading	% Inhibition
FN/wash/DMEM	74 \pm 10.9	0
FN/wash/ganglioside	28 \pm 10.9	62.2
FN/wash/ganglioside/wash	39 \pm 11.5	47.3
Ganglioside/wash/FN/wash	77 \pm 2.4	0

Table 2.2 Ganglioside inhibition of CHO cell spreading

(A) Ganglioside specificity

Linbro wells were incubated for 1h, 37°C with 3µg/ml FN in PBS (250µl/well). Unbound FN was aspirated and replaced by DMEM containing specific gangliosides (Supelco standards, 200µM) as indicated. After incubating for 1h, 37°C freshly trypsinised CHO cells were added (4×10^5 /well in 100µl DMEM) and cell spreading determined after 1h, 37°C as described in Materials and Methods.

(B) Effect of removing unbound gangliosides

Linbro wells were incubated for 1h, 37°C with each of the above additions (250µl/well) in the order shown. Where indicated wells were washed twice with DMEM prior to the next addition. 5×10^4 cells were then added to each well in 100µl DMEM and cell spreading determined as in (A).

FN was 3µg/ml in PBS.

Gangliosides were bovine brain mixed gangliosides and were added in DMEM at a final concentration of 500µg/ml.

cell spreading in linbro wells was FN-dependent with maximal cell spreading occurring at a coating concentration of between 1-3 μ g/ml FN (Fig. 2.7). In the absence of FN a low level of spreading was observed (approx. 10-15%) (Fig. 2.7 and 2.8,E). Incubation of the FN-coated substrata (1 μ g/ml) with 500 μ g/ml bovine brain mixed gangliosides reduced cell spreading to less than 20%, however, at a higher FN coating concentration (5 μ g/ml) the ganglioside apparently had little effect on the number of cells spread (Fig. 2.8 and Table 2.3,B). The morphology of cells spread in ganglioside-containing medium (Fig. 2.8,D) was however slightly different from that in the absence of ganglioside. The significance of this observation is unclear at present. A detailed examination of the concentration dependence of ganglioside inhibition of BHK cell spreading by specific ganglioside is shown in Fig. 2.9. As with CHO cell spreading, GD and GT were consistently the best inhibitors of BHK cell spreading. The concentration of gangliosides GD and GT needed to produce 50% inhibition of spreading on substrates coated with 2 μ g/ml FN were approximately 10 and 35 μ M respectively. However, in some experiments the difference between the activity of GD and GT was less apparent. Similar variability in the inhibition of FN-dependent CHO cell attachment by gangliosides GD_{1a} and GT has previously been reported (Kleinman et al., 1979a). Monosialogangliosides GM₃ and GM₁ were less effective inhibitors of BHK cell spreading than GD and GT and inhibition was incomplete (73%) even at high ganglioside concentrations (200 μ M). As with the CHO cell spreading assay it was important to demonstrate that the ganglioside effect was due to interaction with the FN substrate and not with the cells. Removal of unbound gangliosides from FN-coated wells only partially removed the inhibitory activity (Table 3.3,A) and,

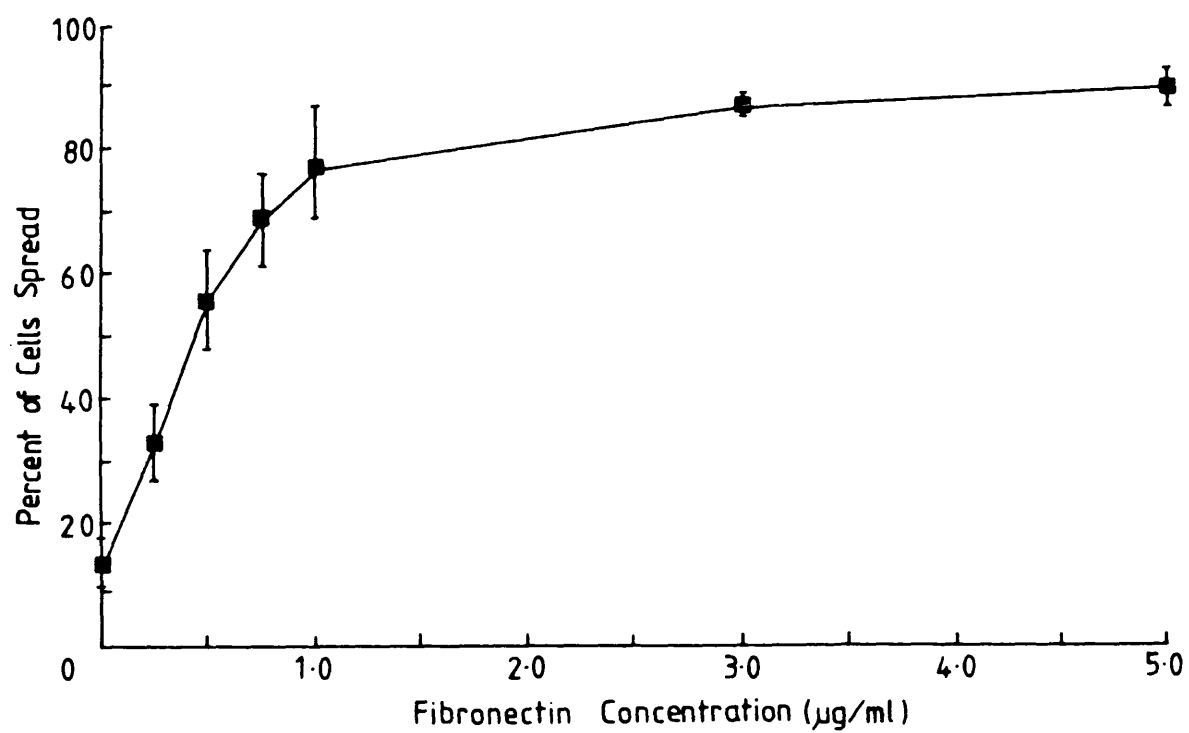
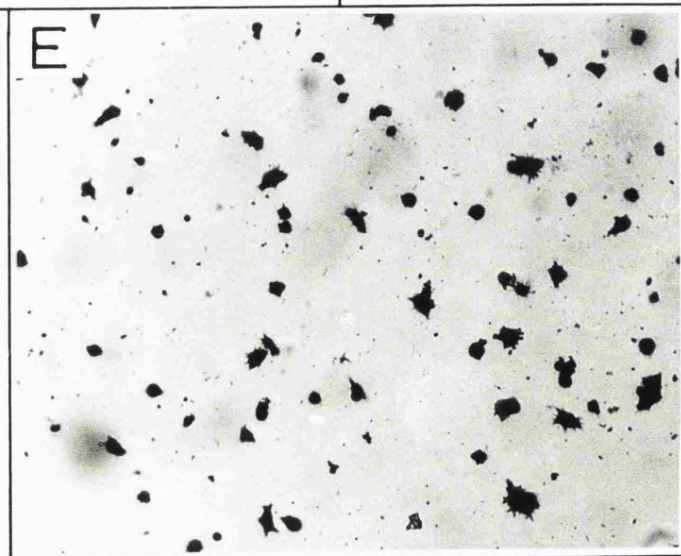
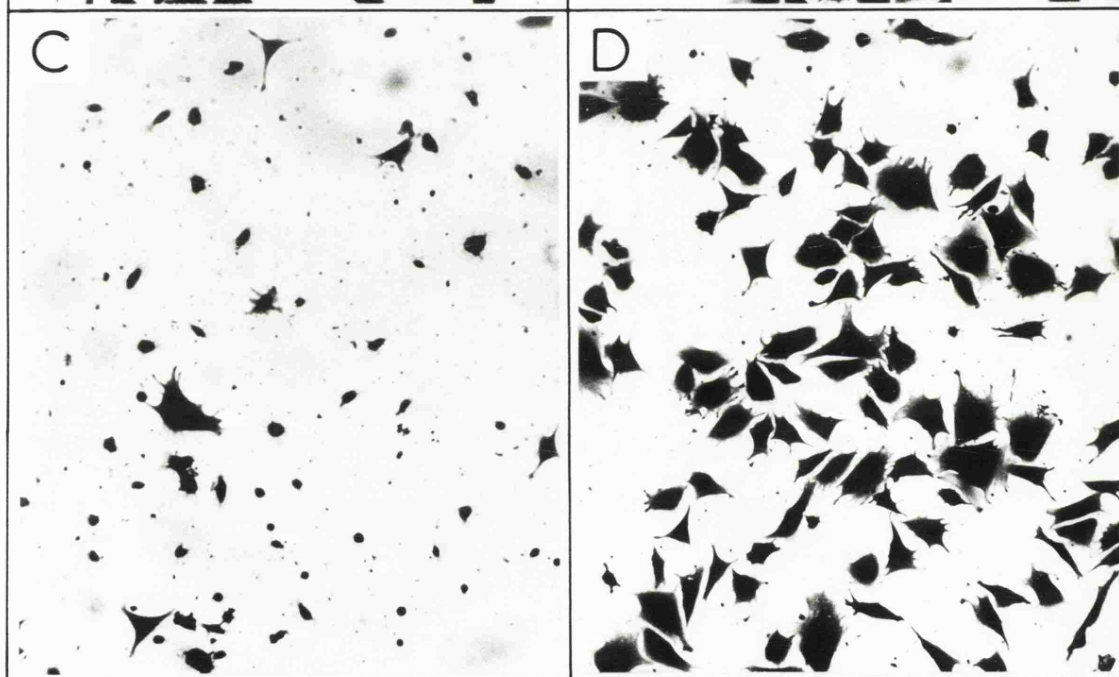
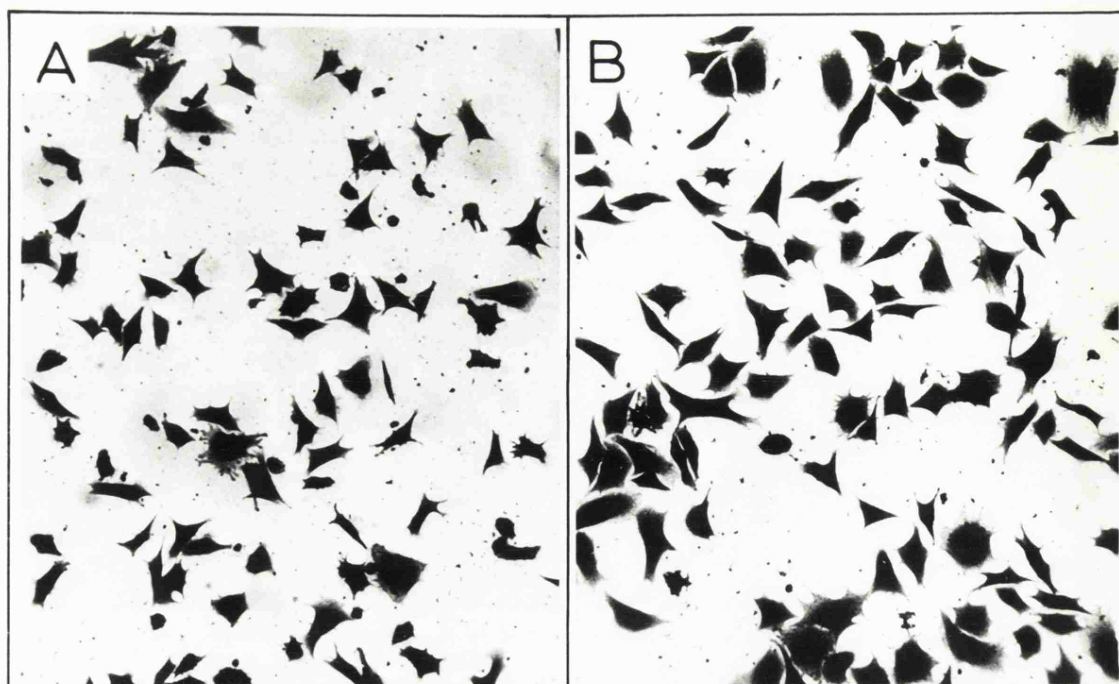


Fig. 2.7 Fibronectin dependent BHK cell spreading;
concentration dependence

Linbro wells were coated with varying concentrations of FN in 250µl of PBS for 1h, 37°C. Unbound FN was then aspirated and replaced by 250µl of DMEM. 5×10^4 BHK cells were then added to each well and allowed to spread for 1h, 37°C. Medium was gently aspirated and cell spreading then quantitated as described in Materials and Methods.



100μm

Fig. 2.8 Ganglioside inhibition of BHK cell spreading -
effect of increasing the serum concentration

Linbro wells were coated with FN as indicated for 1h, 37°C. Unbound FN was aspirated and replaced with 250µl of DMEM +/- 500µg/ml bovine brain mixed ganglioside. After incubating for a further 1h, 37°C BHK cells (5×10^4) were then added in 100µl DMEM and allowed to spread for 1h, 37°C after which the cells were fixed, stained and photographed as described in Materials and Methods.

- A - 1µg/ml FN; no ganglioside
- B - 5µg/ml FN; no ganglioside
- C - 1µg/ml FN; 500µg/ml ganglioside
- D - 5µg/ml FN; 500µg/ml ganglioside
- E - No FN; no ganglioside

(A) Effect of removing unbound gangliosides

Incubation protocol	% Cell spreading	% Inhibition
1. No addition	9.0 \pm 1.2	-
2. FN/wash/DMEM	71.0 \pm 2.6	0
3. FN/wash/ganglioside	14.2 \pm 1.6	80.0
4. FN/wash/ganglioside/wash	45.5 \pm 6.1	35.9

(B) Effect of increasing FN concentration

Incubation protocol	% Cell spreading	% Inhibition
1. 1 μ g/ml FN/wash/DMEM	82.6 \pm 3.3	0
2. 1 μ g/ml FN/wash/ganglioside	7.4 \pm 2.5	91
3. 5 μ g/ml FN/wash/DMEM	90.9 \pm 2.8	0
4. 5 μ g/ml FN/wash/ganglioside	74.5 \pm 4.9	18

Table 2.3 Inhibition of FN-dependent BHK cell spreading
by gangliosides. (A) Effect of removing unbound
gangliosides, (B) effect of increasing FN
concentrations.

Linbro wells were incubated for 1h, 37°C with each of the above additions (250µl/well) in the order shown. Where indicated wells were washed twice with DMEM prior to the next addition. 5×10^4 cells were then added to each well in DMEM and the percentage cell spreading determined after 1h, 37°C as described in Materials and Methods.

FN (3µg/ml) was added in PBS.

Gangliosides (mixed, bovine brain) were added in DMEM to a final concentration of 500µg/ml.

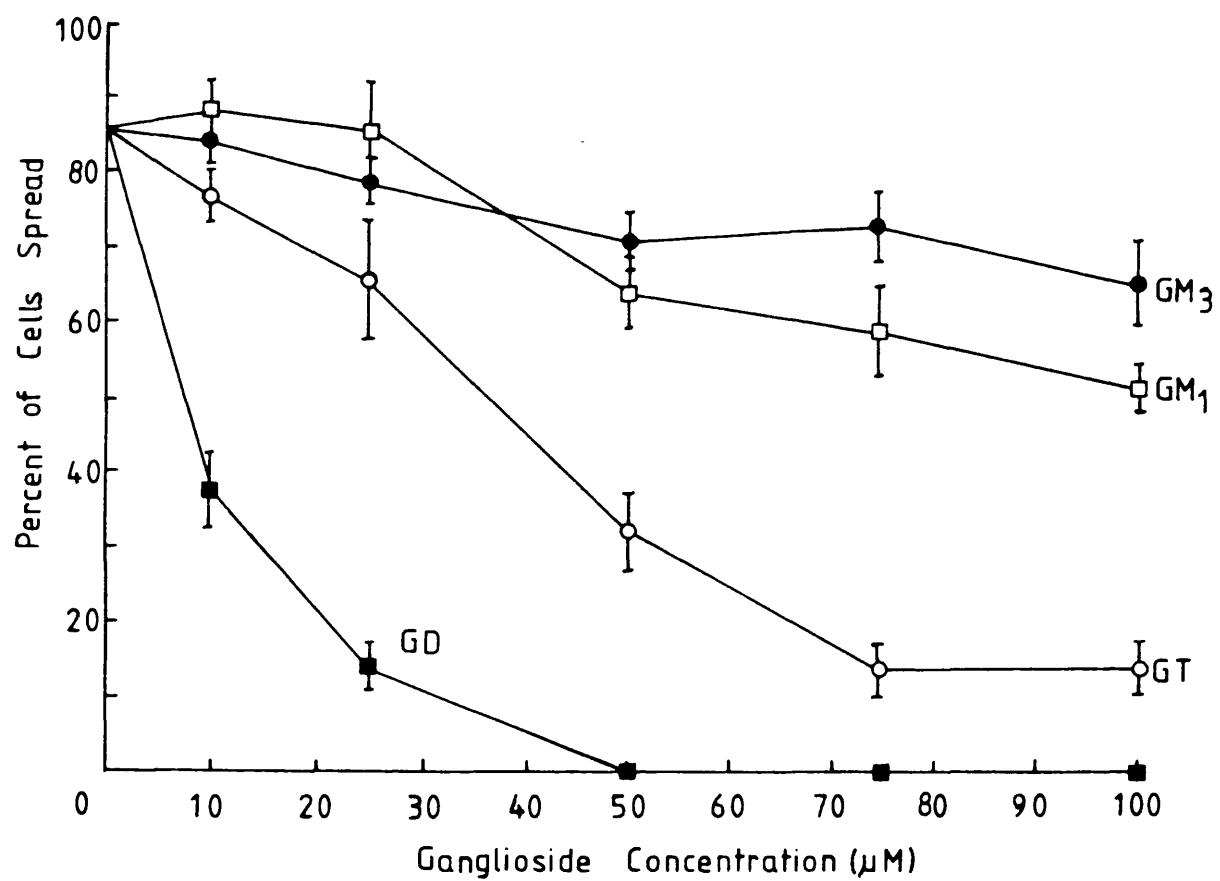


Fig. 2.9 Inhibition of FN-mediated BHK cell spreading by
specific gangliosides - concentration dependence

Linbro wells were coated with 2 μ g/ml FN for 1h, 37 $^{\circ}$ C. Unbound FN was aspirated and replaced by 250 μ l DMEM containing specific gangliosides at varying concentrations for 1h, 37 $^{\circ}$ C prior to the addition of 5×10^4 BHK cells. Cell spreading was quantitated after 1h, 37 $^{\circ}$ C as described in Materials and Methods.

●—●, GM₃; □—□, GM₁; ■—■, GD; ○—○, GT.

similar to CHO cell spreading, pretreatment of wells with gangliosides prior to coating with FN had little effect on subsequent cell spreading (data not shown). It was noted however, that the inhibitory activity remaining following removal of unbound gangliosides varied considerably between experiments. The reason for this variability is not understood although the levels of FN used appears to be important.

Binding of fibronectin to plastic-adsorbed gangliosides

Gangliosides adsorbed to plastic surfaces have been used to investigate the chemical nature of both the cholera and tetanus toxin receptors (Holmgren et al., 1980). We therefore attempted to demonstrate binding of radioiodinated FN to various gangliosides using this technique. However, we were only able to demonstrate trace levels of ^{125}I -labelled FN binding to tubes coated with high levels (200 μM) of trisialogangliosides (Table 2.4). Coating at higher concentrations up to 400 μM did not significantly increase binding. As a positive control for FN biological activity, tubes were coated with 1mg/ml gelatin and treated as for ganglioside coated tubes. In this case greater than 70% of the applied counts remained bound to the tube. In order to establish that the tubes had in fact been coated with gangliosides, control tubes were incubated with various gangliosides and their ability to support the binding of ^{125}I -labelled CT investigated. Ganglioside GM_1 support the binding of ^{125}I -labelled CT (Table 2.4), although little or no binding to other gangliosides was noted in agreement with the known specificity of CT for GM_1 . Subsequent experiments demonstrated that treatment of tubes coated with di- and tri-sialogangliosides with *Vibrio cholerae*

Ganglioside	Ligand binding (CPM/tube)	
	^{125}I -FN	^{125}I -CT
GM ₃ (2μM)	0	0
GM ₁ (2μM)	0	46132
GD (2μM)	0	67
GT (2μM)	39	0
GT (200μM)	251	ND

Table 2.4 Binding of ^{125}I -labelled FN to plastic-adsorbed
gangliosides

Fibronectin binding to polystyrene tubes coated with various gangliosides was determined using ^{125}I -labelled FN (90,000 CPM/tube, specific activity 2354 CPM/fmole). As a positive control for FN binding some tubes were coated with gelatin. Greater than 70% of the applied counts were bound.

To confirm the efficient binding of gangliosides to polystyrene and the specificity of the system ^{125}I -labelled CT (200,000 CPM/tube, 40nM concentration) was added to ganglioside-coated tubes. Non-specific binding to tubes not coated with ligand has, in all cases, been subtracted from the total counts bound.

neuraminidase (0.02 Units/ml) increased CT binding in these tubes to values similar to that for GM₁ indicating that other gangliosides are equally well absorbed to these tubes. Thus, although it was established that gangliosides were bound to the tubes and that they could support the binding of ¹²⁵I-labelled CT only limited binding of ¹²⁵I-FN could be determined. Similarly, when gangliosides were separated by thin layer chromatography and the plates overlayed with iodinated ligand as described by Magnani et al (1982) ¹²⁵I-labelled CT binding to GM₁ was clearly demonstrable although we failed to detect binding of ¹²⁵I-labelled FN to any of the gangliosides tested (data not shown).

Binding of unlabelled FN to gangliosides was reproducibly demonstrated using a rabbit antiserum to FN and ¹²⁵I-labelled protein A as the detection system (Table 2.5). As in experiments using ¹²⁵I-labelled FN, binding could only be demonstrated when tubes were coated with high levels of ganglioside (>200μM) and even then only low levels of binding were observed. Binding of FN to di- and tri-sialogangliosides was consistently higher than to the monosialogangliosides GM₁ and GM₃, although the specificity for the former pair appeared to be variable. Gelatin coated tubes were used as a control for FN binding and high levels of bound FN could be demonstrated in contrast to the low levels seen on ganglioside-coated tubes, cf. 39610 and 3004 CPM of ¹²⁵I-labelled protein A to gelatin- and ganglioside-coated tubes respectively. Similarly, high levels of CT binding could be demonstrated to tubes coated with GM₁ (Table 2.5). Only background levels of bound radioactivity could be detected when rabbit preimmune serum was used to replace either anti-FN or anti-CT antisera.

Immobilised ganglioside	Binding assay	¹²⁵ I-labelled Protein A bound (CPM)	
		A) 2μM Ganglioside	B) 200μM Ganglioside
GM ₃	Fibronectin	0	392
GM ₁	"	0	935
GD	"	0	3004
GT	"	0	1367
GM ₁	Cholera toxin	97039	38732

Table 2.5 Demonstration of the binding of FN to gangliosides
using a solid phase radioimmune assay

Binding of fibronectin (114nM) to polystyrene tubes coated with various gangliosides (2 μ M and 200 μ M) was determined using a rabbit anti-fibronectin antiserum (diluted 1:30 in PBS) and 125 I-labelled protein A (specific activity 350CPM/fmole, 140,000CPM/tube in experiment A; 216CPM/fmole, 110,000CPM/tube in experiment B). As a positive control for binding, tubes were coated with 1mg/ml of gelatin, and fibronectin binding assayed as above. The CPM of 125 I-labelled protein A bound were 73693 (experiment A) and 39610 (experiment B). To establish that gangliosides were adsorbed efficiently to the tubes, cholera toxin (40nM) binding was assayed using a rabbit anti-cholera toxin antiserum and 125 I-labelled protein A.

In all cases, non-specific binding of 125 I-labelled protein A to tubes not coated with gangliosides has been subtracted (1010CPM) from the total counts bound. The results are expressed as the mean of duplicate determinations differing from the mean by <10%.

Ganglioside inhibition of ConA-mediated BHK cell spreading

In order to demonstrate that the ability of gangliosides to inhibit cell spreading was specific for the FN-mediated process we investigated the effect of gangliosides on ConA-mediated cell spreading. A number of workers have shown that the lectin ConA can mediate BHK cell spreading due to its ability to bind to mannose-containing cell surface glycoconjugates (Grinnell *et al.*, 1978; Hughes *et al.*, 1979). Here we demonstrate that coating of linbro wells with ConA induces BHK cell spreading in a concentration dependent manner with maximal spreading occurring at approximately 100µg/ml ConA (Fig. 2.10). Cells spread on ConA coated wells (100µg/ml) were morphologically indistinguishable from cells induced to spread by FN (compare Fig. 2.8,B with Fig. 2.11,B). Incubation of the ConA-coated wells with 500µg/ml bovine brain mixed gangliosides prior to the direct addition of the cells caused a dramatic reduction in the numbers of cells spread (Fig. 2.11,C and Table 2.6). However, if the ganglioside-containing medium was removed and wells washed prior to the addition of the cells the inhibitory activity was lost (Table 2.6). The magnitude of the ganglioside effect was shown to be dependent on the ConA coating concentrations (Table 2.6). Thus, raising the coating concentrations from 25µg/ml to 1mg/ml caused a reduction in the inhibitory activity from 70.6% to 20.0%. As with similar data on the inhibition of FN spreading this argues against a direct cytotoxic effect of gangliosides on the cells being responsible for the inhibitory effect.

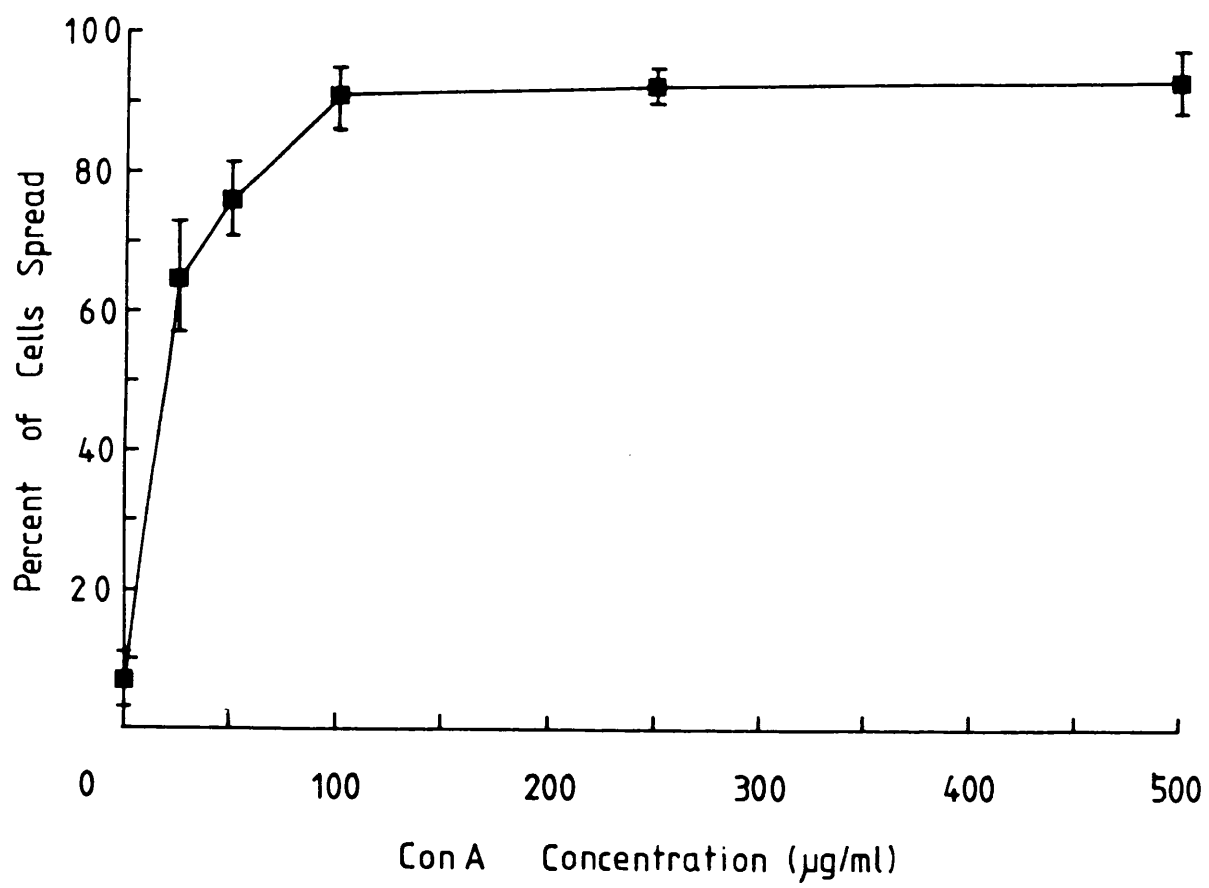


Fig. 2.10 BHK cell spreading on ConA-coated substrata -
concentration dependence

Linbro wells were coated with varying concentrations of ConA in PBS (250µl/well) for 1h, 37°C. Wells were aspirated and washed once with PBS prior to the addition of 5×10^4 freshly trypsinised BHK cells in DMEM. Cells were allowed to spread for 1h, 37°C and cell spreading quantitated as for FN-induced cell spreading.

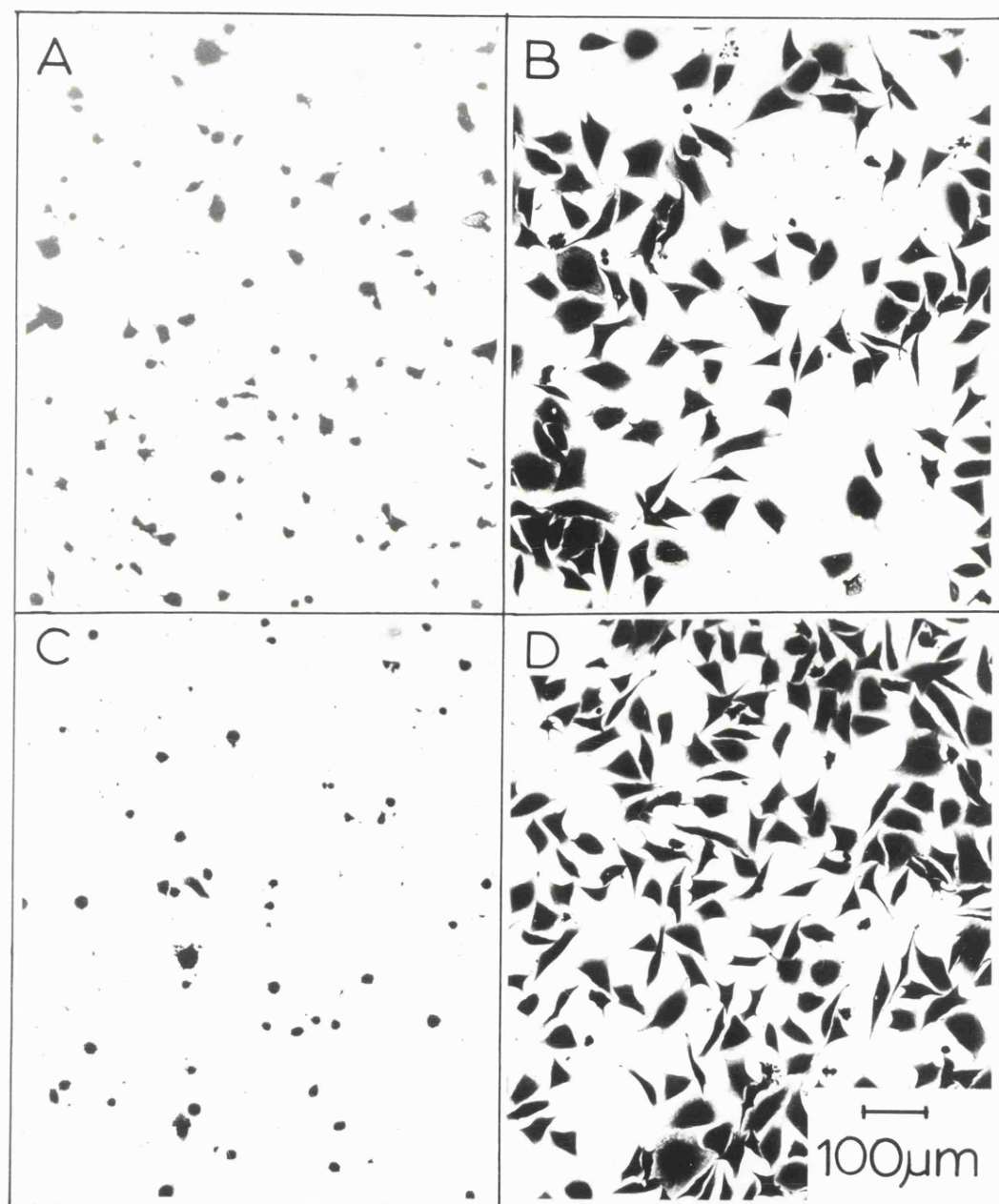


Fig. 2.11 Ganglioside inhibition of ConA mediated BHK cell
spreading

Linbro wells were coated with 100µg/ml ConA in PBS (250µl/well) for 1h, 37°C. Unbound ConA was then aspirated and replaced by DMEM +/- 500µg/ml bovine brain mixed ganglioside (250µl/well). Trypsinised BHK cells (5×10^4) were added to each well and allowed to spread for 1h, 37°C. Cells were then fixed and stained as described in Materials and Methods.

- A - No ConA; no ganglioside; cells
- B - 100µg/ml ConA; no ganglioside; cells
- C - 100µg/ml ConA; 500µg/ml ganglioside; cells
- D - 100µg/ml ConA; 500µg/ml ganglioside; wash; cells.

Incubation protocol	% Cell spreading	% Inhibition
---------------------	------------------	--------------

(A)

1. No additions	8.4 \pm 0.9	-
2. ConA/wash/DMEM	76.9 \pm 4.1	0
3. ConA/wash/ganglioside	24.2 \pm 3.0	68.5
4. ConA/wash/ganglioside/wash	71.8 \pm 4.4	6.6

(B)

1. 25 μ g/ml ConA/wash/DMEM	68.3 \pm 5.4	0
2. 25 μ g/ml ConA/wash/ganglioside	20.1 \pm 6.2	70.6
3. 1mg/ml ConA/wash/DMEM	80.4 \pm 4.3	0
4. 1mg/ml ConA/wash/ganglioside	64.3 \pm 3.4	20.0

Table 2.6 Inhibition of ConA-mediated BHK cell spreading by
gangliosides

- (A) Effect of removing unbound ganglioside.
- (B) Effect of increasing ConA concentration.

Linbro wells were incubated with ConA in PBS (100µg/ml in (A)) for 1h, 37°C, unbound material removed by washing once with PBS and further incubated with DMEM +/- 500µg/ml bovine brain mixed gangliosides for 1h, 37°C. Trypsinised BHK cells (5×10^4) were then added directly and the degree of cell spreading quantitated after 1 h, 37°C as outlined in Materials and Methods. Where indicated, wells were washed twice with DMEM prior to the addition of cells.

A detailed examination of the ganglioside specificity of the inhibitory activity is shown in Fig. 2.12. Linbro wells were coated with 200µg/ml ConA, unbound material removed by washing and then further incubated with increasing concentrations of specific gangliosides in DMEM. BHK cells were then added to the ganglioside containing medium and allowed to spread for 1h, 37°C. Although a relatively high coating concentration of ConA was used (200µg/ml) only 70% cell spreading was achieved in the absence of gangliosides. The reason for this low level of cell spreading is thought to be due to shedding of substratum-adsorbed ConA into the medium during the incubation of wells with ganglioside. The poor binding of ConA to plastic compared to FN has previously been reported by Hughes et al. (1979). Even at higher coating concentrations the level of maximal cell spreading did not exceed 70%. Disialogangliosides were the most effective inhibitors of ConA-mediated cell spreading with a 50% inhibitory concentration of approximately 7µM and complete inhibition occurring at a concentration of 25µM. In contrast to their ability to inhibit FN-mediated spread, trisialogangliosides were only poorly inhibitory in the ConA spreading assay (50% inhibitory concentration approximately 100µM) and were no more effective than the monosialogangliosides, GM₁ and GM₃. Although considerable variation was observed in the level of cell spreading following ganglioside treatment disialogangliosides reproducibility produced a greater inhibitory effect than either the tri- or monosialogangliosides.

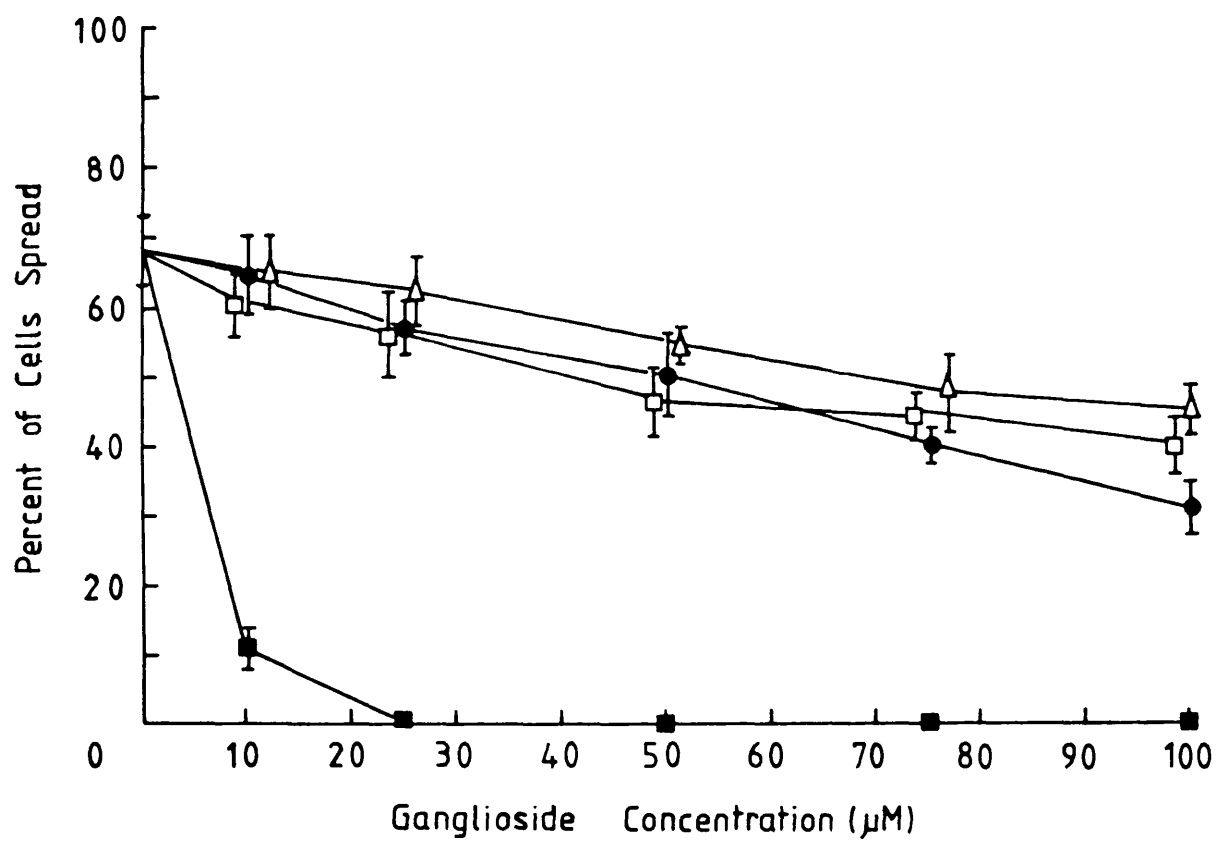


Fig. 2.12 Inhibition of ConA-mediated BHK cell spreading
by specific gangliosides; concentration dependence

Linbro wells were coated with 200µg/ml ConA in PBS for 1h, 37°C, unbound material removed by washing once with PBS and further incubated with specific gangliosides (isolated from human brain) in DMEM for 1h, 37°C. Freshly trypsinised BHK cells (5×10^4) were then added and cell spreading scored after 1h, 37°C.

□—□, GM₁; △—△, GM₃; ●—●, GT; ■—■, GD.

DISCUSSION

At the outset of this project it had recently been demonstrated that di- and tri-sialogangliosides were capable of inhibiting the FN-mediated attachment of CHO cells to collagenous substrata and may thus act as cell surface receptors for FN (Kleinman et al., 1979a). It was important therefore to confirm this report and to investigate whether gangliosides were capable of inhibiting other FN-mediated activities.

Attempts to demonstrate FN-dependent CHO cell attachment to collagen-coated dishes met with two main problems.

i) High levels of CHO cell attachment to collagen in the absence of serum or FN.

ii) Maximal cell attachment in the presence of serum or FN rarely exceeded 50% of the total number of applied cells.

As a result of the above difficulties the attachment assay, in our hands at least, was rather insensitive with as little as a 3-fold promotion of cell attachment by FN often resulting. The cause of these problems is not known, although the preparation of the collagen substrate has been shown to be an important factor in this assay (Kleinman et al., (1979c). The coating of the plastic substratum with collagen is a key step in this assay as it serves to prevent the non-specific attachment of cells to the plastic. In our hands however, considerable cell attachment to the collagen-coated dishes occurred in the absence of FN.

Treatment of the collagen with 8M urea as described previously (Klebe, 1974; Pearlstein, 1976) or the use of bacteriological as opposed to tissue culture dishes did not significantly reduce the levels of FN-independent attachment. Similar results were also obtained using rat tail collagen as described by Kleinman et al. (1979c, data not shown). It has been shown that if high levels of phosphate (above 0.01M) are present during the preparation of the collagen substrate cell attachment, but not cell spreading may occur without FN (Kleinman et al., 1979c). Although care was taken to avoid phosphate-containing buffers during our substrate preparations other, as yet unknown agents may be responsible for the high levels of FN-independent attachment. The demonstration of Harper and Juliano (1981) that CHO cells possess two distinct mechanisms of adhesion further supports the presence of FN-independent adhesion mechanisms in these cells. Attempts to demonstrate ganglioside inhibition of CHO cell attachment to collagen were only partially successful in that although inhibition was observed it was far less than that previously reported (Kleinman et al., 1979a). Cell spreading however proved extremely sensitive to the presence of gangliosides. It seems likely therefore, that the lack of sensitivity of attachment to ganglioside inhibition is due to a proportion of the cells attaching to the substratum by a FN-independent mechanism. If this mode of attachment were insensitive to gangliosides it would explain why only partial inhibition of cell attachment is observed in our assay. As this attachment is not followed by cell spreading in the absence of FN it may be non-physiological and may represent passive adhesion of cells to the substratum. Such adhesion has been shown to be independent of temperature, pH, cellular integrity and cell fixation (Grinnell, 1978).

In contrast to cell attachment to collagen-coated substrata, cell spreading was clearly dependent on the presence of FN. It was found that CHO cells spread on plastic surfaces in a FN-dependent manner without prior treatment of the substrata with collagen. An assay for FN biological activity was thus developed involving CHO cell spreading on plastic linbro wells. Maximal cell spreading occurred at 2.0µg/ml FN with less than 10% cell spreading in the absence of FN. Gangliosides inhibited CHO cell spreading in a similar manner to that previously described for cell attachment (Kleinman et al., 1979a). At a FN coating concentration of 3µg/ml, bovine brain mixed gangliosides produced a 50% reduction in cell spreading at a concentration of approximately 100µM. This is four times less than that required to inhibit CHO cell attachment suggesting a greater sensitivity of cell spreading to gangliosides than cell attachment. Di- and tri-sialogangliosides were the most effective inhibitors of cell spreading with the monosialoganglioside GM₁ showing little inhibition at a concentration of 200µM. That ganglioside inhibition was not due to any obvious direct effect of gangliosides on the cells was shown by, (a) inhibition could be overcome by increasing the concentration of FN used to coat the substrata and (b) removal of unbound ganglioside from the FN-coated substrata prior to the addition of the cells only partially relieved the inhibition. The above data is in agreement with that previously described for CHO cell attachment to collagen-coated substrata (Kleinman et al., 1979a).

Following confirmation of the ability of gangliosides, in particular GD1_a and GT, to block the interaction of FN with CHO cells, apparently through a direct interaction with substrate-bound FN, it was important to

know whether gangliosides were capable of disrupting other FN-mediated activities. It has previously been shown that BHK cell spreading on plastic is a sensitive bioassay for FN activity (Pena and Hughes, 1978). Here I have demonstrated FN-dependent BHK cell spreading using a linbro plate assay. Maximal cell spreading was achieved at a FN coating concentration of 1-3 μ g/ml, similar to that described by Pena and Hughes (1978). Investigation of the effects of gangliosides on this system clearly shows that gangliosides can inhibit BHK cell spreading in a similar manner to that shown for CHO cell spreading. Thus:-

i) 500 μ g/ml mixed gangliosides inhibited spreading (1 μ g/ml FN) by greater than 80% and this inhibition could be overcome by increasing the coating levels of FN to 5 μ g/ml.

ii) di- and tri-sialogangliosides were consistently the best inhibitors of cell spreading, 50% inhibition at 10 and 35 μ M respectively.

iii) removal of unbound gangliosides from FN-coated wells only partially removed the inhibitory activity.

The demonstration that di- and tri-sialogangliosides were the best inhibitor of both CHO and BHK cell spreading clearly indicates these molecules are potential receptors for FN. However, it is possible that some, as yet uncharacterised ganglioside species is capable of inhibiting cell spreading at far lower levels than that required for GD and GT. Therefore, it is important to know if BHK or CHO cells contain novel gangliosides capable of inhibiting FN-mediated spreading at lower

concentrations than that demonstrated with bovine brain gangliosides. If such a species exists in BHK cells it apparently binds to FN with low affinity since ^{125}I -labelled FN failed to bind to any BHK cell gangliosides separated by TLC using the Magnani overlay technique (data not shown). Subsequent to the completion of this work Yamada et al. (1981) similarly demonstrated inhibition of BHK cell spreading by gangliosides. GT1_b and GD1_a were the best inhibitors with half maximal inhibition at concentrations of 10 and $40\mu\text{M}$ respectively. In addition, it was shown that gangliosides were capable of inhibiting FN-mediated haemagglutination as well as the ability of FN to restore a normal morphology to transformed cells. Other carbohydrate containing molecules tested, including hyaluronic acid, heparan sulphate, ovomucoid, transferrin as well as free sialic acid, were inactive. In addition, glycopeptides obtained from the surface of BHK cells were also without effect at the concentration tested (1mg/ml). However, certain negatively charged phospholipids were inhibitory although they were an order of magnitude less effective than gangliosides.

A criticism of the interpretation that ganglioside inhibition of FN mediated cell spreading is due to receptor competition is the relatively high levels of gangliosides required to produce such an effect. For example, Rauvala et al. (1981) has shown that attachment of Balb/c 3T3 cells to FN-coated surfaces was inhibited by relatively high concentrations of GT1_b ($0.25\text{--}0.5\text{mM}$) whereas substantially lower concentrations were required to inhibit adhesion to a sialic acid-specific lectin or *Clostridium perfringens* sialidase ($0.05\text{--}0.1\text{mM}$). In addition, 3T3 cell adhesion to CT-coated substrata is inhibited by

ganglioside GM₁ at concentrations as low as 25-100nM (see Chapter 3). It is possible however that this simply reflects a low affinity of FN for gangliosides.

In an attempt to obtain direct evidence of ganglioside-FN interactions we investigated the ability of FN to bind to plastic absorbed gangliosides. Similar methodology has previously been used to investigate the ganglioside nature of the cholera toxin and tetanus toxin receptors (Holmgren et al., 1980). Although specific binding of ¹²⁵I-labelled CT to GM₁-coated tubes was clearly demonstrated only relatively poor binding of ¹²⁵I-labelled FN to gangliosides was evident. FN binding could only be demonstrated in tubes coated with high levels of GT (200μM) and then less than 0.25% of the applied counts were bound. In addition, it was not possible to demonstrate binding of ¹²⁵I-labelled FN to gangliosides separated on plastic backed TLC plates although binding of ¹²⁵I-labelled CT to ganglioside GM₁ was clearly seen (data not shown). It is possible that the low levels of FN binding were due to inactivation of the protein during radioiodination. However, a number of points argue against this.

i) Extremely mild iodination conditions were used to label the FN, i.e. enzymebeads, so that the protein was not exposed to high concentrations of oxidising or reducing agents.

ii) The protein appeared to be largely undegraded as judged by SDS-PAGE and autoradiography.

iii) Greater than 70% of the labelled FN applied to gelatin-coated tubes was specifically bound.

However, the possibility that the radiolabelling procedure disrupted FN binding to gangliosides without effecting its gelatin-binding ability cannot be excluded. It was not possible to test radiolabelled FN for activity in a cell spreading assay due to the low concentration of labelled protein and also the presence of carrier BSA in the labelled protein preparation. The carrier BSA would compete with the FN for binding to the substratum thereby further lowering the effective concentration of FN.

In an attempt to resolve this possibility a radioimmune assay was developed whereby bound FN was detected using a rabbit antiserum to FN and ^{125}I -labelled protein A. Using this system it was possible to reproducibly demonstrate FN binding to di- and tri-sialogangliosides and to a lesser extent to the mono-sialogangliosides GM_1 and GM_3 , in agreement with the relative effectiveness of these gangliosides to inhibit cell spreading. Because of the qualitative nature of the radioimmune assay used the results do not allow a comparison to be made between the relative binding affinities of FN and CT for gangliosides. However, the fact that we were unable to convincingly demonstrate binding of ^{125}I -labelled FN to ganglioside-coated tubes under conditions where we could readily detect binding of ^{125}I -labelled CT suggests that the ligands bind to gangliosides with widely differing affinities. Similarly, the affinity of FN for gelatin appears to be far greater than for gangliosides. The low affinity of FN for gangliosides in this

system may reflect the low affinity of FN in solution for the cell surface. It has recently been proposed that limited proteolysis or binding of other extracellular matrix components such as heparan sulphate or collagen, significantly improves the ability of FN to bind to its receptor (Johansson and Hook, 1984). It was suggested that this was the result of an alteration in the conformation of the FN such that a previously cryptic cell binding domain became available for binding. It is thus of interest to know if such treatments are able to increase the binding of FN to ganglioside-coated tubes.

It has previously been demonstrated that mild periodate treatment of GD1_a abolished the inhibitory activity of this ganglioside in the CHO cell attachment assay (Kleinman et al., 1979a) suggesting that the sialic acid is important in FN binding. However, it remains to be demonstrated that FN binding to GD1_a-coated tubes can be abolished by periodate or neuraminidase-treatment of the ganglioside. In addition, given the apparent low affinity of FN in solution for its cell surface receptor it is important to investigate the binding of labelled gangliosides to immobilised FN or FN-containing extracellular matrices. However, methodology for labelling gangliosides to high specific activity are generally lacking.

Although it has been shown that gangliosides inhibit CHO and BHK cell spreading on FN-coated substrates and that this inhibition apparently occurs via a low affinity interaction of gangliosides with FN it is possible that some inhibitory activity may result from a deleterious effect on the cell or the cytoskeleton. To test this we

investigated the effect of gangliosides on ConA-mediated cell spreading. BHK cell adhesion to ConA-coated substrata has previously been described (Grinnell et al., 1978; Hughes et al., 1979) and is thought to occur via the interaction of substrate-adsorbed ConA with mannose-containing glycoconjugates at the cell surface. As gangliosides do not contain mannose it is thought that they do not play a part in ConA-mediated cell adhesion.

In agreement with previous reports (Grinnell et al., 1978; Aplin and Hughes, 1981a) BHK cells plated onto ConA-coated substrata spread to a morphology indistinguishable from that induced by FN. In the presence of 500µg/ml mixed brain gangliosides this cell spreading was inhibited by 68.5% and was accompanied by a moderate reduction in cell adhesion (not quantitated). An investigation of the ganglioside specificity of inhibition demonstrated that, as for FN-coated substrata, GD was the most effective inhibitor. The 50% inhibitory concentration of GD for ConA-mediated spreading was 5-10µM a similar concentration to that required for FN-mediated spreading (10µM). Trisialogangliosides were far less effective inhibitors of ConA-mediated spreading than for FN-mediated spreading with 50% inhibitory concentrations of 100 and 35µM respectively. This level of inhibition was similar to that produced by the monosialogangliosides GM₁ and GM₃. The significance of the difference in inhibitory activity displayed by trisialogangliosides on the two substrata is unclear at present. Although considerable variation in the levels of inhibition were observed between experiments the inhibitory specificity GD > GT = GM₃ = GM₁ was consistently observed. In addition to the apparent difference in inhibitory activity of

trisialogangliosides towards ConA- and FN-mediated BHK cell spreading, gangliosides also displayed differing inhibitory activities in experiments where unbound gangliosides were removed from the substratum prior to the addition of cells. In the case of FN-coated substrata a proportion of the inhibitory activity remained following the removal of unbound material. However, on ConA-coated substrata the inhibitory activity was completely removed by such treatment. This may be interpreted in one of two ways.

i) Gangliosides inhibit ConA-mediated spreading by interacting with the substrate-bound ligand but the interaction is of such low affinity that it is not resistant to washing.

ii) Gangliosides inhibit ConA-mediated spreading via a direct interaction with the cell surface. However, this does not represent direct cytotoxicity since raising the concentration of ConA relieves the inhibitory activity.

At present it is not known which of the above interpretations are correct, however, the fact that gangliosides do not contain mannose and are known to incorporate into membranes when cells are placed in ganglioside-containing medium (Fishman, Moss and Vaughan, 1976) supports the latter. In order to satisfactorily resolve the issue it will be necessary to determine directly whether or not ConA can bind to gangliosides. It is interesting to note that Yamada et al. (1981), also investigating the ability of gangliosides to block FN-mediated functions, failed to demonstrate any effect of gangliosides on ConA-

mediated BHK cell spreading. Although it is not possible to explain this discrepancy with certainty it seems likely that the lack of inhibitory activity was related to the relatively high levels of ConA used to coat the substrata (1mg/ml). We clearly show that the inhibitory activity displayed by gangliosides is markedly reduced by raising the coating concentration of ConA from 100µg/ml to 1mg/ml.

In summary, we have demonstrated that gangliosides, in particular di- and tri-sialogangliosides, are capable of inhibiting both CHO and BHK cell spreading on FN-coated substrata in a similar manner to that previously described for FN-mediated CHO cell adhesion. We also provide direct evidence of FN binding to gangliosides although the levels of binding were at least an order of magnitude less than binding of FN to gelatin or CT to GM₁. However, the finding that gangliosides inhibit BHK cell spreading on ConA-coated substrata seriously questions the specificity of the inhibitory effect of gangliosides towards FN-mediated functions. This is further supported by the finding that gangliosides inhibit cell adhesion to gelatin- and soybean agglutinin-coated substrata at similar concentrations to that required to inhibit FN-mediated cell adhesion (Rauvala et al., 1981).

CHAPTER 3

A COMPARISON OF CELL SPREADING ON SUBSTRATA COATED WITH FN, CT AND CON A

INTRODUCTION

A major criticism of the proposed role of gangliosides as cell surface receptors for FN is that being localised to the outer leaflet of the plasma membrane (Hakomori, 1981; Luiza, Barbosa and de Silva, 1983), it is difficult to see how they might mediate the transmembrane interaction of FN with the cytoskeleton. Such an interaction is thought to be essential in FN-mediated cell adhesion (Hynes, 1981). Given this criticism it is important to establish whether the interaction of FN with a cell surface ganglioside could support cell adhesion. We have thus examined the ability of CT, a ganglioside-specific ligand, to mediate cell attachment and spreading.

The interaction of a cell with the underlying substratum is a complex phenomenon which has been divided into four main components (see review by Grinnell, 1978).

1. The nature of the substratum and adsorption of proteins

The adhesive capacity of a given substratum is dependent on a number of physical and chemical characteristics. For example, bacteriological grade polystyrene petri dishes have a low substrate wettability and do not generally support cell adhesion. However, sulphuric acid treatment (Martin and Rubin, 1974) or exposure to a coronal discharge (Amstein and Hartman, 1975) converts this surface into a substratum capable of mediating cell attachment under tissue culture conditions. The addition of cells to a plastic substratum in serum-free

medium generally results in rapid and complete cell adhesion. Such adhesion has however been shown to be independent of temperature, pH, metabolic activity and cell viability and is termed passive adhesion (Taylor, 1961). This process probably represents the direct adsorption of cells to the substratum at sites to which serum proteins are bound under normal culturing conditions. Thus, in serum-containing medium passive adhesion is blocked and active cell adhesion results due to the adsorption of specific adhesion factors onto the substratum.

ii) Cell contact with the substratum

In general an electrostatic barrier exists between a cell and the substratum due to the net negative charge of both the cell surface at physiological pH and the substratum under many conditions. Theoretical considerations suggest that cellular microextensions might facilitate initial cell contact by penetrating this electrostatic barrier (Grinnell, 1978). This has been supported by light- and electron-microscopic studies demonstrating that microvilli are involved in initial contact with the substratum and that larger structures, termed filopodia, are involved in initiating the subsequent cell spreading events (Grinnell, 1978).

iii) Formation of bonds of attachment

Following cell contact with the substratum new bonds of attachment are formed by the extension of filopodial structures from the base of the cell. The filopodia form new distant points of adhesion, presumably by specific ligand-receptor interactions, and thus increase the net

adhesive bond between the cell and the substratum.

iv) Cell spreading

The movement of cytoplasm along and between the filopodial extensions of the main cell body result in broad lamellapodia and a gradual transition from a rounded to a flattened, well spread morphology. Cell spreading is an active, Ca^{2+} -dependent process thought to be brought about by specific cytoskeletal components, namely the actin-containing microfilament system. Disruption of these structures by cytochalasin B effectively inhibits the spreading process (Nath and Srere, 1977). The microtubule system does not appear to be directly involved in cell spreading although it may be important in cellular polarisation (Grinnell, 1974).

Following the initial spreading response whereby a cell has reached a well spread morphology further specialised adhesive structures may develop (Izzard and Lochner, 1976). One such structure is the focal contact or focal adhesion plaque. These small punctate or arrowhead shaped regions represent areas of intimate approach between the ventral cell surface and the substratum (10-15nm) and correspond to sites at which microfilament bundles attach to the plasma membrane. The cytoskeletal protein vinculin has been shown to be concentrated on the cytoplasmic surface of focal contacts and may play a key role in linking the microfilament system to the plasma membrane (Geiger, Tokuyasu, Dutton and Singer, 1980). The presence of FN on the extracellular surface of focal contacts and its ability to promote the formation of

such structures is the subject of some debate at present. Focal contacts in rat dermal fibroblasts have been reported to contain FN (Rees et al., 1978) and coincident localisation of actin, FN, vinculin and focal contacts has been reported in NIL 8 fibroblasts (Singer, 1982). However, other workers have reported the absence (Chen and Singer, 1980) and even the active removal of FN from focal contacts (Avnur and Geiger, 1981). Focal contacts are thought to be involved in the stable adhesion of cells to the substratum and are less evident in actively moving cells (Kolega, Shure, Chen and Young, 1982). A second type of adhesion site, termed close contacts, are characterised by a set of broader areas where the cell surface is further separated from the substratum than in focal contacts (30nm). These structures appear to be important in cell-substratum adhesion related to cell motility (Kolega et al., 1982).

FN is one of the best characterised adhesion factors to date although it is by no means unique. Cell adhesion can be mediated by many ligands which display an affinity for the cell surface, e.g. lectins, glycosidases, antibodies directed at cell surface determinants (Grinnell and Hays, 1978; Carter et al., 1981), as well as other physiologically important adhesion factors such as laminin and chondronectin (Timpl et al., 1979; Hewitt, Kleinman, Pennypacker and Martin, 1980). Detailed analysis of cell adhesion to FN- and other ligand-coated substrata have identified a number of criteria which determine a cell's ability to spread on a given ligand (Aplin and Hughes, 1981a; Rauvala et al., 1981; Carter et al., 1981). These are:-

- i) The number of ligand receptors at the cell surface,
- ii) the concentration of substratum-bound ligand,
- and iii) the valency of the ligand.

An inverse relationship was shown to exist between receptor number and the ligand density required to trigger cell spreading (Aplin and Hughes, 1981a). Thus, a cell which is unable to spread on a given ligand might be induced to do so by raising the number of ligand receptors at the cell surface or alternatively, by increasing the density of substratum-bound ligand. That ligand valency might be important in cell spreading was indicated by data demonstrating that monovalent ConA, although able to promote cell attachment, was unable to induce the spreading response produced by native, tetravalent ConA (Carter et al., 1981). This indicates that cross-linking of cell surface receptors might play an important role in triggering cell spreading.

Carter et al. (1981) showed that although ligand-receptor interactions were important determinants in cell adhesion they alone are not sufficient to promote and maintain a stable adhesion response. It was proposed that following affinity-dependent binding of cell surface receptors to ligand-coated substrata, cell-dependent attachment and spreading processes are stimulated. These are essential for stable cell adhesion to occur and are independent of the stimulating adhesion surface. The capacity of a ligand to promote cell spreading may thus be dependent on its ability to promote the cell-dependent response in addition to the criteria outlined above.

In this study I have investigated the ability of Balb/c 3T3 cells to spread on a substratum coated with the ganglioside-specific ligand CT, and compared this response with that induced by FN and the glycoprotein-binding lectin, ConA. Cell spreading on ConA is of interest because it is thought to be mediated by mannose-containing glycoproteins at the cell surface and is not thought to involve gangliosides. Since glycoproteins as well as gangliosides have been postulated to act as receptors for FN during fibroblast adhesion, the ConA response forms an important comparison to cell spreading on CT and FN. In addition to characterising the morphological response of these cells to the three ligands the role of the cytoskeleton and focal contacts in generating the spread morphologies were also examined.

MATERIALS AND METHODS

Cell culture

Essentially as in Chapter 2. A subclone (S24) of Balb/c 3T3 A31 cells was used throughout this study and grown in DMEM/10% NCS supplemented with penicillin/streptomycin/amphotericin B. Subconfluent monolayers were routinely subcultured twice weekly using 0.25% trypsin. Balb/c 3T3 A31 cells were obtained from the Imperial Cancer Research Fund, London.

Attachment and spreading on plastic substrata

Dynatech microtitre wells (M129B) were incubated for 2h, RT^O, with 100µl of CT, toxin B subunit (Schwarz Mann and Sigma), ConA (Miles Yeda), or FN in PBS followed by 10mg/ml BSA/PBS to block non-specific cell attachment. To quantitate attachment, $2-4 \times 10^5$ trypsinised cells, labelled with [³⁵S] methionine (0.3µCi/ml) for 18h in methionine-free medium and washed in DMEM/1mM PMSF, were added to each well in 100µl of DMEM. After incubation for 2h, 37°C in a humidified CO₂ incubator, unattached cells were removed by gently washing with PBS. Attached cells were solubilised in 1M NaOH and counted in a toluene-based scintillant (4.5l toluene, 27 gms butyl BPD, 2.25l Fisons emulsifier No.1, 675ml water) on a Packard scintillation counter.

The ability of cells to spread on various ligands was examined in a similar manner except that between $1-4 \times 10^4$ unlabelled cells were

used. These were fixed with 3.8% formaldehyde/PBS for 30', RT⁰ and subsequently stained with crystal violet (BDH, 0.02% w/v) 10', RT⁰. Cells were either photographed following staining using a Leitz diavert microscope with Pan F film (50ASA) and an automatic exposure system or spreading quantitated as follows. The clearly different morphologies displayed by these cells on different ligands made it important to clearly define a spread cell so as to include the various spread morphologies. We have thus defined a cell as being "spread" if sufficient cell flattening has occurred to allow visualisation of nuclear outline in crystal violet stained cells. The number of spread cells in 3-6 fields of view (80-100 cells/field) were counted and expressed as a percentage of the total number of attached cells.

Where indicated cells were treated with 0.04units/ml *Vibrio cholerae* neuraminidase (Behringwerke) in DMEM for 1h, 37°C prior to trypsinisation and plating. Monolayers were washed twice with DMEM before enzyme treatment to remove serum since it is known to inhibit enzyme activity.

Radioiodination of ligands

ConA was radioiodinated as described by Burridge (1978). 50µg of ConA in 10µl PBS containing 0.23M α-methylmannoside, 5µl ¹²⁵I (240µCi) and 2.5µl aqueous chloramine T (5mg/ml) were incubated for 1.5', RT⁰ and the reaction terminated by the addition of 5µl of sodium metabisulphite (10mg/ml in PBS) followed by 50µl 0.1M sodium iodide/0.1M Tris pH 7.4, / 0.1% BSA. Iodinated lectin was separated from unreacted iodine and inhibitory saccharide ^{by} gel filtration on a Biogel P-10 column

equilibrated with PBS/0.1% BSA. The specific activity of the labelled lectin was 4 μ Ci/ μ g.

CT (Sigma) was iodinated using iodogen (Pierce). 10 μ g of iodogen (in 50 μ l of chloroform) was dried onto the surface of a small glass iodination tube. 10 μ l of toxin (0.5mg/ml), 55 μ l 0.2M phosphate buffer, pH 7.4 and 2.5 μ l $^{125}\text{I}^-$ (250 μ Ci, carrier free, Radiochemical Centre, Amersham) were then added and incubated for 15', RT^O. The reaction was terminated by the addition of 50 μ l of saturated tyrosine solution (aqueous) and transferring the reaction mix into 100 μ l of 0.1M Tris/0.25% gelatin, pH 7.4. Free $^{125}\text{I}^-$ was removed using a Sephadex G-25 column. Specific activities of 20 μ Ci/ μ g were obtained using this method.

FN was iodinated by a chloramine T procedure described by Ruoslahti et al. (1982). Briefly, 10 μ l of gelatin-Sepharose purified FN (1mg/ml) was mixed with 10 μ l of 1M phosphate buffer, pH 7.0, 1mCi of $^{125}\text{I}^-$ and 10 μ l of 4mg/ml chloramine T and incubated for 1', RT^O. The reaction was terminated with 10 μ l of sodium metabisulphite (2.4mg/ml) followed by the addition of 500 μ l of PBS/1mg/ml BSA. Free $^{125}\text{I}^-$ was then separated from labelled protein on a Sephadex G-25 column. A specific activity of 30 μ Ci/ μ g protein was obtained.

In all cases the efficiency of labelling was determined by taking 4 x 2 μ l aliquots from the reaction mix following iodination and determining the percentage of TCA precipitable counts.

Quantitation of ligand binding to substrata

Ligand binding to microtitre wells and glass coverslips was quantitated using ^{125}I -labelled ligands. Substrata were incubated with varying concentrations of ^{125}I -labelled ligand (constant specific activity) as described for cell spreading experiments and unbound material removed by extensive washing with PBS. The bases of the microtitre wells were removed with a hot scalpel and counted on a Beckman gamma counter together with ligand derivitised glass coverslips.

Derivitisation of glass coverslips

For immunofluorescence and interference reflection microscopy (IRM) cell spreading was carried out on 12mm diameter glass coverslips (Chance Proper) derivitised with ligands essentially as described by Aplin and Hughes (1981b). Coverslips were soaked overnight, RT° , in 20% H_2SO_4 , washed twice with distilled water and once with 0.1M NaOH. Excess NaOH was removed by blotting and coverslips transferred to a Pyrex crystallising dish where they were incubated with 3-aminopropyltriethyloxysilane (Sigma) for 4', RT° , ensuring complete coverage. After washing with excess water and PBS, coverslips were immersed in 0.25% glutaraldehyde/PBS for 45', RT° , and then further washed three times with PBS. 100 μl of protein in PBS was then added and allowed to couple for 1h, RT° in a humidified atmosphere followed by incubation with 10mg/ml BSA overnight, 4°C to block any unreacted sites and thereby preventing non-specific cell attachment and spreading. In order to monitor ligand binding coverslips were derivitised with radioiodinated

ligand and the counts/min remaining following three washes in PBS determined using a Beckman gamma counter. At a derivitisation concentration of 100µg/ml, 390fmoles FN, 1510fmoles CT and 1740fmoles ConA were bound per cm².

Effects of cytoskeletal disrupting agents on cell spreading

Microtitre wells were coated with ligand as described above and 2×10^4 trypsinised cells added to each well. Where indicated, cells were pretreated in suspension, with cytochalasin B (5µg/ml, Sigma) for 30', RT⁰. The effect of sodium azide on spreading was investigated by adding cells to wells containing azide such that the final concentration was 5mM. The stability of the spread morphologies to cytochalasin B (5µg/ml) and demicolcemid (Sigma, 10µg/ml) was investigated by applying these drugs to cells after being allowed to spread for 2h, 37⁰C. After a further 1.5h cells were fixed with 3.8% formaldehyde/PBS and stained with crystal violet.

Immunofluorescent staining of F-actin

Cells spread on derivitised glass coverslips were fixed with 3.8% formaldehyde/PBS for 30', RT⁰, and then permeabilised with 0.25% Triton X-100/PBS for 5', RT⁰. After washing gently three times with PBS F-actin was visualised by staining for 30', RT⁰, with 0.1µM NBD-phalloidin (7-nitrobenz-2-oxa-1,3-diazole phalloidin; Molecular Probes Inc., Texas) as previously described by Barak et al. (1980). Stock solution of NBD-phalloidin were stored in methanol at -20⁰C and

reconstituted in PBS prior to use. Coverslips were then washed three times with PBS (30' per wash) and mounted in 50% (v/v) glycerol/PBS. Stained cells were examined on a Zeiss Standard-16 microscope equipped with epifluorescence using filter set No. 9. Photographs were taken on Ilford HP5 35mm film (400ASA) uprated to 800ASA during developing. An exposure of 30secs was routinely used since after this time NBD-phalloidin staining was largely bleached out.

Visualisation of focal contacts

Two methods were used to look at specialisation of the underside of cells spread on various ligands, (A) a modified version of an antibody exclusion method previously described by Grinnell (1980) and (B) interference reflection microscopy (IRM) the standard technique for visualisation of focal and close contacts (Abercrombie and Dunn, 1975).

(A) Antibody exclusion technique. 12mm coverslips were derivitised with FN, CT or ConA as described above. Freshly trypsinised cells (10^4) were then added to each coverslip in 100 μ l of DMEM and allowed to attach for approximately 10-15', 37°C in a CO₂ incubator. Coverslips, in 6cm petri dishes, were gently flooded with DMEM and cells allowed to spread for 4h, 37°C. After gently washing once with PBS cells were fixed with 3.8% formaldehyde for 40', RT⁰ or overnight, 4°C and further washed three times with PBS to remove fixative. Coverslips were then incubated with a rabbit antiserum against the coupled ligand, diluted 1:100 in PBS (100 μ l/coverslip) for 40', RT⁰. After further washing with PBS cells were permeabilised with 0.25% Triton X-100/PBS for 5', RT⁰, washed twice

with PBS and F-actin visualised by staining for 30', RT⁰ with 0.1 μ M NBD-phalloidin as described above. Bound primary antibody was visualised with rhodamine-labelled goat anti-rabbit immunoglobulin (Nordic Ltd.) diluted 1:50 in PBS, at the same time as F-actin staining.

(B) Interference reflection microscopy (IRM). This technique, originally developed by Curtis (1964) and subsequently improved by Abercrombie et al. (1975), is an optical technique used to visualise regions of close apposition between the ventral cell surface and the substratum.

A Zeiss Standard-16 microscope was modified as follows for IRM. A narrow waveband filter (254nm) and polariser were fitted between the 50W tungsten lamp and the beam splitter housing from which the standard barrier fillers had been removed. An analysing filter was placed between the beam splitter and eye piece. This arrangement is essentially as described by Abercrombie and Dunn, (1975). Cells were then viewed using a x63 antiflex oil emersion objective or occasionally a standard x100 phase objective. The latter objective lens gave a less well defined image but allowed phase images to be seen without changing objectives, unlike the x63 antiflex lens.

Live cells were used throughout the study as fixation has been suggested to reduce the quality of the image obtained and also makes interpretation difficult (Dr. R.A. Badley, personal communication). Cells were allowed to spread for 4h, 37⁰C on derivitised coverslips in a humidified CO₂ incubator and then viewed directly as follows.

Excess DMEM was removed from the coverslip by blotting and the cell-free surface cleaned by gentle wiping on a damp tissue. The coverslip was then inverted onto a 13mm silicone gasket (Millipore Cat. No. SX00 01301) sealed to a standard microscope slide with a small amount of vaseline. The gasket prevented direct contact of the coverslip with the slide, an arrangement which gave better IRM images than simply inverting the coverslip onto the slide. The space between the slide and the coverslip was filled with DMEM by placing a drop of medium in the centre of the gasket prior to applying the coverslip. Excess medium was then gently blotted off. IRM images were photographed using Ilford Pan F film (50ASA) and an automatic exposure system.

Scanning electron microscopy (SEM)

Cells were plated onto glass coverslips derivitised with the above ligands and allowed to spread for 2h, 37°C. After fixing overnight in Karnovsky's fixative (3% formalin (v/v), 4% glutaraldehyde (v/v), 0.07M sodium cacodylate, 0.05% calcium chloride (w/v), pH 7.4) cells were washed for 2h, RT^O in 0.05M sodium cacodylate, pH 7.4 and then post-fixed with cacodylate-buffered osmium tetroxide (1% v/v) for 45', RT^O. After dehydration in a graded ethanol series coverslips were stored overnight in 100% acetone before being critical point dried, mounted and gold coated by standard methods (Karnovsky, 1965).

RESULTS

Cell attachment to ligand-coated substrata

Dynatech microtitre wells coated with FN (10µg/ml), CT-B subunit (100µg/ml), whole toxin (100µg/ml, or ConA (100µg/ml) were all capable of supporting stable cell attachment of Balb/c 3T3 cells with only minimal attachment occurring in the absence of the ligand (Table 3.1). Cell attachment to B subunit-coated wells was effectively blocked in the presence of 500 µg/ml bovine mixed brain gangliosides indicating that CT-induced attachment was indeed due to an interaction with cell surface gangliosides. Although CT effectively promoted cell attachment it is known that its addition to Balb/c 3T3 cells causes cell rounding due to an increase in cAMP levels in response to activation of adenylate cyclase by the toxin A-subunit. The ability of cells to spread on the B subunit (referred to as CT from this point on) was thus investigated.

Cell spreading on ligand-coated substrata

In an attempt to demonstrate whether or not a cell surface ganglioside is capable of mediating the spreading response we examined whether, as a result of cell attachment to CT-coated substrata, cells then went on to adopt a spread morphology as has previously been demonstrated on FN- and ConA-coated substrata. Cells were found to be able to spread on substrata coated with FN (10µg/ml, 22.5nM) and ConA (100µg/ml, 0.91µM), although the morphology of the spread cells was clearly different (Fig. 3.1 A,B). Cells spread on FN displayed a typical

Table 3.1 Attachment of Balb/c 3T3 cells to substrata coated
with FN, ConA and CT

Ligand	No. of cells attached per well ($\times 10^4$)
None	0.22
FN (10 μ g/ml)	2.30
ConA (100 μ g/ml)	3.20
CT (100 μ g/ml)	3.00
CT-B subunit (100 μ g/ml)	2.40
CT-B subunit plus ganglioside	0.49

3.75×10^4 cells (labelled with ^{35}S -methionine, 1400CPM/ 10^3 cells) were added to Dynatech microtitre wells coated with various ligands, and the number of cells attached after a 2h incubation at 37°C determined as described in Materials and Methods. The results represent the mean of triplicate determinations.

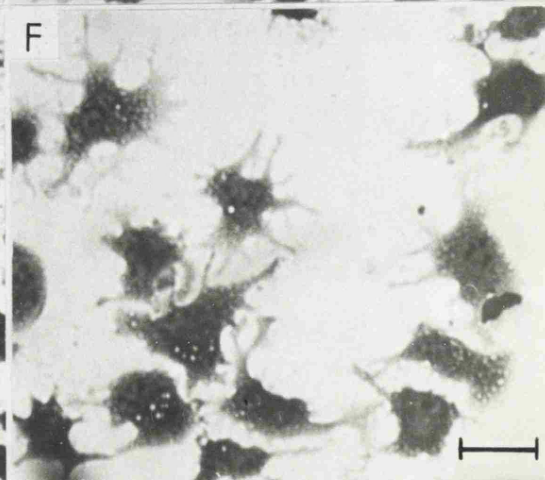
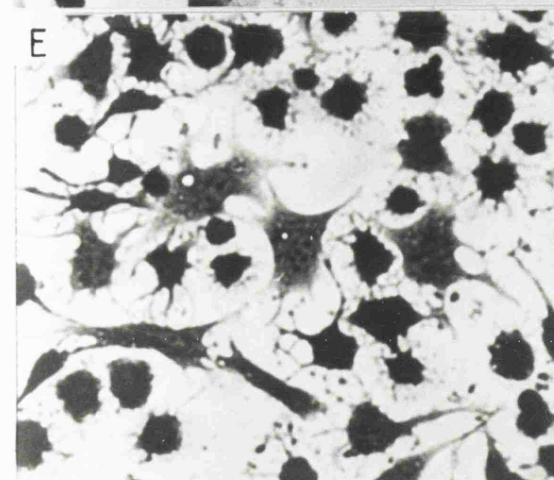
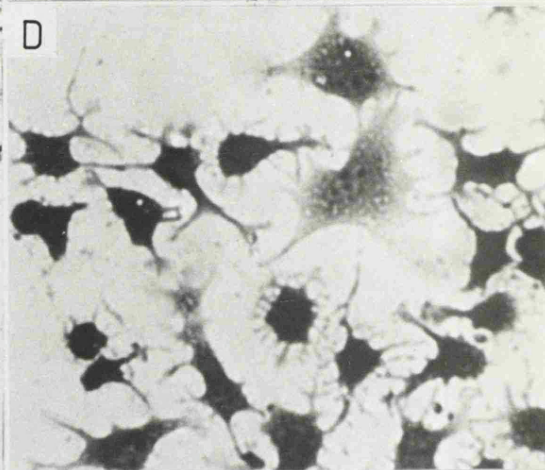
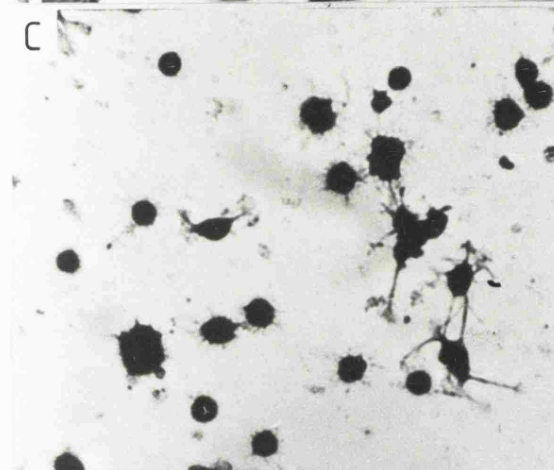
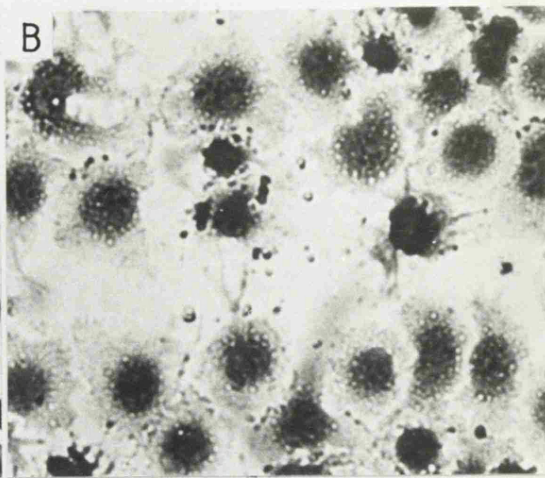
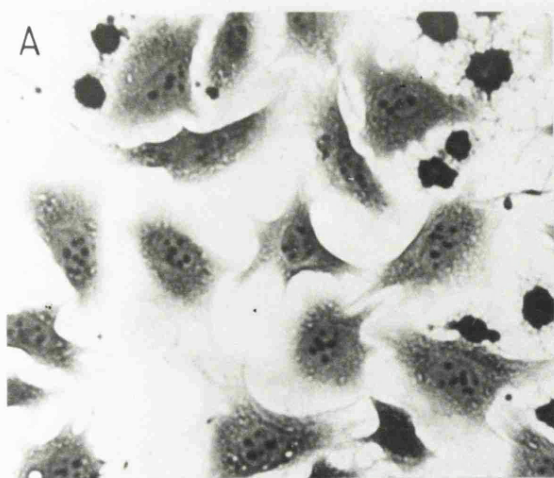


Fig. 3.1. Cell spreading on FN-, ConA- and CT-coated substrata

Cells were plated onto Dynatech microtitre wells coated with the above ligands and incubated for 2h, 37°C before fixing and staining.

(A) 10µg/ml (22.5nM) FN; (B) 100µg/ml (0.91µM) ConA; (C, D) 10µg/ml (0.182µM) CT; (E, F) 100µg/ml (1.82µM) CT. In (D) and (F) cells were pretreated with 0.04units/ml of neuraminidase for 1h, 37°C prior to trypsinisation and plating onto wells coated with CT. (Molarities expressed assuming the following M.wts. FN 440000; ConA 110000; CT 55000)

fibroblastic morphology while cells on ConA appeared to be circularly spread with an ill-defined outline due to poor staining of the thin cytoplasm at the cell periphery. In contrast, cells plated onto CT-coated wells were only poorly spread at coating concentrations up to 100µg/ml (1.82µM) (Fig. 3.1 C and E). However, many cells showed evidence of extensive cytoplasmic processes indicating that they were in a preliminary stage of spreading (Fig. 3.1, E). As the number of ligand receptors is known to affect the ability of the cell to spread at a fixed ligand concentration the effect of neuraminidase treatment of Balb/c 3T3 cells on their ability to spread on CT was investigated. It has previously been demonstrated that the toxin binding capacity of Balb/c 3T3 cells can be increased by 2-5 fold by pretreatment of the cells in monolayer with neuraminidase due to conversion of the higher gangliosides GD_{1a} and GT into GM_1 . Neuraminidase treatment markedly increased the ability of cells to spread on both low (10µg/ml) and high (100µg/ml) concentrations of CT although even at the high concentration cell spreading on CT did not resemble that on either FN or ConA (Fig. 3.1 D,F).

Analysis of the time course of cell spreading (Fig. 3.2) demonstrated that cell spreading on CT is generally slower than on either FN or ConA with full cell spreading occurring after approximately 2 hours on CT, 80 mins on FN and 60 mins on ConA. Cells spread on CT and ConA retained a distinctive morphology, dissimilar from cells spread on FN, even after prolonged incubation times (4h). The differing time courses of cell spreading on each ligand might reflect quantitative differences in ligand receptor interaction or alternatively a qualitative difference in the

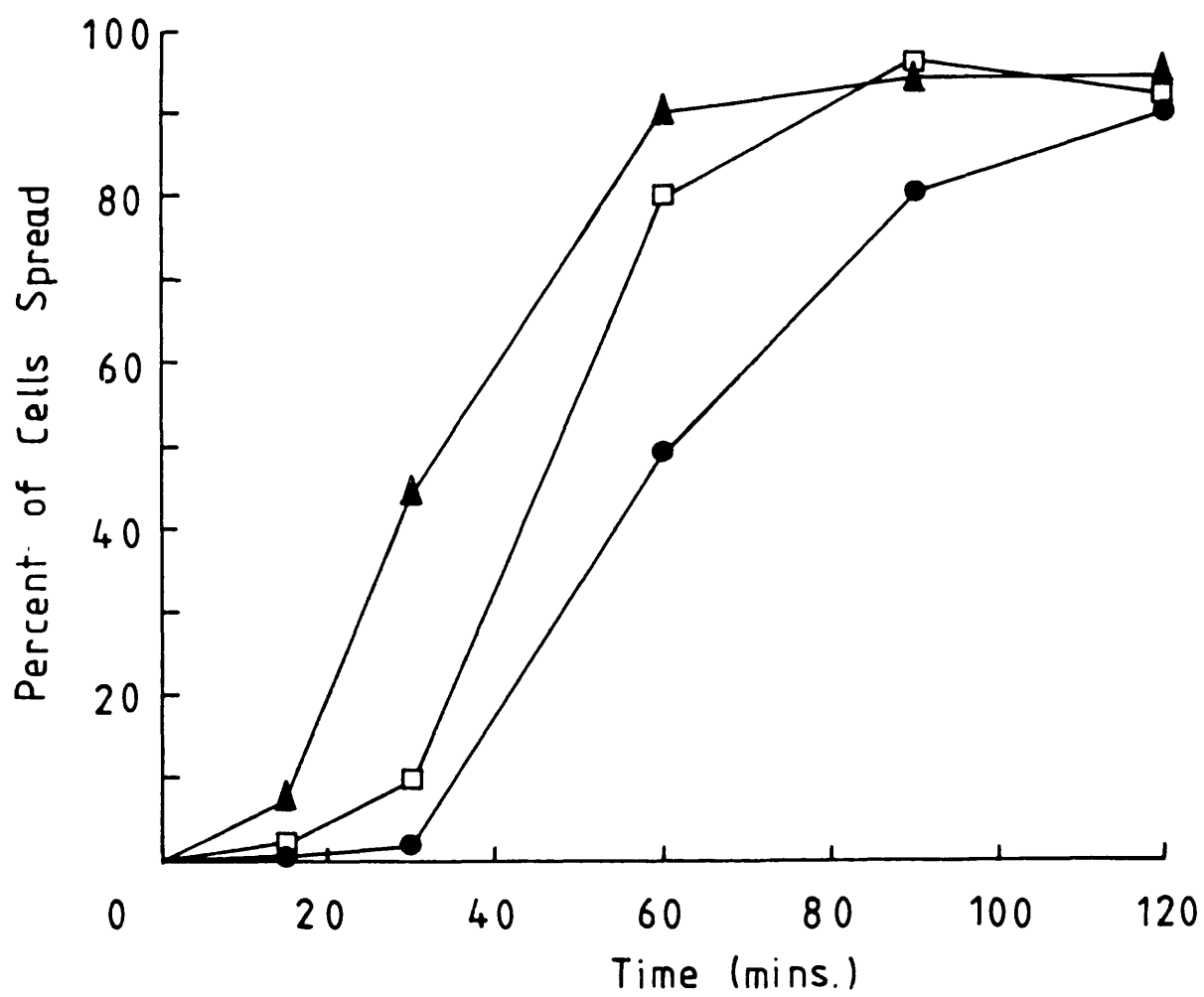
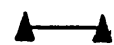
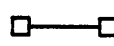



Fig. 3.2 Time course of Balb/c 3T3 cell spreading on FN-, CT-
and ConA-coated substrata

Glass coverslips were derivitised with either fibronectin, concanavalin A or cholera toxin B subunit at a concentration of $1.8\mu\text{M}$. Coverslips were then treated with 1mg/ml BSA for 1h, 37°C to block non-specific cell spreading. Neuraminidase-treated Balb/c 3T3 cells were then added and cell spreading quantitated at various times after addition.

	Concanavalin A
	Fibronectin
	Cholera toxin B subunit

mechanisms of cell spreading induced by each ligand.

Specificity of ligand-cell interactions

To establish the specificity of cell spreading on each ligand the ability of ligand-specific antibodies to inhibit the cell spreading was investigated (Table 3.2). Antibodies to FN almost completely inhibited cell spreading on FN-coated substrata, but did not effect spreading on CT or ConA (see Appendix II for characterisation of anti-FN antiserum). Similarly, antibodies to CT and ConA only inhibited cell spreading on the respective ligand. Due to the longer time course of CT spreading compared to FN or ConA, it was possible that this cell spreading might be due to secretion of endogenous FN onto the substratum. The demonstration that anti-FN antibodies do not affect CT spreading argues against such a possibility. In addition, immunofluorescent staining of CT- and ConA-spread cells failed to detect any secreted FN even after permeabilisation of the cells with 0.25% Triton X-100/PBS (data not shown).

It has previously been shown that cell spreading on ConA-coated substrata was inhibited by preincubating the substratum with α -methyl-mannoside (Grinnell and Hays, 1978) demonstrating the specificity of ConA towards mannose-containing glycoconjugates. In order to demonstrate that cell spreading on CT was due only to an interaction with ganglioside GM₁ at the cell surface, the ability of various gangliosides to inhibit CT-induced cell spreading was investigated (Fig. 3.3). CT-coated microtitre wells were incubated with specific gangliosides at various concentrations and unbound material removed by washing. Cells were plated

Ligand	Antibody	Percent of cells spread
FN	Anti-FN	4.8
	Anti-ConA	97.6
	Anti-CT	96.0
	None	95.7
ConA	Anti-FN	84.4
	Anti-ConA	0
	Anti-CT	80.9
	None	98.1
CT	Anti-FN	91.8
	Anti-ConA	88.1
	Anti-CT	6.3
	None	93.4

Table 3.2 Ligand specificity of cell spreading. Effect of
anti-ligand antibodies on cell spreading

Microtitre wells were coated with either FN (10µg/ml), ConA (200µg/ml) or CT (100µg/ml). 10^4 neuraminidase-treated cells were then added to the wells in the presence of anti-ligand antibody 1gG and allowed to spread for 2h, 37°C. The final antibody concentration was equivalent to a 1/50th dilution of the whole serum.

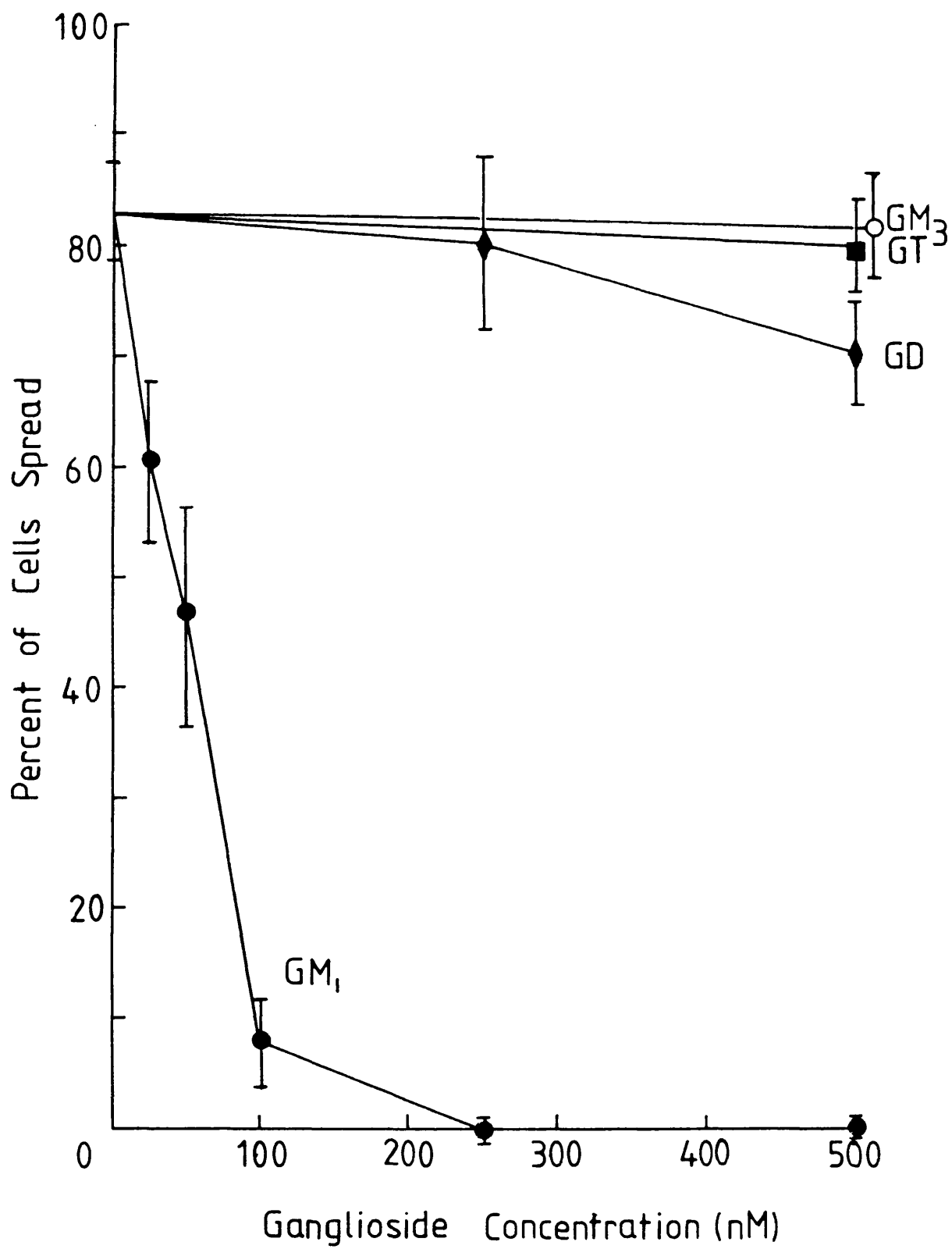


Fig. 3.3 Ganglioside inhibition of cell spreading on CT-coated
substrata

Microtitre wells were coated with 100µg/ml CT in PBS for 2h, 37°C and blocked for a further 1h with 10mg/ml BSA. Gangliosides (Supelco) at various concentrations in PBS were incubated with wells for 1h, 37°C, aspirated, and the wells washed once with 200µl PBS prior to the addition of 10^4 neuraminidase-treated cells. After 2h, 37°C cells were fixed, stained and the percent of cells spread quantitated.

onto the wells and allowed to spread for 2h, 37°C. It was decided to remove any unbound ganglioside prior to the addition of cells so as to avoid the possible direct effect of gangliosides on the cells, as described in Chapter 2. Ganglioside GM₁ produced a 50% reduction in cell spreading at a concentration of 50nM whereas all the other gangliosides tested (GM₃, GD, GT) were ineffective even at ten-fold higher concentrations. This is consistent with the proposal that cell spreading on CT is due to the interaction of the ligand with ganglioside GM₁. The absence of any CT-binding glycoproteins in these cells has previously been demonstrated (Critchley et al., 1982).

SEM analysis of the spreading induced by FN, CT and ConA

Although the morphology of cells spread on FN could be successfully demonstrated by crystal violet staining, the morphology of cells spread on ConA and CT was only poorly defined. This was largely due to the thin cytoplasmic projections at the cell periphery. In an attempt to better characterise the morphologies described above, cells spread on either FN, CT or ConA were analysed by SEM (Fig. 3.4). Freshly trypsinised, neuraminidase-treated cells were plated onto 12mm glass coverslips derivitised with each ligand and allowed to spread for 2h, 37°C. SEM analysis of fixed, dried and gold-coated coverslips emphasised the differing morphologies induced by each ligand. Cells spread on FN adopted a characteristic fibroblast-type morphology with a flattened cell body and well defined concave edges, often raised above the substratum (Fig. 3.4 A,B). This morphology closely resembles that displayed by fibroblasts in serum-containing medium. In contrast, cells

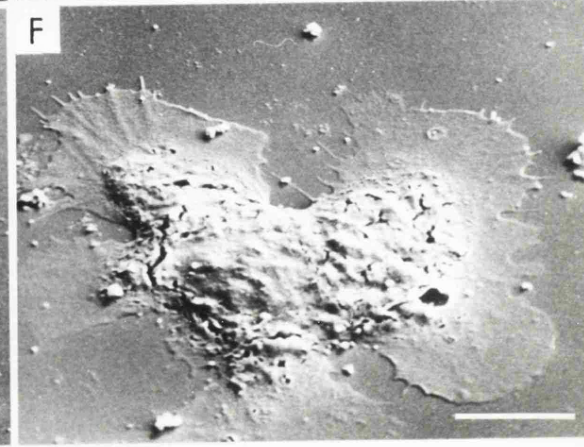
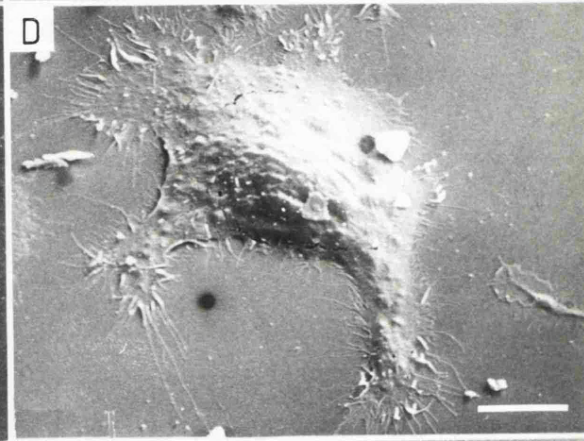
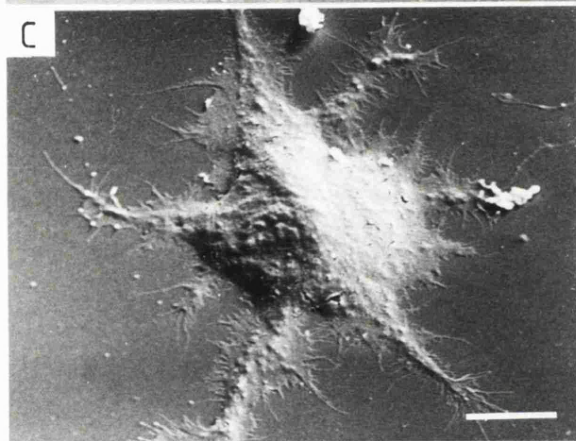
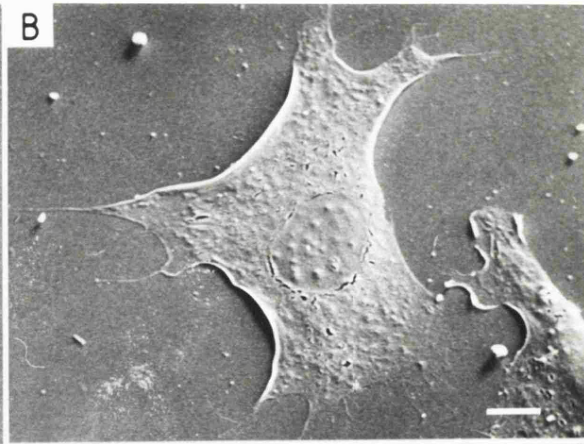
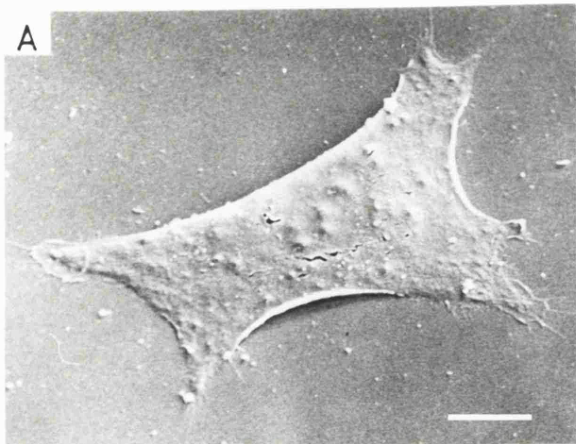


Fig. 3.4 Scanning electron microscopy of cells spread on FN-,
CT- and ConA-coated substrata

Cells were plated onto 12mm glass coverslips derivitised with either 0.5mg/ml FN (1.1 μ M, A, B); 0.1mg/ml CT (1.8 μ M, C,D) or 1mg/ml ConA (9 μ M, E, F) and allowed to spread for 4h, 37°C. Cells were then fixed and prepared for SEM as described in Materials and Methods. All cells were pretreated with neuraminidase prior to trypsinisation and plating onto substrata.

(Bar, 10 μ M)

spread on CT or ConA displayed an irregular, less flattened morphology lacking the well defined concave edges seen in FN-spread cells. Cells spread on ConA showed characteristic broad, fan-shaped lamellae extending from the main cell body apparently consisting of extremely thin regions of cytoplasm (Fig. 3.4 E,F). Small microspikes were often seen extending from the periphery of this thin cytoplasmic regions. Cells spread on CT generally lacked the fan-shaped lamellae but produced a large number of filopodia-like structures extending from large, irregular extensions of the main cell body (Fig. 3.4 C,D).

A possible reason for the irregular morphology of cells spread on CT was that the B subunit preparation contained sufficient A subunit contamination to activate adenylyl cyclase in these cells resulting in the previously described morphological changes. To test this possibility I took two approaches. Firstly, it is known that radioiodination of the whole toxin results in preferential incorporation of the radiolabel into the A subunit and thus provides a sensitive method of detecting the A subunit. Radioiodination of the whole toxin and B subunit preparation used in the spreading experiments followed by SDS-PAGE and autoradiography failed to detect any contaminating A subunit (Fig. 3.5 insert). Furthermore, slicing of the gel tracks and gamma counting also demonstrated a lack of specific counts in the region of the gel corresponding to the A subunit (Fig. 3.5). Secondly, I tested the B subunit preparation for A subunit using a morphological assay. As stated above the A subunit of CT is able to activate adenylate cyclase resulting in an increase in cAMP and an associated morphological change. Addition of whole toxin at concentrations as low as 0.1ng/ml (1.2pM) to

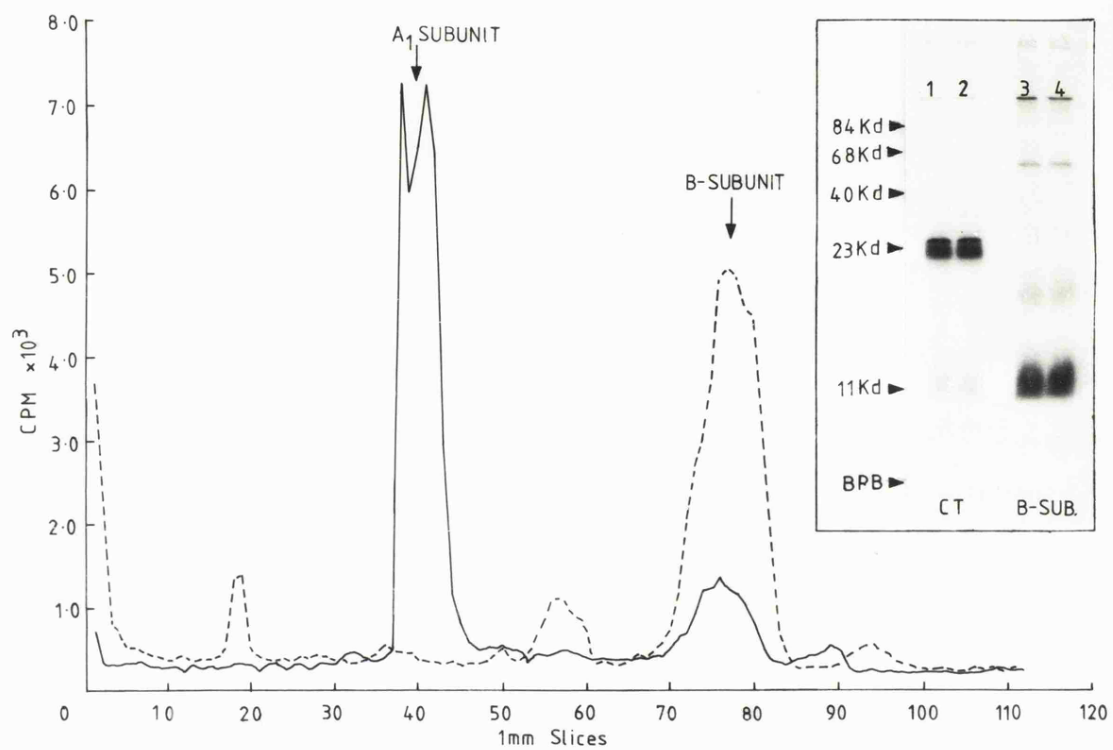


Fig. 3.5 Lack of CT-A subunit in B subunit preparation.
Analysis of ^{125}I -labelled material by SDS-PAGE

10 μg of whole toxin or B subunit preparation were iodinated by the chloramine T method. Samples of each were then run on a 12% SDS-polyacrylamide gel under reducing conditions. The gel was then either dried down and autoradiographed (insert) or sliced and counted on a Beckman gamma counter. Note that iodination of the whole toxin results in preferential labelling of the A subunit, however, no evidence of labelling in this region of the gel is evident following iodination of the B subunit preparation.

a monolayer of Balb/c 3T3 cells results in retraction of the cytoplasm around the nucleus within 1h, 37°C (Fig. 3.6 C-F). This change can be mimiced by dibutyl cAMP, phosphodiesterase inhibitors, and prostaglandin E1 which also increase intracellular cAMP. However, the addition of the B subunit preparation at 100µg/ml (1.82µM) produced no such morphological effect (Fig. 3.6 B). Calculation shows that contamination of the B subunit by intact toxin or free A subunit is thus less than $6.6 \times 10^{-5}\%$ by weight.

Adsorption of ligands to microtitre wells

The differing morphologies of Balb/c 3T3 cells spread on FN-, ConA- and CT-coated substrata may be a reflection of variable adsorption of these ligands to the substratum. Quantitation of the binding of FN, CT and ConA to microtitre wells during a 2h incubation is shown in Fig. 3.7. All three ligands were effectively adsorbed onto the plastic substrata with approximately 1640fmoles FN, 1470 fmoles CT and 2400fmoles ConA adsorbed per well at a coating concentration of 100µg/ml (0.22µM FN, 1.82µM CT, 0.91µM ConA). The different spreading responses triggered by these ligands does not therefore appear to be the result of differences in the ability of each ligand to adsorb to plastic substrata.

The effect of ligand density and receptor number on cell spreading

The density of substratum-bound ligand and the number of cell surface receptor molecules are important factors in determining a cell's ability to spread (Aplin and Hughes, 1981a). As shown in Fig. 3.1

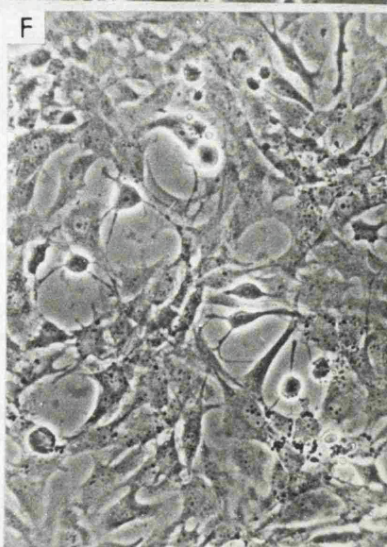
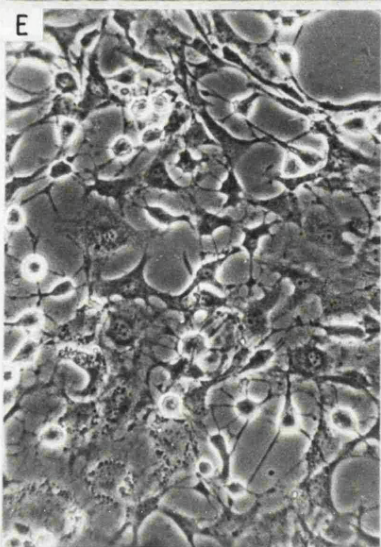
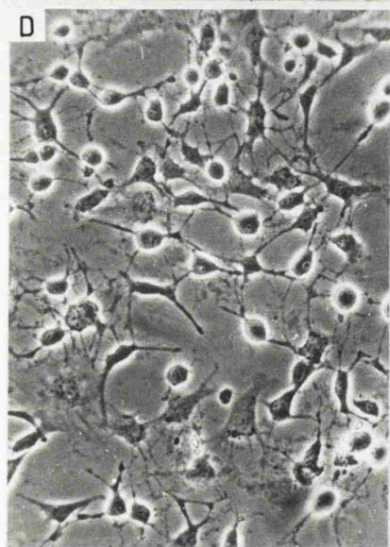
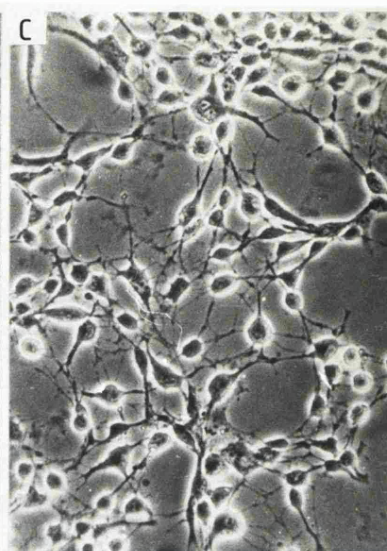
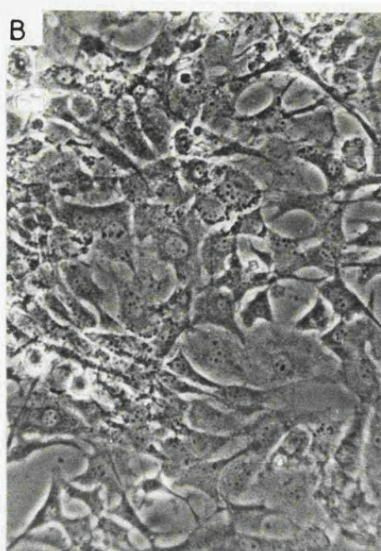
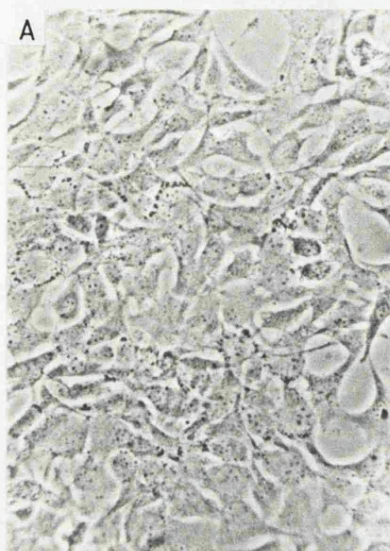


Fig. 3.6 Lack of CT-A subunit in B subunit preparation.

Morphological evidence

Balb/c 3T3 cells were grown on 12mm glass coverslips and then incubated with either whole toxin or B subunit in DMEM for 1h, 37°C. Coverslips were then fixed with 3.8% formaldehyde/PBS (45', RT⁰) and photographed using a Leitz diavert microscope and Ilford Pan F film (50ASA).

- | | | |
|----|----------|-----------|
| A. | No toxin | |
| B. | 100µg/ml | B subunit |
| C. | 100µg/ml | CT |
| D. | 100ng/ml | CT |
| E. | 1ng/ml | CT |
| F. | 0.1ng/ml | CT |

increasing the number of CT receptors on Balb/c 3T3 cells increased cell spreading at a fixed ligand concentration. Using Fig. 3.7 to convert ligand coating concentrations into ligand densities adsorbed to the substratum I have quantitated the relationship between ligand density and cell spreading for each ligand (Fig. 3.8 A). FN was able to promote cell spreading at a far lower ligand density than either CT or ConA, maximal cell spreading occurring at ligand densities of 100-200 fmoles/cm² for FN, 700-800fmoles/cm² for ConA and greater than 2000fmoles/cm² for CT. These figures correspond to coating concentrations of approximately 2.5µg/ml FN, 10µg/ml ConA and greater than 100µg/ml CT. On FN-coated substrata there is a sharp increase in cell spreading at ligand densities between 40-100fmoles/cm². However, on ConA and CT this transition was over a far greater range, 50-800fmoles/cm² for ConA and 300 to greater than 2000fmoles/cm² for CT.

It has previously been demonstrated that the threshold ligand concentration required to induce cell spreading is inversely related to the number of ligand receptors at the cell surface (Aplin and Hughes, 1981a). It is known that neuraminidase treatment of Balb/c 3T3 cells results in a 2-5 fold increase in the number of CT binding sites (normally $0.8-1.6 \times 10^6$ /cell). The quantitative effect of such treatment on the ability of Balb/c 3T3 cells to spread on FN, CT and ConA is shown in Fig. 3.8 B. It can be seen that following neuraminidase treatment the ligand density required for 50% cell spreading on CT is reduced from 1500fmoles/cm² to 500-600fmoles/cm² while that on FN and ConA remained unaltered (50% cell spreading at 60fmoles/cm² for FN and 150fmoles/cm² for ConA). Cell spreading on CT-coated substrata is thus dependent on

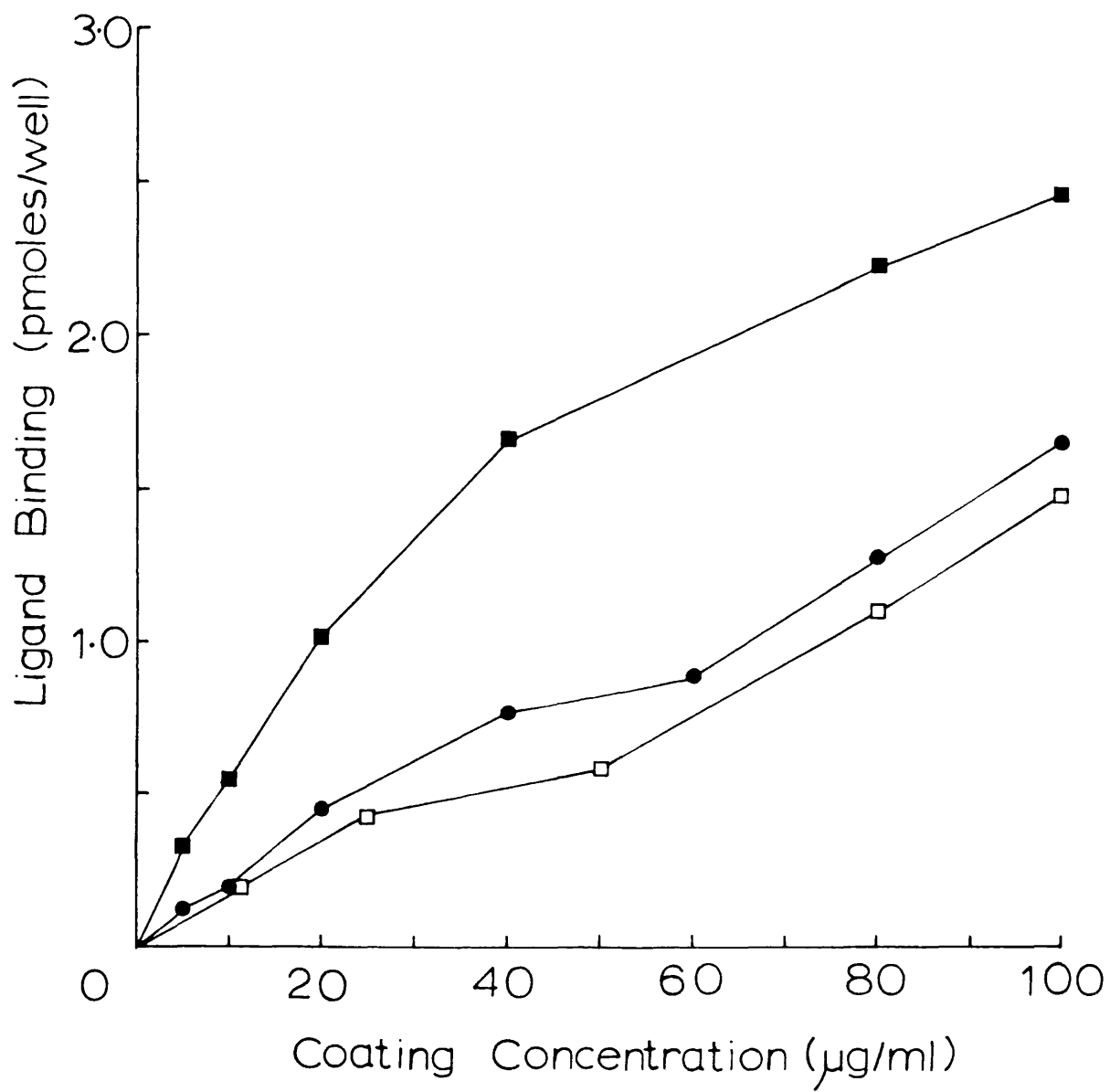


Fig. 3.7 Ligand adsorption to microtitre wells

Ligand binding to Dynatech microtitre wells was quantitated using radioiodinated ligand as described in Methods. 100 μ l of radiolabelled ligand (specific activity 10CPM/fmole) was added to each well (triplicate wells per coating concentration) and incubated for 2h, RT⁰. Unbound ligand was then removed by washing x5 with 200 μ l of PBS. Bound material was then quantitated by removing the base of each well with a hot scalpel and counting on a Beckman gamma counter.

■—■ ConA
●—● FN
□—□ CT (B subunit)

Each point is the mean of triplicate results.

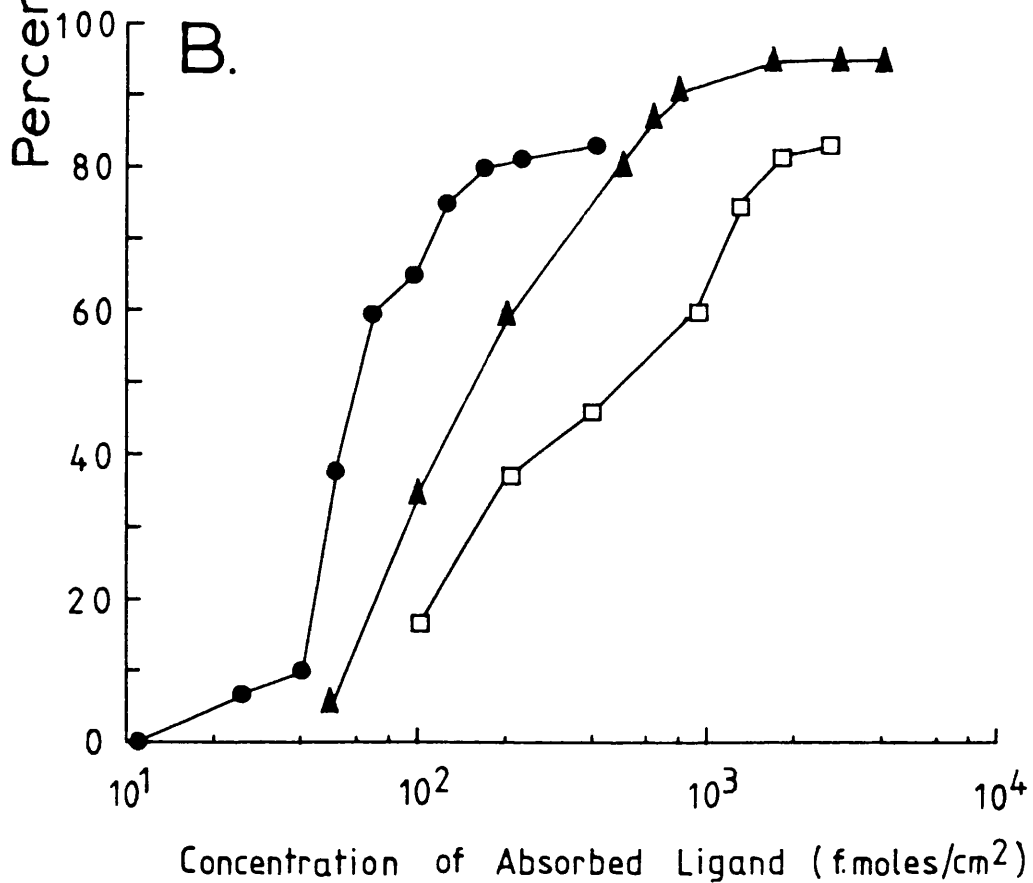
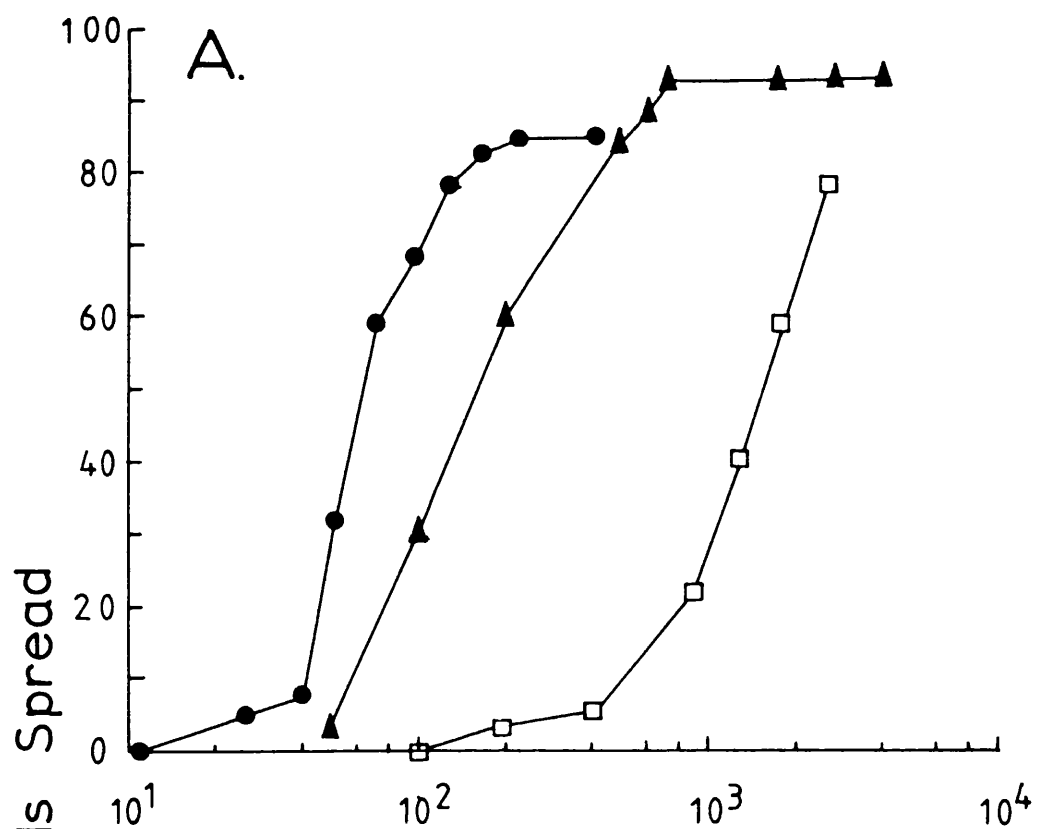


Fig. 3.8 The effect of ligand-density and neuraminidase
treatment on cell spreading

Cells were plated onto microtitre wells coated with varying concentrations of FN (●—●), ConA (▲—▲) or CT (□—□) and allowed to spread for 2h, 37°C. Cell spreading was quantitated from fixed and stained cells and related to the levels of substrate-bound ligand determined in parallel experiments using radiolabelled ligand. Where indicated cells were pretreated with 0.04units/ml neuraminidase for 1h, 37°C prior to trypsinisation and plating onto ligand-coated wells. A, no neuraminidase treatment; B, plus neuraminidase treatment.

the concentration of substrate-adsorbed ligand and the number of cell surface receptors. The finding that neuraminidase treatment of Balb/c 3T3 cells had no effect on their ability to spread on FN while increasing their ability to spread on CT clearly indicates that neuraminidase-sensitive structures are not directly involved in FN-mediated cell spreading. (The effect of neuraminidase treatment on cell spreading is discussed in Chapter 4.)

The effects of cytoskeletal disrupting agents on cell spreading

The cytoskeleton, in particular the microfilament system, has been implicated as an essential element in cell spreading on serum- or FN-coated substrata (Goldman, Schloss and Starger, 1976). Disruption of these structures or inhibition of their function with metabolic inhibitors can inhibit, or to varying degrees reverse, cell spreading on various substrata (Grinnell and Hays, 1978). The effect of such agents on Balb/c 3T3 cell spreading on FN-, CT- and ConA-coated substrata is shown in Table 3.3.

Treatment of cells spread on FN-coated microtitre wells with cytochalasin B or a combination of cytochalasin B and demicolcemid (a microtubule-disrupting agent) reduced the number of spread cells by 90.4% and 96.0% respectively. Many cells although not spread displayed a characteristic arborized morphology with retraction fibres extending from the cell body. Treatment with demicolcemid alone also reduced the number of spread cells (30.5% reduction) although the predominant effect was to abolish cell polarity with circularly spread cells resulting.

Additions	Ligand		
	FN	CT	ConA
None	80.9 (0) *	86.9 (0)	96.5 (0)
Cytochalasin-B (5µg/ml)	7.8 (90.4)	59.5 (31.5)	76.9 (8.3)
Demicolcemid (10µg/ml)	56.2 (30.5)	79.1 (9.0)	87.9 (8.9)
Cytochalasin-B + Demicolcemid	3.2 (96.0)	19.2 (87.9)	73.1 (24.2)
Cyto.B Pretreat (5µg/ml)	0.9 (98.9)	4.3 (95.1)	14.4 (85.1)
NaN ₃ (5mM)	15.7 (80.6)	9.1 (89.7)	59.7 (38.2)

* Percentage inhibition/reduction of cell spreading.

Table 3.3 Inhibition of cell spreading by cytoskeletal
disrupting agents

Microtitre wells were coated with either FN (10µg/ml) or CT (100µg/ml for 2h, 37°C and blocked with 10mg/ml BSA in PBS for a further 1h. Neuraminidase treated cells were then allowed to spread for 2h, 37°C before the addition of cytochalasin B or demicolcemid to a final concentration of 5-and 10µg/ml respectively. Cell spreading was quantitated 1.5h after the addition of the drug. Where indicated cells were pretreated with cytochalasin B (5µg/ml) (Cyto B Pretreat) for 30' at RT⁰ prior to plating on ligand-coated substrata. The effect of sodium azide (NaN₃) on cell spreading was investigated by the direct addition of cells to ligand-coated wells such that the final concentration of azide was 5mM. Unless stated otherwise cell spreading was quantitated 2h after plating.

Pretreatment of cells with cytochalasin B prior to plating onto FN-coated wells resulted in almost complete inhibition of cell spreading (98.9% inhibition) together with evidence of reduced cell attachment (not quantitated). Cell spreading was also effectively blocked in the presence of the metabolic inhibitor sodium azide.

Cells spread on CT-coated wells and treated with cytochalasin B or demicolcemid alone showed a less dramatic reduction in spreading than seen on FN-coated wells (31.5% and 9.0% reduction respectively) although a combination of the two dramatically increased the effect (87.9% reduction). Again sodium azide and pretreatment with cytochalasin B both markedly inhibited cell spreading (89.7% and 95.1% inhibition respectively).

Treatment of cells spread on ConA-coated wells with cytochalasin B or demicolcemid had little effect on the number of spread cells (8.3% and 8.9% reduction respectively). In combination however, these drugs caused a significant reduction in cell spreading (24.2%) although this was not as pronounced as similar treatment of cells spread on FN or CT. Interestingly however, pretreatment of cells with cytochalasin B effectively inhibited cell spreading on ConA (85.1% inhibition) perhaps suggesting that cell spreading on ConA requires a functional micro-filament system while the maintenance of the spread state is less dependent on this system. As with FN and CT induced cell spreading sodium azide also inhibited cell spreading on ConA-coated substrata although the degree of inhibition was apparently less than for the other two ligands (38.2% inhibition).

Thus, cell spreading on all three ligands requires the cell to be metabolically active and to have a functional microfilament system.

F-actin organisation of cells spread on FN-, CT- and ConA-coated substrata

The involvement of the actin-containing microfilament system in cell spreading on FN-, CT- and ConA-coated substrata was further examined by immunofluorescent staining of F-actin using NBD-phalloidin as previously described by Barak *et al.* (1980). Two hours after plating onto FN-derivitised coverslips most of the cells were well spread and displayed a typical fibroblastic morphology. The majority of F-actin was localised at the periphery of the cells although thin microfilament bundles were also present in many cells running in the direction of the long axis of the cell (Fig. 3.9 A, B). There was little evidence of any perinuclear staining although small fluorescently stained aggregates were common throughout the cytoplasm. In contrast to cells spread on FN-coated substrata cells spread on CT showed no evidence of microfilament bundles (Fig. 3.9 C, D). Instead, thin actin-containing filaments were found running concentrically around the nucleus and extending radially to protrude beyond the general cell outline. The cell periphery between these filaments also displayed actin staining giving rise to a webbed appearance. Cells spread on ConA also lacked any well defined microfilament cables but contain thinner actin-containing structures often concentrated into an arc around the nucleus (Fig. 3.9 E). In addition, strongly stained nucleation sites for actin polymerisation were often seen with these filaments radiating from them (Fig. 3.9 F). Many ConA spread cells

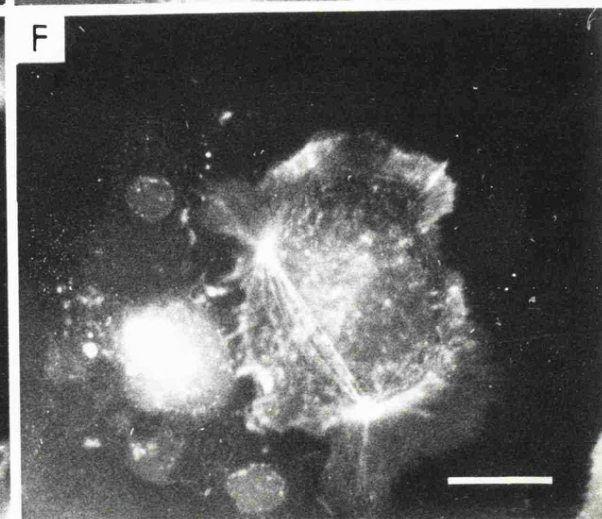
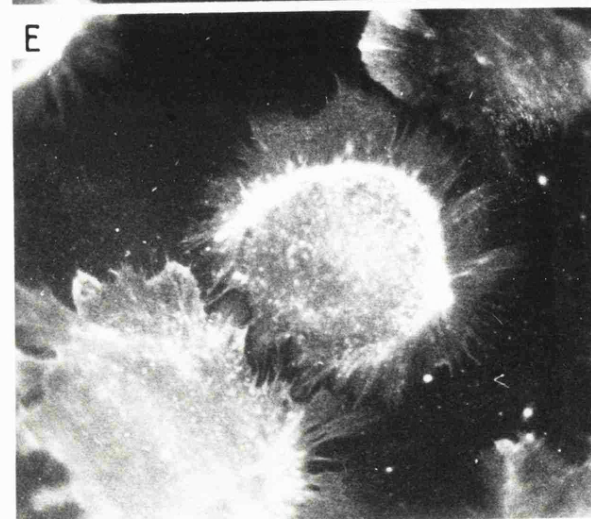
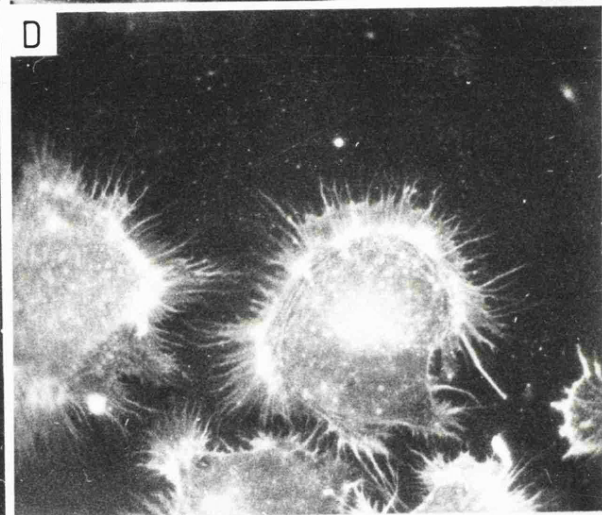
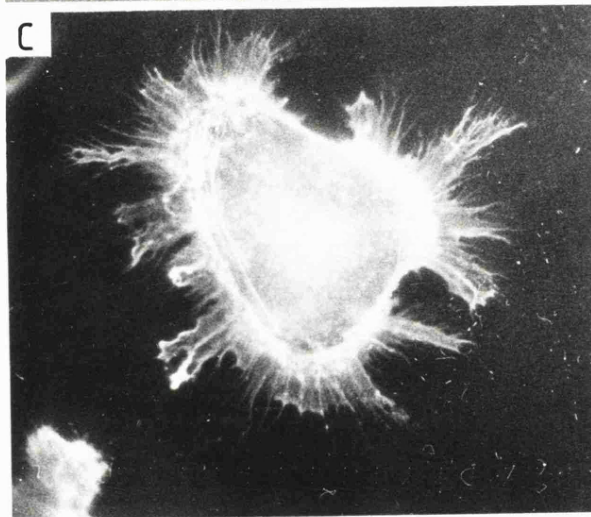
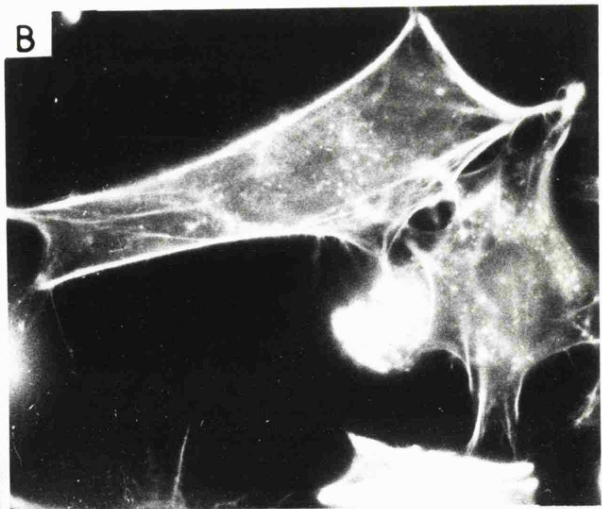
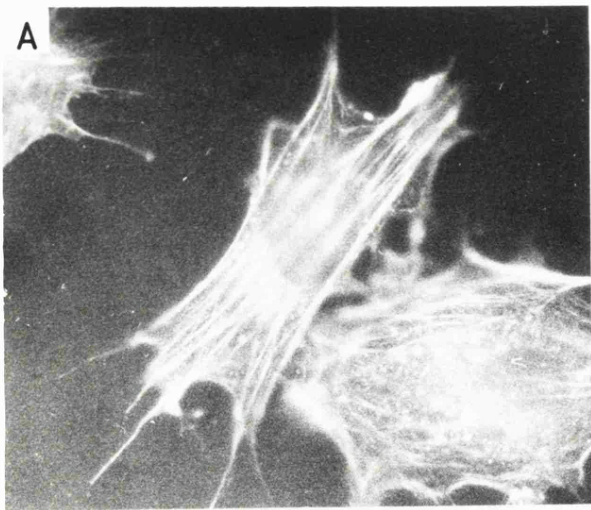


Fig. 3.9 Comparison of F-actin organisation in cells spread
on FN, ConA and CT

Cells plated onto glass coverslips derivitised with various ligands and incubated for 2h, 37°C were fixed and stained for F-actin using NBD-phalloidin as described in Materials and Methods. Coverslips were derivitised with (A, B) FN, 0.5mg/ml (1.1µM); (E, F) ConA, 1mg/ml (9µM); (C, D) CT, 0.1mg/ml (1.82µM). All cells were pretreated with neuraminidase. Two micrographs of cells spreading on each substrate are presented to demonstrate the different morphologies and staining profiles within each population. Bar = 10µM.

also showed evidence of short actin-containing structures in fan-shaped regions of cytoplasm at the cell periphery. These may correspond to the microspikes seen by SEM extending from the edge of thin cytoplasmic lamellapodia (see Fig. 3.4 F).

Ability of each ligand to trigger focal contact formation

The morphology and cytoskeletal organisation of Balb/c 3T3 cells spread on an FN-coated substratum is clearly not reproduced when the same cells spread on CT- or ConA-coated substrata suggesting that FN-mediated cell spreading may differ mechanistically from that on CT or ConA. It was therefore of interest to know if CT- and ConA-spread cells had a similar degree of cell-substratum specialisation as cell spread on FN. We thus examined cells for the presence of focal and close contacts following spreading on each ligand using interference reflection microscopy (IRM) and an antibody exclusion technique modified from that of Grinnell (1980b).

The technique of IRM is used to visualise regions of close apposition between the ventral cell surface and the underlying substratum (Curtis, 1964; Abercrombie et al. 1975). Three types of image are commonly described;

- i) focal contacts, corresponding to areas of close apposition (< 10-15nm) between the cell and the substratum, usually well defined with a black image,

- ii) close contacts, larger areas of substratum contact with a separation of between 20-100nm and corresponding to grey, less well defined regions,
- and iii) areas of cell substratum separation greater than 100nm corresponding to a white image.

An alternative method for the visualisation of focal contacts has been described previously by Grinnell (1980b) and is used here in a slightly modified form. The technique relies on the exclusion of anti-substratum antibodies from areas of close cell-substratum separation while other areas remain accessible. The technique is well suited to the visualisation of focal contacts in fixed cells and in conjunction with immunofluorescent staining of actin-containing microfilaments.

Balb/c 3T3 cells were plated onto either FN (0.5mg/ml), CT (0.1mg/ml), or ConA (1mg/ml) derivitised glass coverslips and allowed to spread for 4h, 37°C in DMEM without serum. Focal contacts were then visualised by either antibody exclusion (Fig. 3.10, 3.11, 3.12) or IRM (Fig. 3.13). Additionally cells prepared for antibody exclusion were simultaneously stained with NBD-phalloidin to visualise the microfilament organisation. It can be seen that cells plated onto FN-derivitised coverslips form extensive microfilament bundles or stress fibres which terminate at areas of antibody exclusion supporting the identity of these images as focal contacts (Fig. 3.10). The accessibility of the anti-ligand antibody to other regions under the cell appeared to be variable, usually showing partially restricted access, particularly under the nucleus. These areas of reduced fluorescent intensity were clearly distinguishable from the black areas

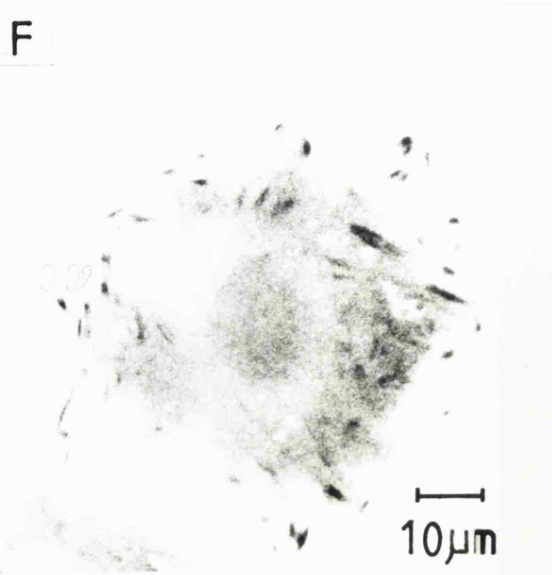
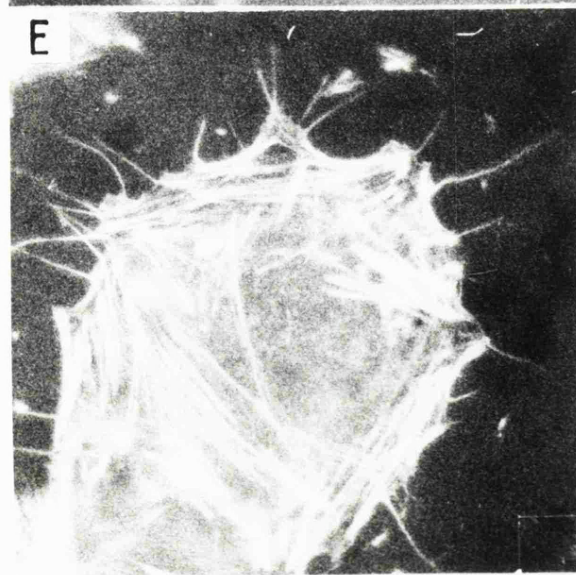
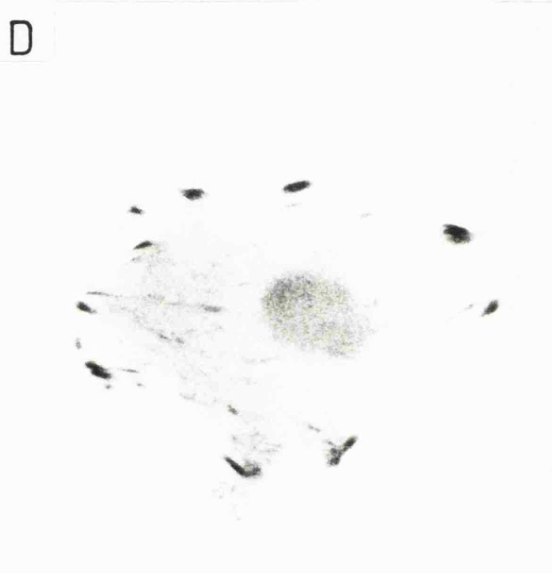
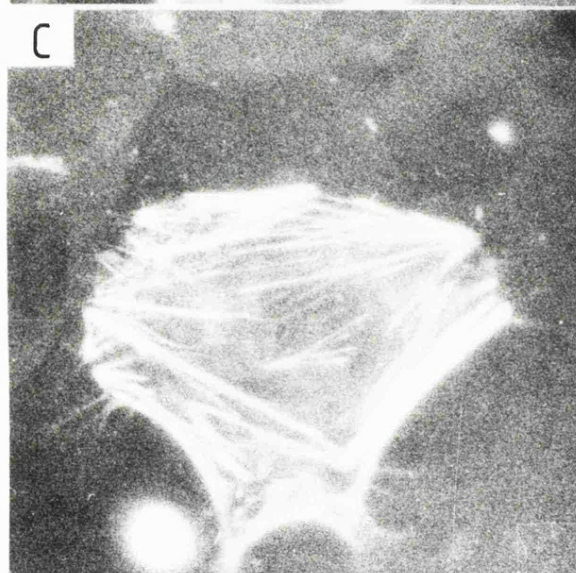
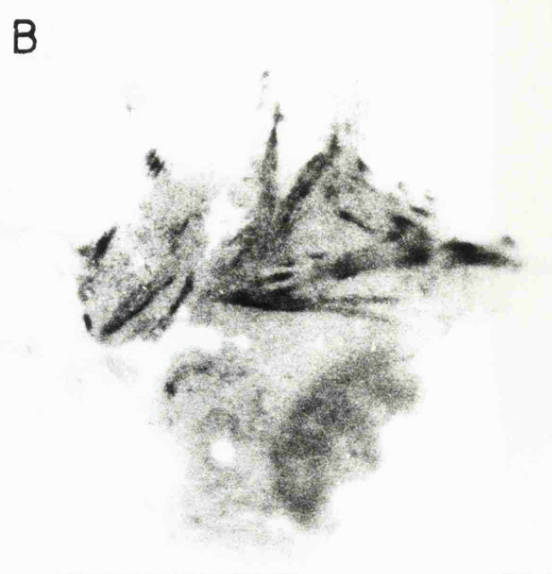
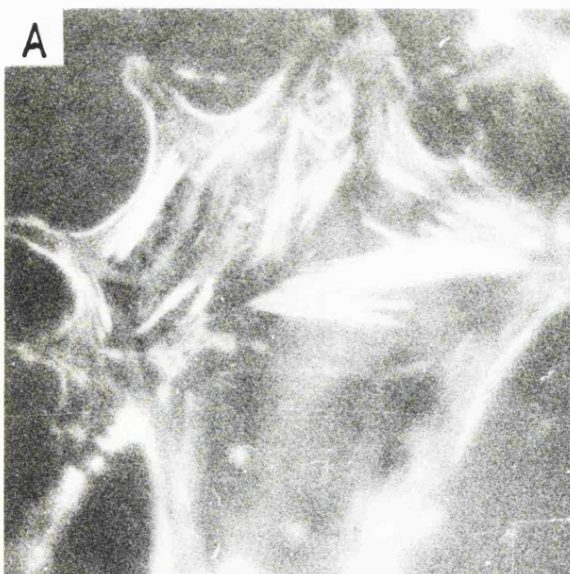


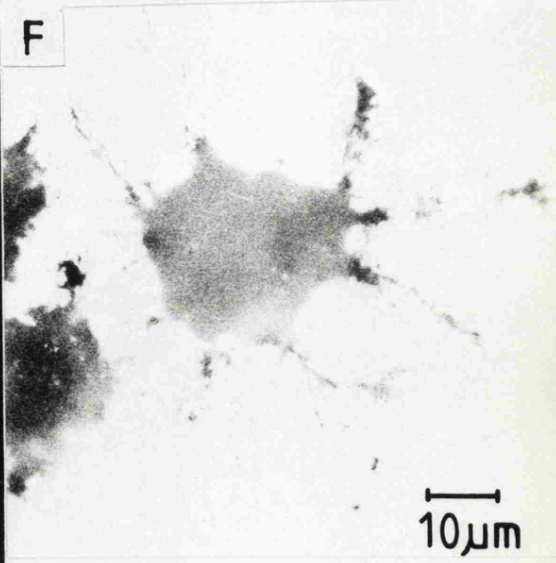
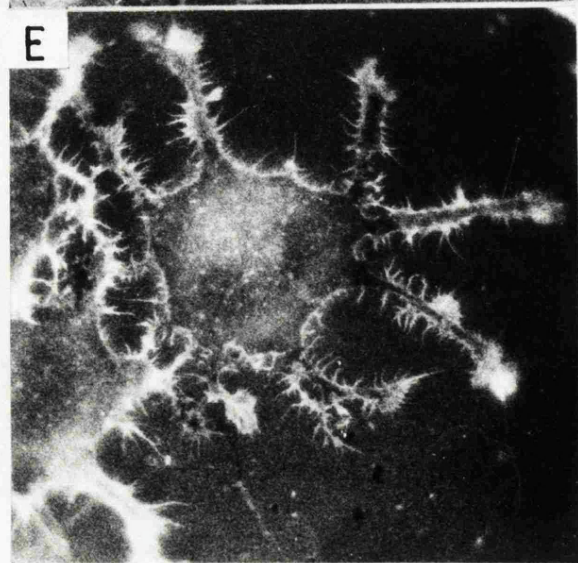
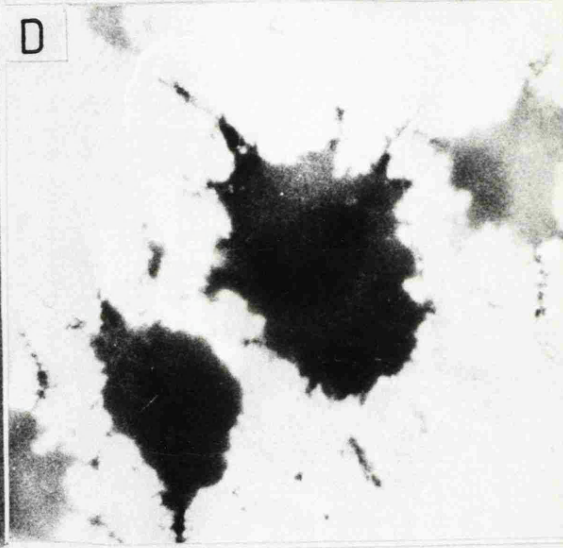
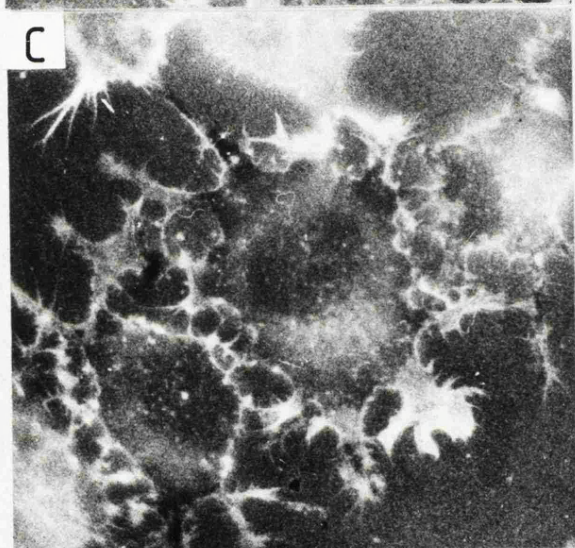
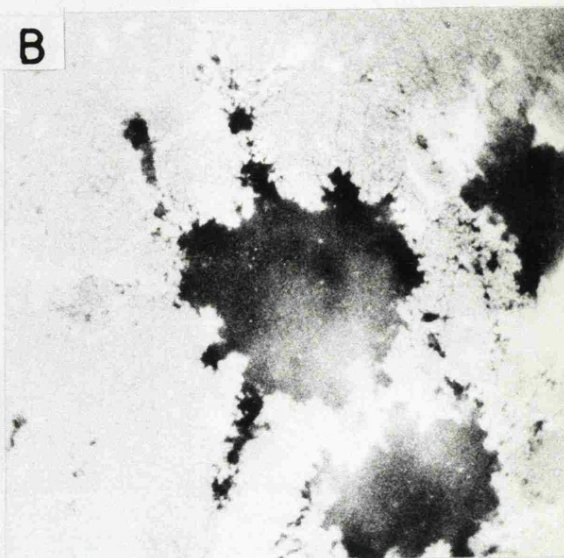
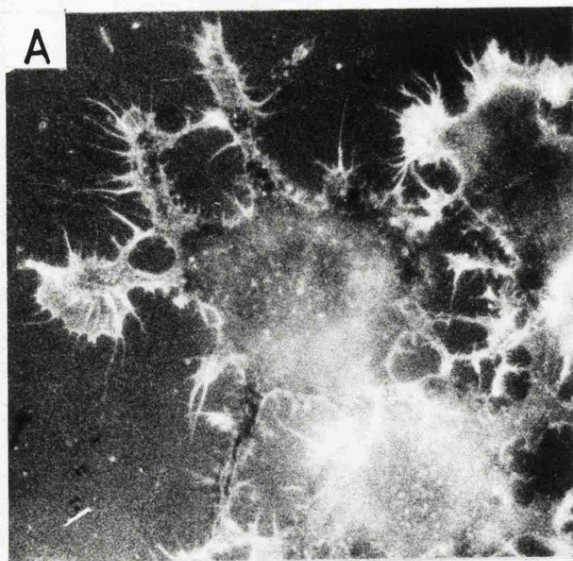
Fig. 3.10. Ability of cells spread on FN to form focal contacts
as determined by antibody exclusion

12mm Glass coverslips were derivitised with FN (500µg/ml) as described in Materials and Methods. Freshly trypsinised neuraminidase-treated Balb/c 3T3 cells were then plated onto the derivitised coverslips in 100µl of DMEM and allowed to attach for 15', 37°C. The petri dish containing the coverslips was then carefully filled with DMEM and the cells further incubated for 2h, 37°C. Cells were fixed with 3.8% formaldehyde/PBS for 40', RT^O and coverslips incubated with a 1:100 dilution of a rabbit anti-substrate antiserum for 40', RT^O. To allow staining of intracellular actin (A, C, E) cells were permeablised with 0.25% Triton X-100/PBS for 5', RT^O and stained with 0.1µM NBD-phalloidin for 30', RT^O. Visualisation of the antibody exclusion image (B, D, F) was achieved by incubating coverslips with rhodamine-conjugated goat anti-rabbit second antibody (diluted 1:50) at the same time as the NBD-phalloidin. Coverslips were then viewed by epifluorescence using a Zeiss Standard 16 microscope.

Bar = 10µM.

where antibody had been totally excluded and corresponding to focal contacts. Permeabilisation of cells with Triton X-100 prior to incubation with anti-FN antiserum did not allow access to focal contacts although it did allow staining of intracellular FN which tended to obscure antibody excluded regions on the substratum. Therefore the permeabilisation of cells, required for F-actin staining, was left until after incubation with the anti-ligand antibody.

In contrast to cells spread on FN, cells spread on CT showed no evidence of stress fibre or focal contact formation 4 hours after plating (Fig. 3.11). Even after prolonged incubation (up to 6 hours) there was no evidence of focal contacts. The antibody exclusion image of cells spread on CT showed that the majority of the underside of the cell was closely apposed to the substratum preventing access of antibody to the substratum beneath the cell (Fig. 3.11 B,D,F). As described previously, the only evidence of F-actin staining in these cells was at the periphery of the cell corresponding to filopodia-like structures seen by SEM (Fig. 3.11 A,C,E). Cells spread on ConA similarly showed no evidence of specialised adhesion structures such as focal contacts and did not develop long stress fibres as seen in cells spread on FN. Again the majority of the substratum under the cell was inaccessible to antibody as described in cells spread on CT (Fig. 3.12 B,D,F) and F-actin staining was predominantly at the cell periphery or in fan-like structures (Fig. 3.12 A,C,E). From the antibody exclusion data it is apparent that cells plated onto FN-coated substrata can form well developed microfilament bundles which terminate in clearly visualised focal contacts. However, cells plated onto either CT- or ConA-coated



10μm

Fig. 3.11 Ability of cells spread on CT to form focal contacts
as determined by antibody exclusion

As Fig. 3.10 except coverslips were derivitised with 100µg/ml CT.

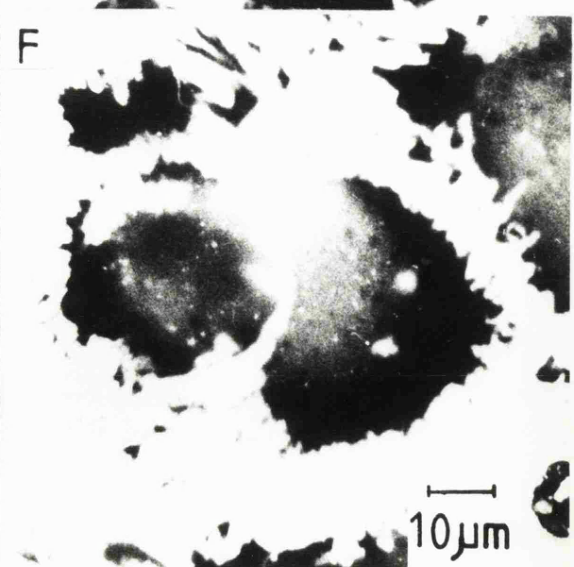
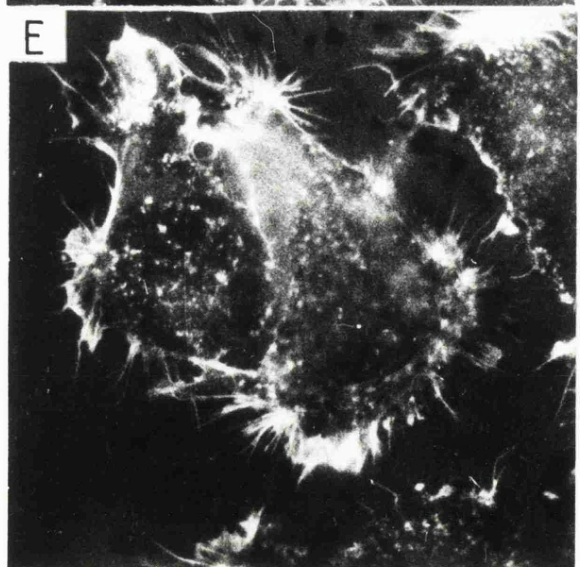
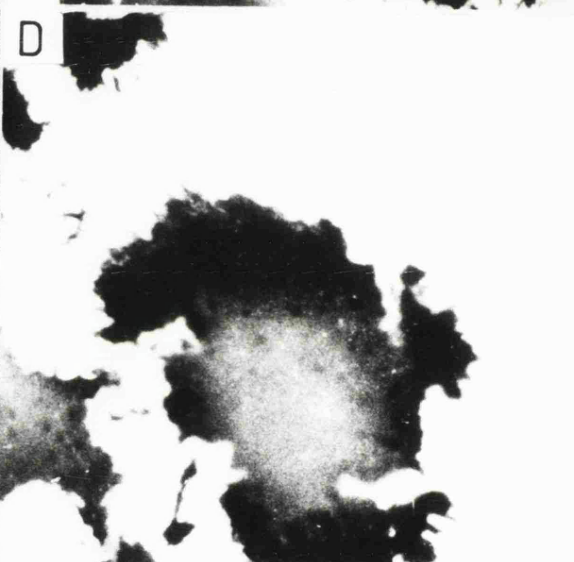
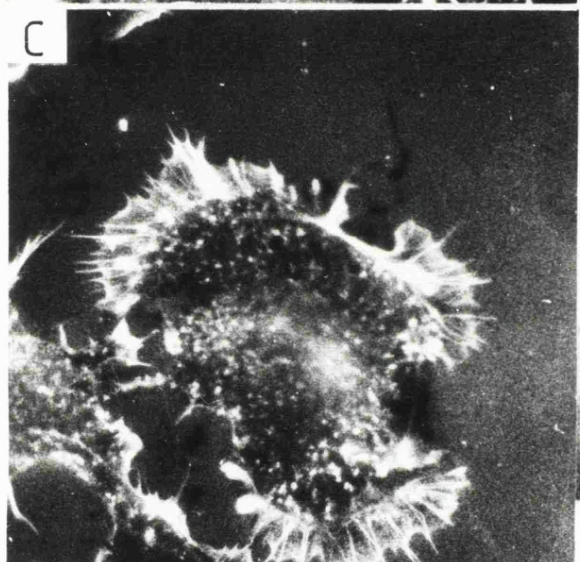
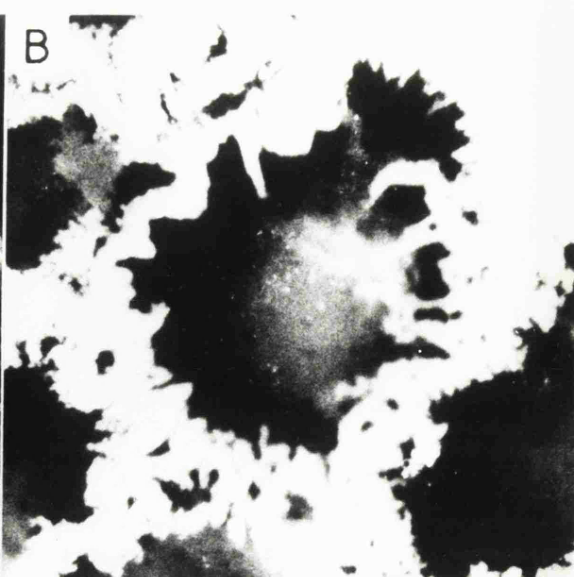
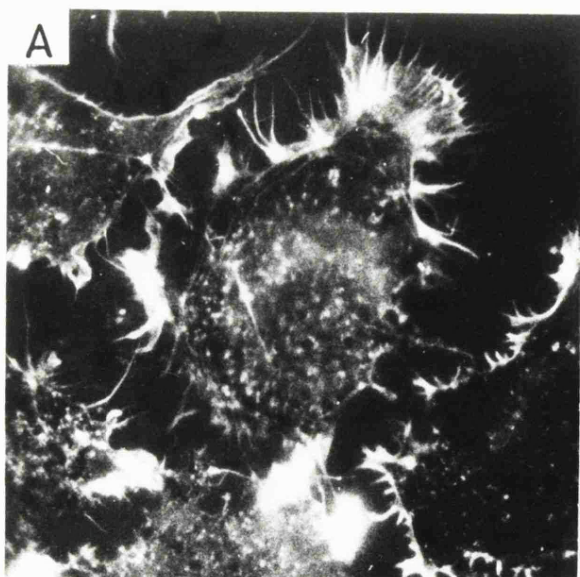


Fig. 3.12 Ability of cells spread on ConA to form focal contacts
as determined by antibody exclusion

As Fig. 3.10 except coverslips were derivitised with 1mg/ml ConA.

substrata were apparently incapable of forming either of these structures.

A more detailed analysis of cell-substratum specialisation following spreading on FN, CT and ConA by IRM confirmed the antibody exclusion data showing the formation of focal contacts in cells spread on FN but not on CT or ConA (Fig. 3.13). Examination of live cells spread on FN-derivitised glass coverslips by IRM showed the presence of numerous focal contacts both at the periphery of the cell and under the main body of the cell (Fig. 3.13 D,G,H arrowed). Large areas of less well defined close contacts (grey, mottle image) were also present in FN spread cells together with areas of greater cell-substratum separation (white image). The large areas of close contact tended to obscure the focal contact image making them less well defined than seen in cells grown in serum-containing medium (see Chapter 4, Fig. 4.7). In contrast to cells plated onto FN the IRM image of cells plated onto CT- or ConA-derivitised coverslips showed no evidence of focal contacts but gave a mottle grey appearance similar to areas of close contact (Fig. 3.13 E,F). On CT-derivitised coverslips a continuous black IRM image could be seen around the periphery of the cell and extending along the long filopodial extensions (Fig. 3.13 B,E). This type of image was also prominent at the periphery of cells spread on ConA, especially at thin fan-shaped cytoplasmic extensions (Fig. 3.13 C,F). Such images have previously been shown not to be due to regions of focal contacts but are formed by a double membrane effect occurring at ultra-thin cytoplasmic processes (Gingell, 1981; Gingell and Vince, 1982). Under the main body of cells spread on CT or ConA a grey mottled IRM image was seen suggesting

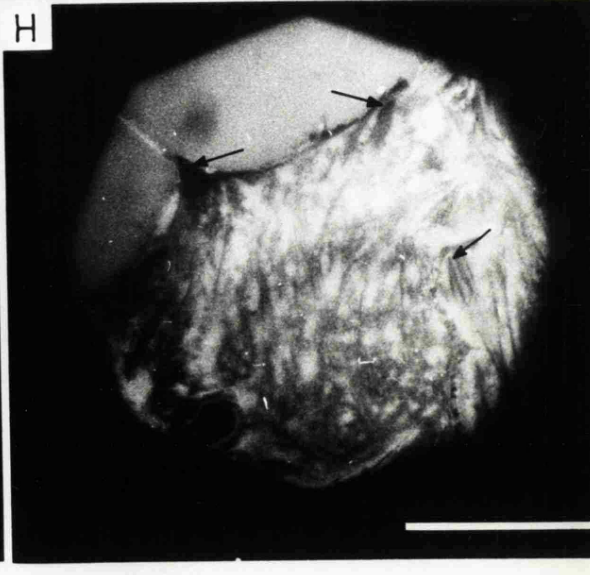
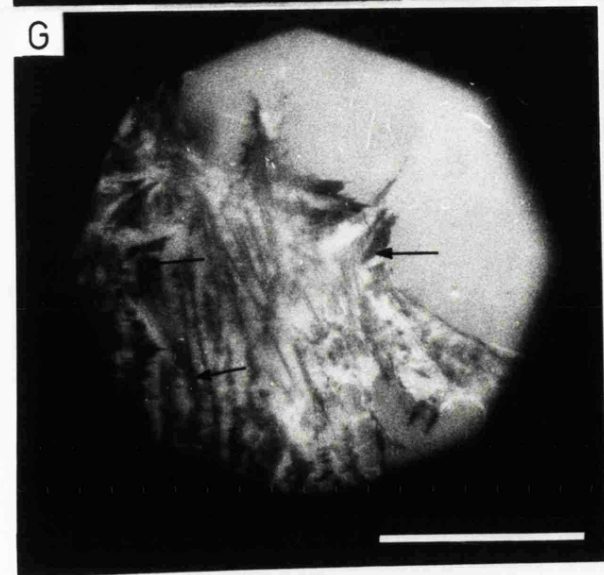
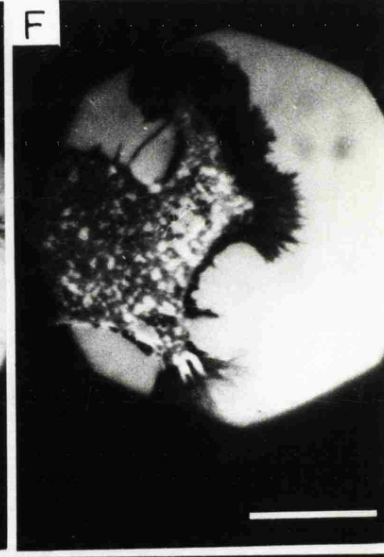
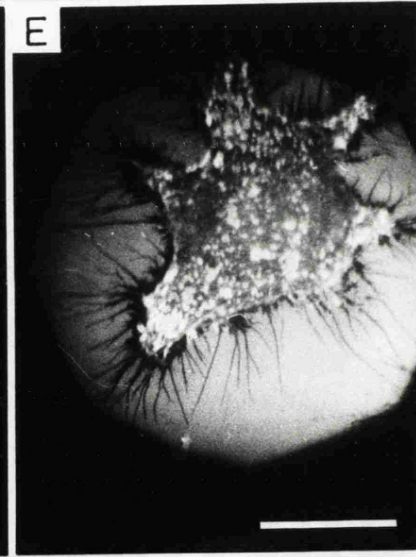
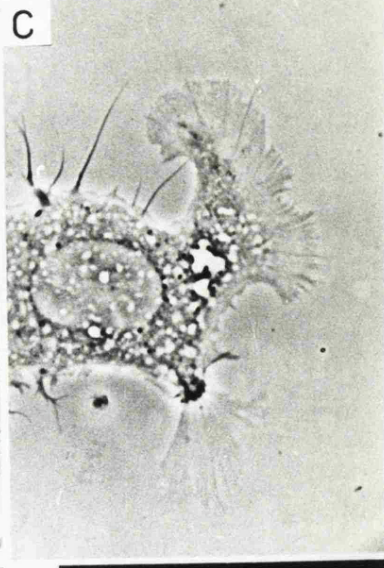
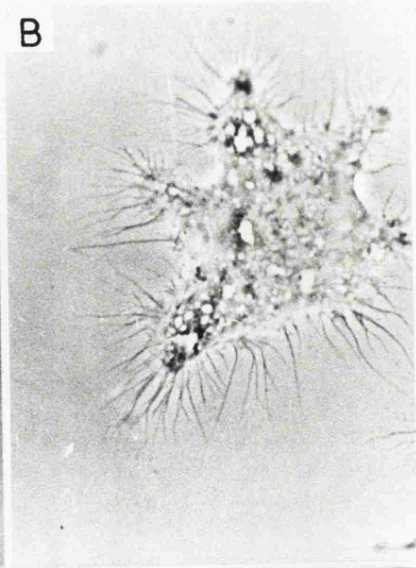
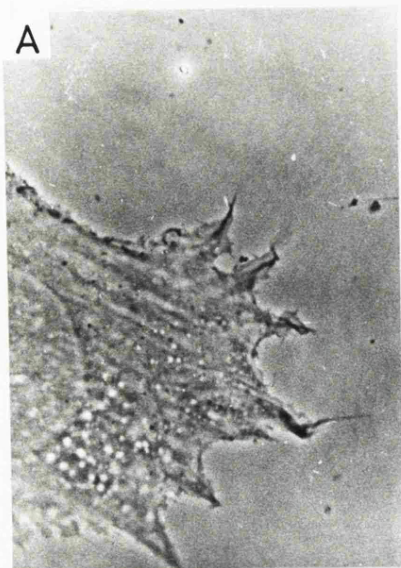


Fig. 3.13 Ability of cells spread on FN, ConA and CT to form
focal contacts as determined by IRM

Neuraminidase-treated Balb/c 3T3 cells were plated onto 12mm glass coverslips derivitised with either FN (500µg/ml), CT (100µg/ml) or ConA (1mg/ml) and incubated in DMEM for 4h, 37°C. Live cells were then viewed on a Zeiss Standard 16 microscope modified for IRM as described in Materials and Methods.

- | | |
|------|---|
| A, D | FN-derivitised coverslip |
| B, E | CT-derivitised coverslip |
| C, F | ConA-derivitised coverslip |
| G, H | FN-derivitised coverslip high magnification |

A, B and C corresponding phase contrast image of D, E and F respectively.

Bar = 25µM.

large areas of close contact. The mottle appearance was due to small white areas interrupting the grey image. These areas appear to correspond to vesicular structures seen in the phase image (Fig. 3.13 B,E,C,F).

In an attempt to further characterise the cytoskeletal organisation of cells spread on FN-, CT- and ConA-coated substrata and also to confirm the presence of focal contacts in cells spread on FN the localisation of vinculin was investigated following cell spreading. Vinculin is known to be concentrated at the cytoplasmic face of focal contacts associated with the termini of actin-containing microfilaments. Extraction of cells in 1% Triton X-100 and immunofluorescent staining of vinculin as described by Geiger (1979) showed that only the cells spread on FN had detergent-resistant vinculin complexes (Fig. 3.14). In these cells vinculin was localised mainly at the periphery of the cell in discrete locations reminiscent of the pattern of focal contacts visualised by antibody exclusion and IRM. In contrast, cells plated onto CT or ConA showed no distinct areas of vinculin staining presumably due to its solubilisation by the detergent-containing extraction buffer.

Thus, FN-coated substrata are able to trigger Balb/c 3T3 cells to form vinculin-containing focal contacts and associated microfilament bundles in serum free medium. However, CT- or ConA-coated substrata are unable to induce the formation of such structures.

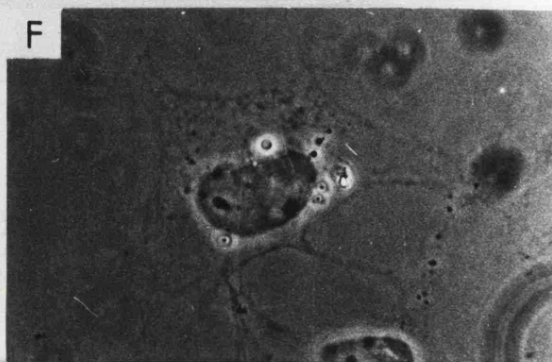
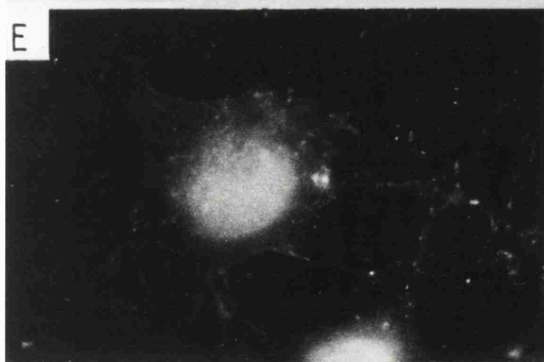
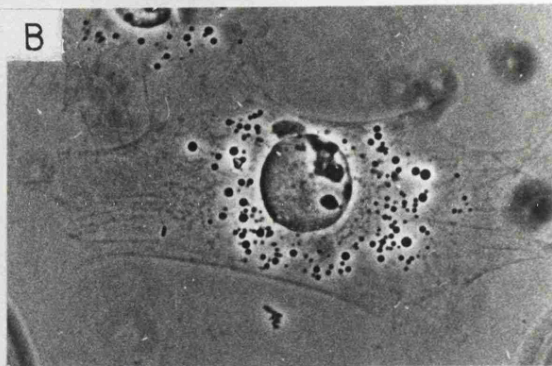
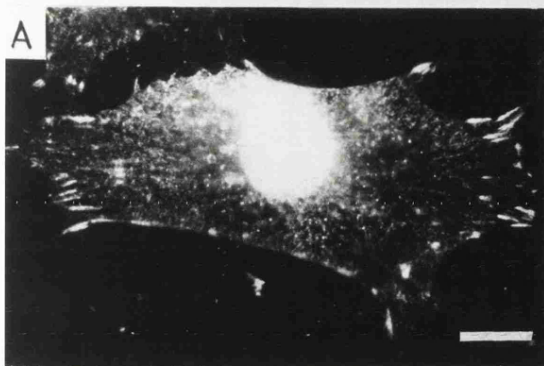


Fig. 3.14 Immunofluorescent localisation of vinculin in detergent-
extracted cells spread on FN, CT and ConA coated substrata

Cells were plated onto derivitised coverslips and allowed to spread for 4h, 37°C, detergent extracted (5mM MgCl₂, 3mM EGTA, 50mM KCl, 50mM MES, 0.2% Triton X-100 pH 6.0 for 5', 0°C) washed twice with ice cold PBS and finally fixed with 3.8% formaldehyde/PBS. Vinculin was then visualised by sequential incubation of the coverslips with affinity purified rabbit anti-vinculin (40µg/ml) and a 1:50 dilution of rhodamine-labelled goat-anti-rabbit IgG. Rabbit anti-vinculin antisera was prepared by Dr. S. Kellie using chicken gizzard vinculin as the immunogen. This antibody specifically stains the Z-line of mouse skeletal muscle myofibrils and was specific for vinculin as determined by immune precipitation and immunoblotting.

a, c, e anti-vinculin immunofluorescence
 b, d, f phase contrast
 a, b FN substrate
 c, d ConA substrate
 e, f CT substrate
 Bar = 10µM

DISCUSSION

In this study I have attempted to answer the question of whether a cell surface ganglioside is capable of mediating the transmembrane events known to occur during FN-mediated cell spreading. To do this a detailed study was made of the ability of CT, a ganglioside-specific ligand, to promote Balb/c 3T3 cell spreading. In addition, by comparing the spreading response triggered by CT with spreading on ConA, a glycoprotein-reactive ligand, and FN it was hoped to be able to assess the relative importance of gangliosides and glycoproteins in FN-mediated cell spreading.

A number of lines of evidence suggest that FN is capable of interacting with the intracellular microfilament system. Addition of exogenous FN to cultures of transformed fibroblasts results in a dramatic reorganisation of cytoplasmic actin into microfilament bundles (Ali et al, 1977). Similarly, attachment of many cell types to FN-coated substrata is followed by active cytoskeletal reorganisation resulting in cell spreading and the formation of actin-containing microfilaments. At an ultrastructural level FN has been shown to have a coincident distribution to intracellular actin microfilaments on the ventral cell surface (Hynes and Destree, 1978). Such close (8-20nm) association of extracellular FN fibrils with actin microfilaments has been termed the fibronexus (Singer, 1979). As FN itself is not thought to be a membrane protein it is assumed that the transmembrane interaction of FN with the cytoskeleton occurs via its receptor structure. Given that gangliosides are incapable of spanning the plasma membrane (Hakomori, 1981) and are thus unable to directly link FN to the cytoskeleton it is important to

establish whether gangliosides are capable of mediating such transmembrane events. Indirect evidence suggests that gangliosides are capable of cytoskeletal interaction although their localisation to the outer leaflet of the plasma membrane does not seem in question. Firstly, it has been demonstrated that ganglioside GM₁ remains associated with Triton X-100 cytoskeletons of Balb/c 3T3 cells (Streuli et al., 1981). Under conditions where greater than 80% of total cellular protein is solubilised approximately 80% of ¹²⁵I-labelled CT bound prior to extraction remained associated with the detergent insoluble material. However, bound toxin could be almost quantitatively extracted from liposomes prepared from total cellular lipid (Sahyoun, 1981). In addition, greater than 50% of the capacity of intact cells to bind ¹²⁵I-labelled CT was retained following detergent extraction (Streuli et al., 1981). Interestingly CT-GM₁ complexes extracted from rat erythrocytes with high detergent concentration ran as a macromolecular complex on gel permeation columns suggesting an interaction of CT-GM₁ complexes with other membrane proteins (Sahyoun et al., 1981). Further evidence of ganglioside interactions with the cytoskeleton comes from data demonstrating that GM₁ can be induced to patch and cap in lymphocyte preparations by the addition of CT (Revesz and Greaves, 1975). Furthermore, the capping process is inhibited by cytochalasin B and is accompanied by co-capping of the cytoskeletal protein α actinin suggesting an interaction of membrane GM₁ with polymerised actin on the inner surface of the plasma membrane (Kellie et al., 1983). There is thus substantial but indirect evidence demonstrating an association of gangliosides with the cytoskeleton. However, the possibility that detergent insolubility might not necessarily reflect cytoskeletal association and the proposal that the capping process

might be the result of lipid flow rather than cytoskeleton-induced movement (Bretscher, 1976; Bretscher, 1984) makes it important to establish more directly whether the interaction of FN with a membrane ganglioside could be sufficient to mediate the cell spreading response.

Substrata coated with either FN, CT or ConA were all able to promote stable cell attachment and spreading. Cell spreading was related to the density of substratum-bound ligand although maximal cell spreading on CT and ConA required approximately 15- and 3-fold higher ligand densities respectively than on FN. A similar relationship has been demonstrated for BHK cell spreading on ricin and ConA where the ligand density required to trigger cell spreading was 10- and 40-fold higher respectively than for FN (Aplin and Hughes, 1981a). The higher levels of these ligands required to trigger cell spreading was suggested to be due to only a small percentage of the ricin or ConA binding sites at the cell surface being functionally active in triggering cell spreading. This receptor population might represent the FN receptor. That a common molecular species is involved in cell spreading on the above three ligands was further supported by the demonstration that a 47Kd glycoprotein can be cross-linked to ConA, ricin and FN following BHK cell spreading on these ligands coupled to a photoactivatably cross-linking reagent (Aplin et al., 1981c). Since CT binding to the cell surface is not thought to involve a 47Kd glycoprotein directly, it would be interesting to determine if this component becomes cross-linked to CT during CT-mediated cell spreading. Aplin et al. (1981a) proposed that an inverse relationship existed between the ligand density required to trigger cell spreading and the number of ligand receptors at the cell surface. Increasing the

receptor number for CT by neuraminidase treatment of Balb/c 3T3 cells caused a 3-fold reduction in the level of CT required for 50% cell spreading in agreement with the above relationship. Interestingly, neuraminidase treatment of cells had no effect on their ability to spread on FN suggesting that neuraminidase-sensitive structures are not involved in FN-mediated adhesion (see Chapter 4 for a more detailed discussion of this phenomenon). The threshold FN density required to trigger Balb/c 3T3 cell spreading (40fmoles/cm^2) was similar to a figure of 30fmoles/cm^2 reported to be required to trigger BHK cell spreading (Hughes et al., 1979).

Although cell spreading was demonstrated on all three ligands the resultant spread morphologies were clearly different suggesting that the spreading response on FN was mechanistically different from that on CT or ConA. Whilst cells spread on FN closely resembled the typical fibroblastic morphology of cells under normal tissue culture conditions, cells spread on ConA produced wide, thin lamellar structures and cells spread on CT showed irregular cytoplasmic processes associated with numerous filopodial like structures. The different morphologies expressed by these cells on each ligand was surprising since BHK cells spread on ConA and other ligands are morphologically identical to cells spread on FN (Grinnell and Hays, 1978). The atypical morphology of cells spread on CT was shown not to be due to adenyl cyclase activation by contaminating CT A subunit since addition of high concentrations of the B subunit preparation ($100\mu\text{g/ml}$) to Balb/c 3T3 cell monolayers produced no morphological effect while whole toxin at concentrations as low as 0.1ng/ml still produced a significant effect. In addition,

radioiodination of the B subunit preparation showed no evidence of A subunit contamination. The possibility that the different morphologies was simply a reflection of variations in the adsorption of the ligand to substratum was also ruled out since the binding of each ligand to both plastic and glass substrata were of a similar level. The amounts of FN and ConA bound to microtitre wells are also in agreement with previously published figures (Rauvala et al., 1981).

The specificity of the spreading response for the individual ligands was shown by the use of ligand-specific antibodies. It was further demonstrated that CT-induced cell spreading was due to an interaction with ganglioside GM₁ by inhibiting cell spreading on CT with low levels of this ganglioside (50-100nM) but not by other gangliosides. It is interesting to note that the concentration of ganglioside required to inhibit CT-mediated cell spreading is approximately 200-fold lower than the levels of ganglioside required to inhibit FN-mediated cell spreading (10 μ M GD; Perkins et al., 1982).

FN, CT and ConA, through interaction with their respective receptor structures, thus appear to trigger three morphologically distinct spreading responses. All three morphologies appear to be generated by an active, microfilament-mediated process, since sodium azide and cytochalasin B are both potent inhibitors of cell spreading on each ligand. Similar morphologies to that displayed by CT and ConA spread cells have been reported to occur during the early stages of fibroblastic cell spreading in serum-containing medium or on FN-coated substrata (Rajaraman et al., 1974; Hughes et al., 1979). This might suggest that

CT and ConA are able to trigger the early stages of cell spreading but not the subsequent stages. We thus examined the ability of each ligand to trigger the formation of focal contacts and the development of microfilament bundles in these cells.

Within 2 hours of plating onto FN-coated substrata most of the cells were well spread and many had well organised actin staining in the form of long microfilament bundles. By 4 hours after plating, extensive microfilament organisation was evident in the majority of cells. In contrast, neither CT- or ConA-coated substrata were capable of inducing the formation of long microfilament bundles even 4 hours after plating. Limited F-actin staining at the periphery of the spread cells was however apparent. When further examined for the presence of focal contacts by IRM and an antibody exclusion technique it became apparent that cells developed focal contacts on FN but not on CT or ConA. Immunofluorescent staining for vinculin, a cytoskeletal protein known to be concentrated at focal contacts clearly demonstrated the presence of vinculin-containing focal contacts in cells spread on FN-coated substrata. No such staining was evident in cells spread on either ConA- or CT-coated substrata. It has previously been suggested that FN is not capable of promoting the formation of focal contacts unless additional serum factors are present (Thom, Powell and Rees, 1979). More recently however Laterra et al. (1983a) demonstrated focal contact formation in Balb/c 3T3 cells plated onto FN-coated glass and Couchman et al. (1983) has similarly shown focal contacts in human embryonic skin fibroblasts plated onto FN. Here we confirm that Balb/c 3T3 cells are capable of forming focal contacts on FN-coated substrata in serum-free medium

although in our hands the focal contacts appear less well developed than in serum-containing medium. The formation of focal contacts has been suggested to require secretion of endogenous cell FN although not required for cell spreading alone (Virtanen, Vartio, Badley and Lehto, 1982). Interestingly we have noted the appearance of extracellular FN in cells spread on FN-coated substrata but not on ConA or CT. However, it was not possible to determine whether this was endogenous, secreted FN or FN from the substratum.

The difference in morphology resulting from spreading on FN, CT and ConA may thus be due to the latter two ligands being unable to trigger the formation of well developed microfilament bundles or vinculin-containing focal contacts. It is known that BHK cell spreading on ConA is morphologically similar to that on FN. However, preliminary results in this laboratory suggest that although morphologically similar, cells spread on ConA-coated substrata do not contain well developed actin cables or focal contacts. This suggests that although a number of ligands capable of binding to cell surface components are able to promote cell spreading they may not necessarily trigger the formation of specialised adhesive structures such as focal contacts. To date only FN and the basement membrane protein laminin have been shown to promote focal contact formation (Couchman et al., 1983) although Morgan and Garrod (1984) have recently demonstrated that focal contact formation in Hela cells is independent of FN or LN suggesting that these adhesive protein are not essential components of such cell-substratum adhesion sites.

One possible explanation for the inability of CT to promote cell spreading similar to FN is that CT alone is not capable of cross-linking its receptor into large macromolecular structures due to the fact that one GM₁ molecule can bind only one CT molecule. It has previously been suggested that cross-linking of cell surface receptors and thus ligand valency may play a key role in triggering the spreading response (Carter et al., 1981). One way to get around this problem is to bind CT to the cell surface and look at cell spreading on substrata coated with anti-CT antiserum. However, preliminary experiments using a 1gG fraction of a rabbit anti-CT antiserum failed to give cell spreading of Balb/c 3T3 cells pretreated with CT although specific cell attachment could be demonstrated. The problem of ligand valency does not appear to be the cause of the morphological differences however, since ConA, which is capable of cross-linking its cell surface receptors, is also unable to trigger the same morphological and cytoskeletal responses as FN.

By a comparison of cell spreading on CT, ConA and FN it was hoped to be able to make some comment on the relative importance of cell surface glycoproteins and glycolipids in mediating cell spreading on FN. In making such a comparison one has to make the assumption that the interaction of a ligand with the FN receptor will illicit some or all of the physiological effects of FN itself. Thus, it might be predicted that BHK cell spreading on ConA is the result of ConA binding to the FN receptor at the cell surface. During this study it was found that to achieve good cell spreading on CT, the cells had to be pretreated with neuraminidase so as to increase the number of CT receptors

Under such conditions much of the proposed FN receptors, di- and tri-sialogangliosides, are converted into CT receptor, GM₁. If these gangliosides are in fact receptors for FN one might expect that CT should induce cell spreading similar to FN itself. This clearly does not occur.

In a recent series of experiments attempting to evaluate the potential importance of cell surface heparan sulphate in FN-mediated cell spreading Laterra et al. (1983b), taking a similar approach to us, compared Balb/c 3T3 cell spreading on FN with the heparan sulphate-binding protein, PF4. Interestingly PF4-coated substrata were only able to induce partial cell spreading, similar to ConA and CT, and were unable to trigger the formation of microfilament bundles or focal contacts (Laterra et al., 1983a). It was also demonstrated that removal of cell surface heparan sulphate with heparinase inhibited cell spreading on FN although such treatment did not affect cell attachment. It was proposed that complete adhesion of Balb/c 3T3 cells requires binding of FN to both its receptor and cell surface heparan sulphate proteoglycans. It would thus be interesting to examine whether a combination of CT or ConA with PF4 might induce cell spreading similar to FN itself. In addition, it is important to demonstrate whether the cell binding domain of FN is capable of inducing the formation of microfilament bundles and focal contacts in these cells since this has no heparan sulphate binding component.

In conclusion, it has been demonstrated that both cell surface glycoproteins and gangliosides are capable of mediating cell spreading

and a limited degree of cytoskeletal reorganisation. However, neither of these components alone were capable of mediating the formation of the specialised adhesive structures associated with cells spread on FN. This suggests that a cooperative interaction with two or more cell surface components, possibly heparan sulphate, may be required for complete spreading of Balb/c 3T3 cells.

CHAPTER 4

THE RELATIONSHIP BETWEEN **LEVELS OF CELLULAR** **GANGLIOSIDES AND THE** **ABILITY TO INTERACT** **WITH FN**

INTRODUCTION

If complex gangliosides (GD1a/GT) are physiologically active receptors for FN it should be possible to demonstrate a correlation between cellular ganglioside levels and the ability to interact with FN. I have attempted to identify such a correlation by the following approaches.

- (i) Comparison of the ability of Balb/c 3T3 cells and variants deficient in complex gangliosides to spread on FN and to organise it in a matrix form.
- (ii) Add back experiment using cell lines lacking, or containing low levels of complex gangliosides. Does increasing the levels of plasma membrane gangliosides by addition of exogenous gangliosides increase the ability to interact with FN?
- (iii) Enzymatic conversion of cellular GD1a and GT to GM₁ by neuraminidase. Is such treatment accompanied by a reduced ability to interact with FN?

A number of workers have used lectin-resistant mutant cell lines to assess the significance of alterations in cell surface carbohydrate to various cellular recognition and control functions (see review by Briles, 1982; Gottlieb and Kornfeld, 1976; Finne, Burger and Prieels, 1982). A number of such lectin-resistant mutants have been demonstrated to display

altered adhesion characteristics. Thus, a series of ricin-resistant mutants of BHK cells have been isolated which have reduced levels of ricin-binding sites (galactose-containing glycoconjugates) at the cell surface and also show reduced adhesion to the substratum (Meager, Ungkitchanukit, Nairn, and Hughes 1975; Edwards, Dysart and Hughes, 1976). It was subsequently demonstrated that a number of these mutant cell lines displayed a reduced ability to spread on FN-coated substrata and had lower levels of cell-associated FN compared to the parental BHK cell line. (Pena and Hughes, 1978). However, due to the pleiotropic cell surface changes associated with ricin-resistance, it has not been possible to identify which particular alteration is responsible for the impaired ability to bind to FN. A number of other mutant cell lines, defective in some aspect of cell surface glycosylation, have been identified which also display altered adhesion characteristics (Pouyssegur, Willingham and Pastan, 1977; Barnhart, 1979). In the past, support for a correlation between the levels of gangliosides and the ability to interact with FN came from a comparison of normal and virally-transformed cell lines. Such transformants frequently possess reduced levels of gangliosides and generally display a reduction in cell-associated FN. (Mora, Brady, Bradley and McFarland, 1969; Brady and Mora, 1970; Hynes, 1973; 1976). However, no strict correlation between the levels of specific gangliosides and the ability to bind FN could be demonstrated. In this study I have used four variant cell lines derived from Balb/c 3T3 cells and deficient in complex gangliosides to investigate the relationship between cellular ganglioside levels and the ability of these cells to interact with FN. The variant cell lines

were isolated in this laboratory by Dr. C.H. Streuli using a selection procedure based on the known specificity of CT for ganglioside GM₁. CT binding cells were specifically killed using anti-toxin antisera and complement mediated lysis. Extensive investigation of the nature of the CT receptor in Balb/c 3T3 cells clearly identified GM₁ as the sole receptor species with no evidence of any CT-binding glycoproteins. (Critchley et al. 1982; Streuli, 1982). Analysis of these variants demonstrated a marked reduction in the number of CT binding sites together with greatly reduced levels of complex gangliosides (Streuli, Griffiths and Critchley, 1984). In addition, no evidence of any gross alterations in the glycoprotein profiles of these cells was detected using cell surface labelling techniques. (Griffiths, Perkins, Streuli and Critchley, in preparation).

As a second approach, I have utilised the known ability of exogenously applied gangliosides to become functionally incorporated into the plasma membrane of recipient cells (Laine and Hakomori, 1973; Keenan et al. 1975; Fishman, Moss and Vaughan, 1976; Callies et al. 1977; Radsak et al. 1982). The transformed mouse fibroblast cell line NCTC 2071A is deficient in complex gangliosides and unresponsive to CT (Moss, Fishman, Manganiello, Vaughan and Brady, 1976). However, it can be converted to CT sensitivity by growth of the cell line in ganglioside-containing medium (Fishman et al. 1976). That ganglioside had in fact become functionally incorporated into the plasma membrane was demonstrated by the finding that bound CT was able to activate adenyl cyclase and thus increase cAMP levels (Fishman et al. 1976). The ability to incorporate exogenous gangliosides into cell

membranes has been used to investigate the significance of the loss of certain gangliosides following cell transformation. Addition of exogenous gangliosides to cultures of NIL hamster fibroblasts has been shown to result in a reduced growth rate (Laine and Hakomori, 1973). Similarly, addition of gangliosides, in particular monosialogangliosides, to cultures of both normal and SV40-transformed Swiss 3T3 cells results in a significantly reduced growth rate and saturation density (Keenan et al. 1975). Detailed analysis of the ability of gangliosides to incorporate into cell membranes and artificial lipid bilayers has been made by Wiegandt's group (Kanda, Inone, Nosima, Utsumi and Wiegandt, 1982; Callies, Radsak, Siebert and Wiegandt 1977; Radsak, Schwarzmann and Wiegandt, 1982). It was demonstrated that plasma membrane-associated gangliosides existed in three forms, (i) a serum- and serum albumin-sensitive form, (ii) a trypsin-sensitive form and (iii) a trypsin- and serum-resistant form (Radsak et al. 1982). It was proposed that the latter, trypsin resistant form, which accounts for approximately 10% of the total gangliosides bound, probably represented gangliosides inserted in the membrane via its ceramide moiety. Once inserted into the cell membrane the gangliosides appear to be relatively stable and not significantly metabolised (O'Keefe and Cuatrecasas 1977). Here I have investigated the effect of exogenous gangliosides on the ability of NCTC 2071A cells and BHK cells to interact with FN.

In a third approach I have looked at the effect of neuraminidase-treatment of Balb/c 3T3 and BHK cells on their ability to spread on FN-coated substrata. Neuraminidase treatment has previously been shown to increase CT binding to cells as a consequence of the conversion of di- and

tri-sialogangliosides into GM₁ (Haksar, Maudsley and Peron, 1974; Critchley and Vicker, 1977). Given the inverse relationship between the number of ligand receptors and the ligand density required to trigger cell spreading (Aplin and Hughes, 1981a) and the proposed nature of the FN receptor as di- and/or tri-sialogangliosides, one would expect neuraminidase to reduce cell spreading on FN-coated substrata.

MATERIALS AND METHODS

Cell Culture

A subclone of NCTC 2071 murine cell line previously shown to be deficient in gangliosides (Moss et al. 1976) was obtained from Dr. J. Moss, N.I.H., Bethesda, U.S.A. (NCTC 2071A). Cells were grown in serum-free NCTC 135 medium and subcultured by scraping with a sterile rubber policeman. A subclone (S24) of the Balb/c 3T3 A31 murine cell line, shown to closely resemble the parent line in ganglioside content and growth characteristics, was used to isolate variants defective in ganglioside biosynthesis (Streuli, 1982; Streuli, Griffiths and Critchley, 1984). Ethane methane sulphonate (EMS) mutagenised S24 cells were selected for variants defective in the synthesis of gangliosides more complex than GM₃ using CT, anti-toxin and complement-mediated lysis (Streuli, 1982). Variants were cloned from populations surviving 5-6 rounds of selection. Cells were routinely cultured in DMEM containing 10% heat-inactivated NCS plus antibiotics/antimycotics and subcultured twice weekly with 0.25% trypsin. BHK 21 cells were cultured as described for Balb/c 3T3 cells above. All media and tissue culture reagents were obtained from Gibco Bio-cult.

Ganglioside analysis

Ganglioside analysis was performed either using unlabelled material and resorcinol detection or metabolically labelled material and autoradiography. For metabolic labelling of cellular lipid, exponentially growing

cells were cultured for 48 h. in complete medium containing 1 μ Ci/ml 1 - 14 C - palmitic acid (55 mCi/mole, Radiochemical Centre, Amersham). Cell monolayers were washed twice with PBS and scraped into PBS containing 1mM PMSF with a rubber policeman. Cell pellets were resuspended in 2 ml methanol and probe sonicated (4 x 15 secs. 0°C) and aliquots taken for protein estimation and isotope incorporation. Lipids were extracted by the addition of 2 vols. of chloroform and the pellet re-extracted twice with 1:2 (v/v) chloroform:methanol. The extract was dried under N₂, re-dissolved in chloroform:methanol (2:1) and partitioned against 0.2 vols. of water. The organic phase was washed three times with chloroform:methanol:water (3:48:47) and the combined aqueous phases dried, then desalted on a Sephadex G25 (Superfine) column (0.75 gms), equilibrated and diluted with chloroform:methanol:water (60:30:4.5). Extracted gangliosides were separated by thin layer chromatography on precoated Silica Gel G plates (Merck) with the solvent chloroform:methanol:water (60:35:8), and detected by spraying with resorcinol as described in Chapter 2, Materials and Methods.

Cell spreading on ligand-coated substrata

Spreading of Balb/c 3T3 cells and variants on FN and CT coated substrata was assayed as described in Chapter 3. BHK cell spreading on FN was assayed as described in Chapter 2. Where indicated, BHK cell monolayers were treated with *Vibrio cholerae* neuraminidase (0.04 units/ml of DMEM; Behringwerke) for 1 h, 37°C. prior to trypsinisation and plating onto FN-coated libro wells. Cell monolayers were washed twice with DMEM prior to enzyme treatment to remove serum components known to inhibit

neuraminidase activity. Crystal violet stained cells were photographed on a Leitz diavert microscope using Ilford Pan F film (50ASA).

Incubation of cells with ganglioside-containing medium

For experiments involving growth of NCTC 2071A cells in ganglioside containing medium, bovine brain mixed gangliosides (Sigma) were dissolved in water at 1 mg/ml and incubated at 37°C for 1 h prior to addition to culture medium. Scraped NCTC 2071A cells were plated onto glass coverslips in 35 mm petri dishes containing 1 ml of NCTC 135 medium and allowed to attach for 4 h , 37°C. Where appropriate, stock gangliosides were added directly to the culture medium, the cells further incubated for 18 h , and then fixed and stained for FN as described below.

Incorporation of gangliosides into BHK cell membranes was performed as described previously by Beckner et al. (1981). BHK cell monolayers were washed twice with DMEM and then incubated with bovine brain mixed gangliosides (250 µg/ml, 2 ml DMEM per 10 cm petri dish) for 1 h , 37°C. Monolayers were further washed with DMEM, trypsinised and plated onto FN-coated linbro wells.

Immunofluorescence

Cells grown on glass coverslips were washed twice with PBS and fixed with 3.8% formaldehyde/PBS for 45', RT⁰. After rinsing 3 times with PBS over 30', coverslips were incubated for 1 h , RT⁰, with rabbit anti-FN

diluted 1:50 with PBS (see Appendix II for antibody production and characterisation). Coverslips were further washed three times with PBS over 30' and bound primary antibody detected using an FITC-conjugated goat-anti-rabbit antiserum (Miles biochemicals) diluted 1:25-1:50 with PBS (1 h , RT⁰).

For localisation of CT bound to cell surfaces the toxin (1 µg/ml in PBS containing 0.1% BSA) was incubated with washed monolayers (5', 37°C) grown on coverslips before washing and formaldehyde fixing. Bound CT was then visualised using a rabbit anti-CT antiserum diluted 1/30 with PBS and an FITC-conjugated goat anti-rabbit antiserum diluted 1/50 with PBS. Coverslips were washed with PBS and mounted on glass microscope slides using 50% glycerol in PBS as mountant. Immunofluorescence was visualised using a Zeiss Standard 16 microscope equipped with epifluorescence. Photographs were taken on Ilford HP5 film 400 ASA uprated to 800 ASA.

Interference reflection microscopy

Essentially as described in Chapter 3, S24 cells and ganglioside-deficient variants were plated onto glass coverslips in DMEM containing 10% heat-inactivated NCS and incubated overnight, 37°C, in a CO₂ incubator. After gentle washing in DMEM, live cells were viewed on a modified Zeiss Standard 16 microscope with an X63 antiflex objective lens as described previously. Images were photographed on Ilford Pan F film (50 ASA).

RESULTS AND DISCUSSIONS

The ability of ganglioside-deficient variants of Balb/c 3T3 cells to interact with FN

Balb/c 3T3 cell variants deficient in complex gangliosides have been isolated and characterised in this laboratory (Streuli, 1982; Streuli et al. 1984; Griffiths, Perkins, Streuli and Critchley, in preparations). It has been demonstrated that these variants have a greatly reduced ability to bind CT (Table 4.1) and have a simplified ganglioside profile. A dramatic reduction in the levels of GM₁ and disialogangliosides was evident in the variants compared to the parental 3T3 cell line, S24 (Fig.4.1 and 4.2). Separation of cellular gangliosides by thin layer chromatography clearly shows that gangliosides more complex than GM₃ are not detectable in variants M1, M3, M6 and M7 by metabolic labelling with ¹⁴C-palmitate (Fig.4.1; D, E, F, G,) or resorcinol spraying of unlabelled gangliosides (Fig.4.2; B, C, D, E). Although GM₁ was consistently visualised in metabolically labelled material from S24 cells, the levels of GDla were somewhat variable (compare Fig.4.1; C and H). GDla was, however, consistently reduced or absent in the variant cell lines as determined by either metabolic labelling or resorcinol spraying. The reason for the variable levels of GDla in S24 cells is not clear at present although a transient increase in the levels of GDla has been reported to occur in Swiss 3T3 cells during the early stages of cell contact (Yogeeswaran and Hakomori, 1975). The absence of GM₁ in the variant cell lines has recently been confirmed by the use of the Magnani overlay technique (Magnani et al. 1980).

Table 4.1 - Quantitation of CT binding to Balb/c 3T3 cells and ganglioside-deficient variants¹

<u>Cell line</u>	<u>Number of CT molecules bound per cell x 10⁻⁶</u>
S24	2.2
M1	0.18
M3	0.1
M6	0.1
M7	0.1
S24 + N*	11.0
M3 + N	0.1
M6 + N	0.3

*Cells pretreated with neuraminidase (0.01 I.U. per ml DMEM/0.1% BSA/PMSF, 1 h., 37°C).

¹ This figure is taken from Streuli, (1982).

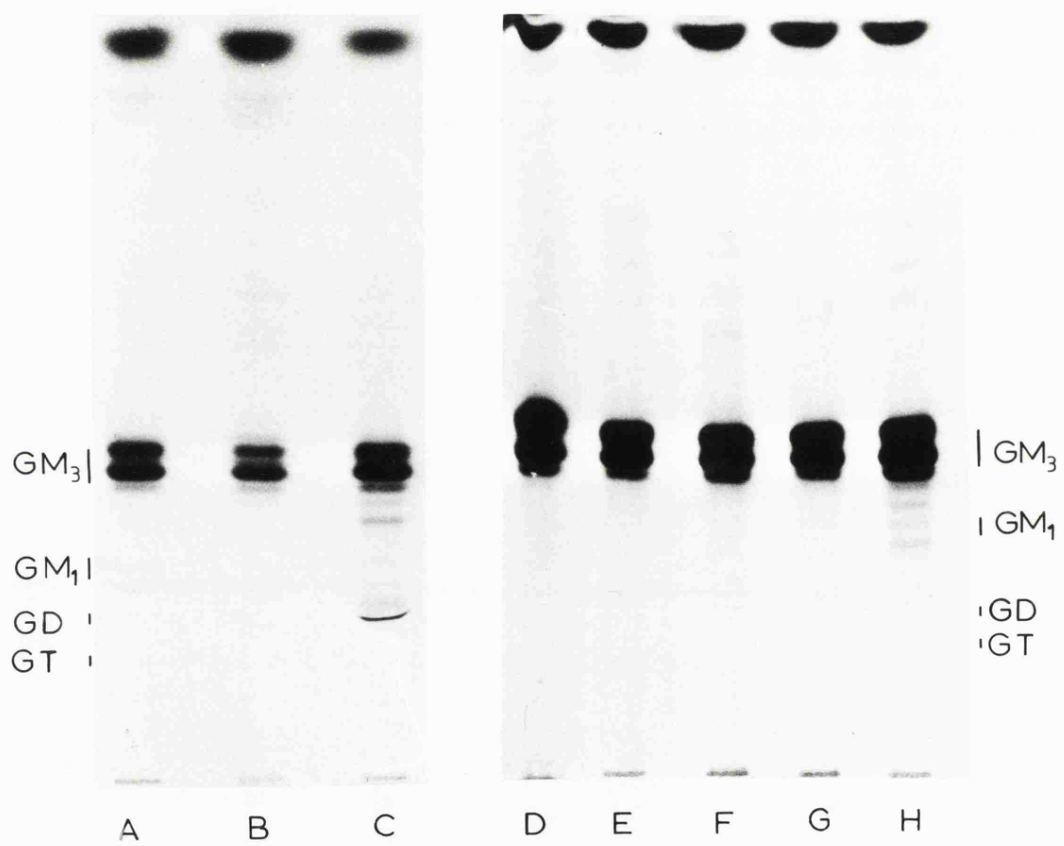


Fig. 4.1 - ^{14}C -palmitate-labelled ganglioside profiles of BHK, Balb/c 3T3 and ganglioside-deficient variant cells

Exponentially growing cells were labelled with $1\text{-}^{14}\text{C}$ palmitic acid to specific activities of $4\text{-}8 \times 10^6$ CPM/mg of protein. Monolayers were then scraped and gangliosides analysed as described in Materials and Methods. Approximately 100,000 CPM of aqueous phase lipids were loaded onto each track. Where indicated, prelabelled BHK cells were treated with *Vibrio cholerae* neuraminidase (0.04 U/ml DMEM 1 h , 37°C) prior to ganglioside analysis.

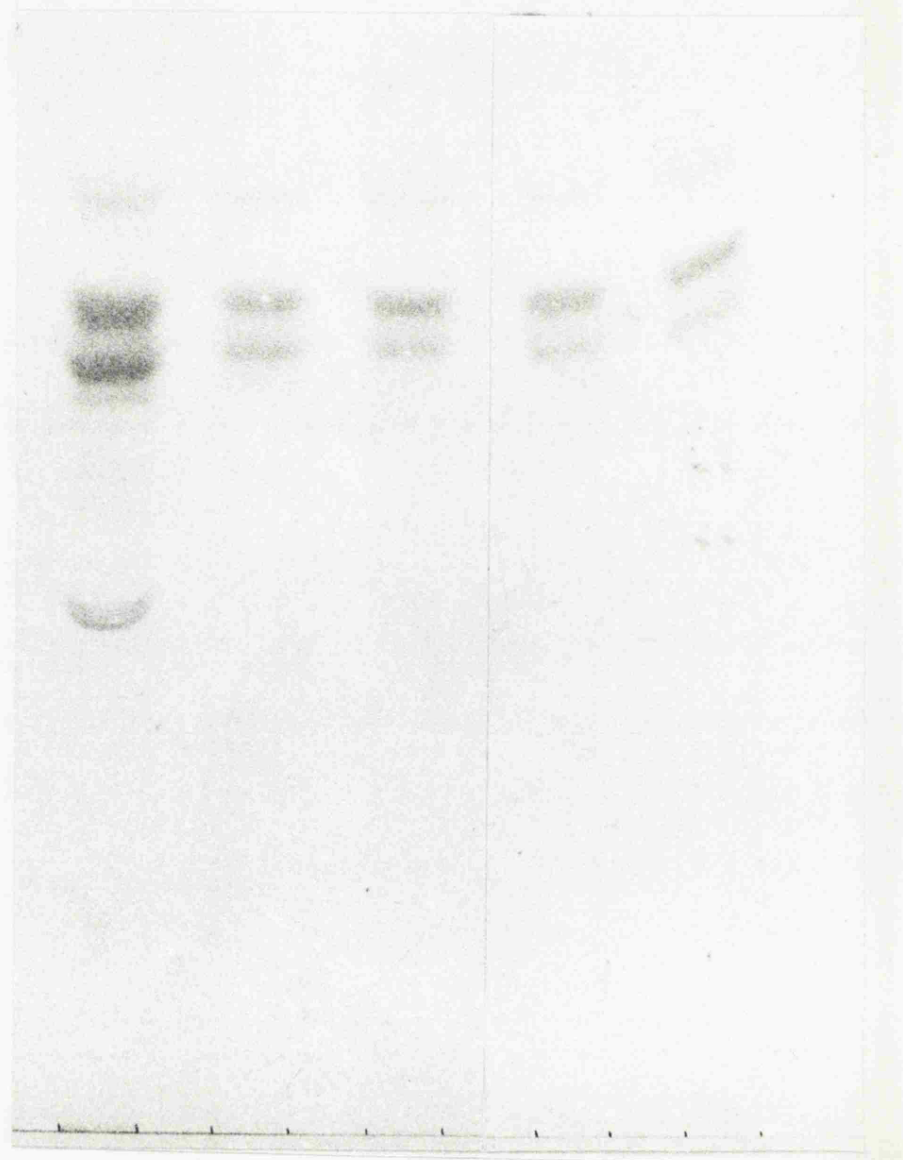
Track A,	BHK cells plus neuraminidase
Track B,	BHK cells minus neuraminidase
Track C,	Balb/c 3T3 (S24) cells
Track D,	M7 cells
Track E,	M6 cells
Track F,	M3 cells
Track G,	M1 cells
Track H,	Balb/c 3T3 (S24) cells.

GM3 |

GM1 |

GD |

GT |



A

B

C

D

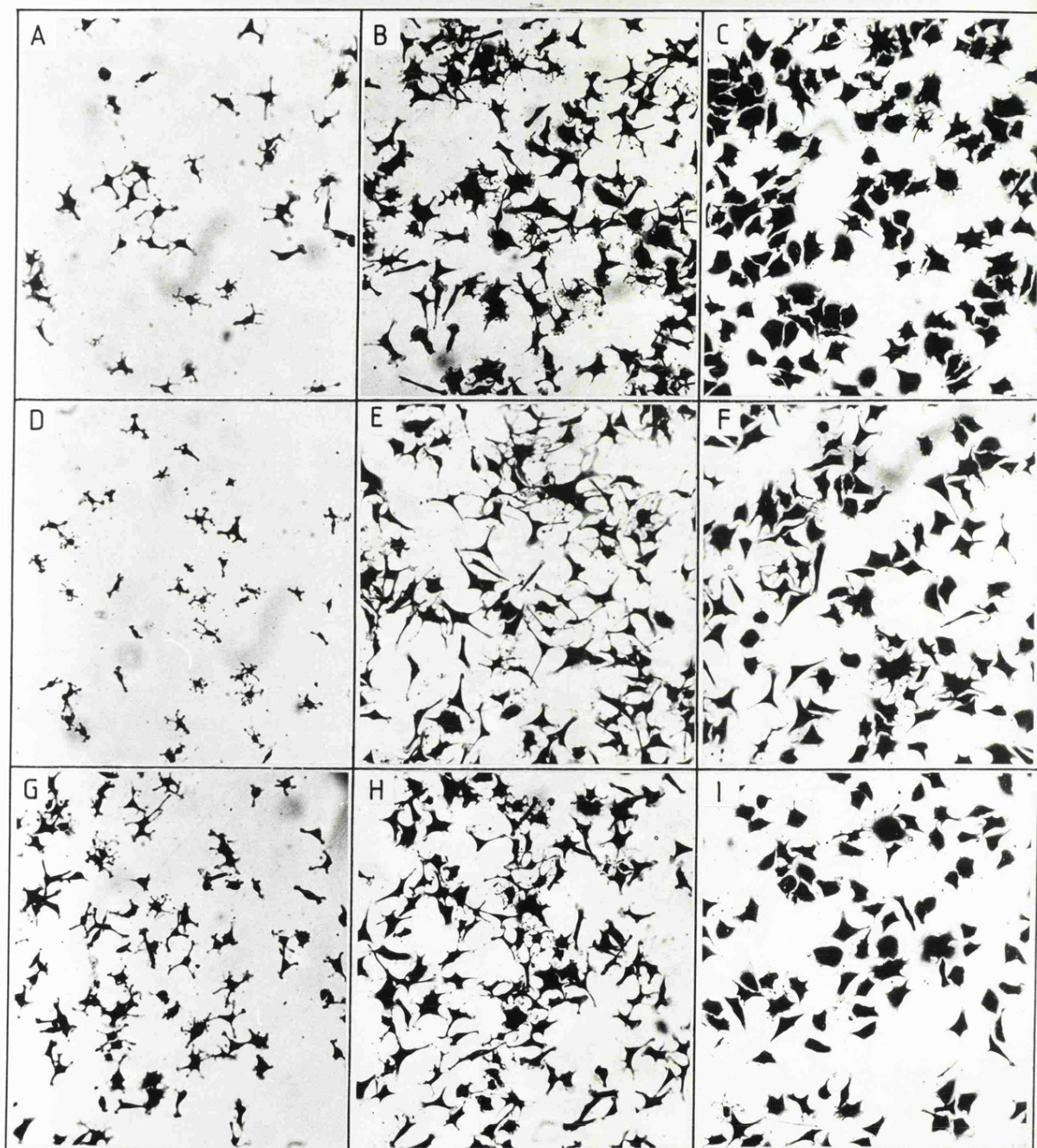
E

Fig 4.2 - Ganglioside profile of Balb/c 3T3 and ganglioside-deficient cells as detected by resorcinol spraying

Unlabelled gangliosides were isolated and separated by TLC as described in Materials and Methods. Separated gangliosides were then detected with resorcinol reagent as described in Chapter 2. Tracks were normalised for protein (900 mg protein equivalents per track).

Track A	Balb/c 3T3 (S24) cells
Track B	M1 cells
Track C	M3 cells
Track D	M6 cells
Track E	M7 cells.

Gangliosides from S24 and the four variant cell lines were separated by thin layer chromatography and then incubated with ^{125}I -labelled CT. Although strong binding was demonstrated to a ganglioside present in S24 cells with a similar mobility to GM_1 , no such binding was evident to gangliosides from the variant cells. (Griffiths et al. in preparation). Given that these cells are able to synthesise normal levels of GM_3 but much reduced levels of GM_1 , a defect in the enzymatic pathway which converts GM_3 into GM_1 seems likely. From the proposed biosynthetic pathway for the main gangliosides in animal cells (Critchley and Vicker, 1977), a lesion at this position would lead to an inability to synthesise the di- and trisialogangliosides (GD1a , GT1a , GD1b , etc.). Further support for a reduction in the levels of di- and trisialogangliosides in the variant cells is demonstrated in Table 4.1. Neuraminidase-treatment of at least two of the variants (M3 and M6) does not result in any appreciable increase in CT binding while similar treatment of S24 cells results in a 5-fold increase in binding. This increase in CT binding is believed to be due to conversion of disialogangliosides into GM_1 by neuraminidase. (Critchley and Vicker 1977). There is thus good evidence that the variant cell lines possess significantly reduced levels of complex gangliosides. However, if these cells are to be used to assess the importance of gangliosides as receptors for FN, it is important to demonstrate that no significant alterations in the glycoprotein profile of these cells has occurred during their selection. Cell surface labelling by lactoperoxidase-catalysed iodination or periodate/borotritide and ^{125}I -ricin overlays of SDS-PAGE separated cell protein failed to detect any gross alterations in the



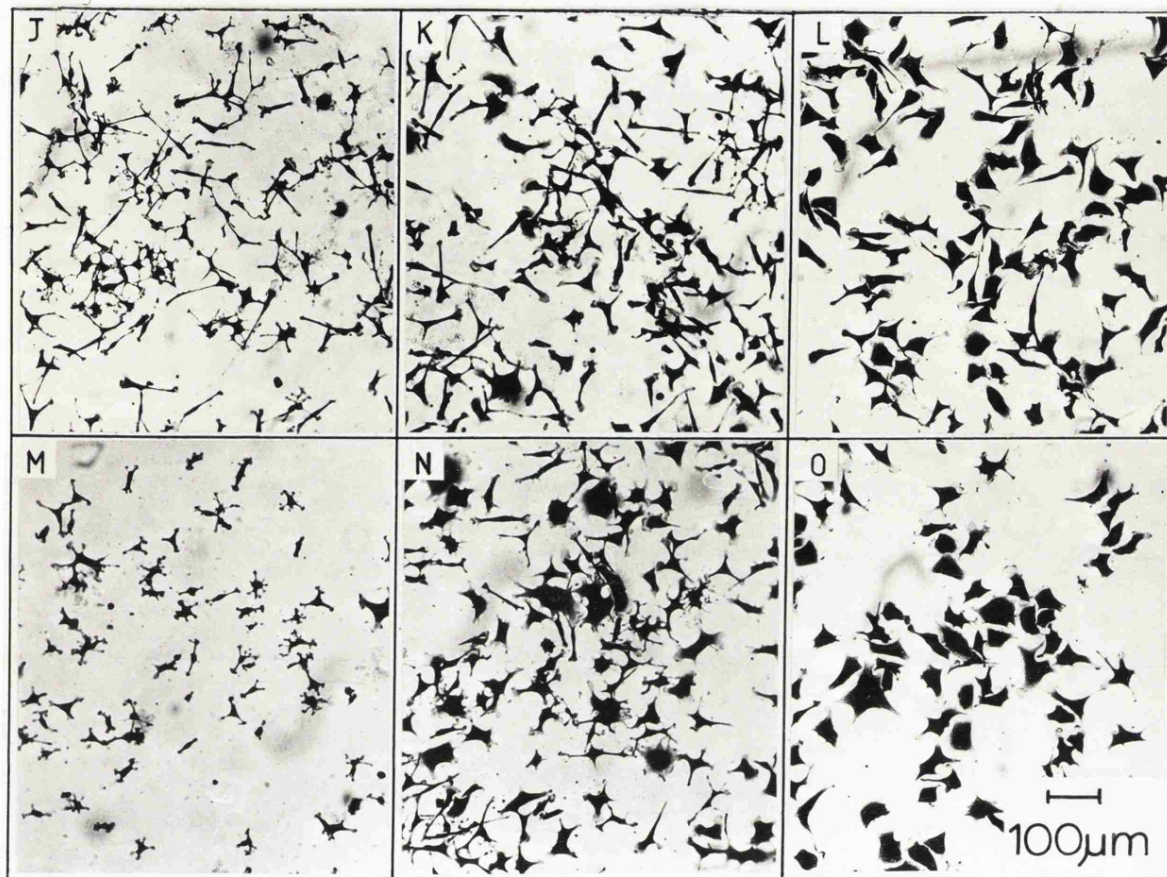


Fig. 4.3 - Ability of ganglioside-deficient variant cells to spread on FN-coated substrata

Microtitre wells were coated with varying concentrations of FN for 2 h , 37°C and subsequently blocked with 10 mg/ml BSA/PBS for 1 h , 37°C. Wells were washed twice with PBS, 1×10^4 cells added in 100 µl DMEM and allowed to spread for 1.5 h 37°C in a CO₂ incubator. Cells were then fixed and stained as described in Materials and Methods.

A,D,G,J,M, 0.75 µg/ml FN; B,E,H,K,N, 1.5 µg/ml; C,F,I,L,O, 10 µg/ml FN.

A - C,	Balb/c 3T3 (S24) cells
D - F,	M1 cells
G - I,	M3 cells
J - L,	M6 cells
M - O,	M7 cells.

glycoprotein profile of the variant cells compared to S24 cells (Griffiths et al. in preparation). These variants thus form an ideal system for analysing the role of complex gangliosides in FN binding to the cell surface.

When plated onto FN-coated microtitre wells both S24 and the variant cells (M1, M3, M6 and M7) attached and adopted a well spread, fibroblastic morphology within 2 hours of plating (Fig.4.3). At low FN-coating concentration (0.75 $\mu\text{g/ml}$), little or no cell spreading was evident in either S24 or the variant cells although they had become attached and formed filopodial processes (Fig.4.3; A, D, G, J, M). In the absence of any FN-coating, no cell attachment was observed. The cell line M6, although not being able to spread at an FN concentration of 0.75 $\mu\text{g/ml}$ showed a greater tendency to adopt an elongated bi-, or tri- polar morphology than S24 or the other variant cells (Fig.4.3; J). At a coating concentration of 1.5 $\mu\text{g/ml}$, many of the cells were well spread while others, apparently in the early stages of spreading, were less well spread with long, tapering cell processes (Fig.4.3; B, E, H, K, N). No difference between S24 and the variant cells was apparent. At 10 $\mu\text{g/ml}$ the majority of cells were well spread in all cell lines although M6 appeared to have a slightly more elongated morphology than S24 or the other variants (Fig.4.3; L).

Quantitation of the ability of these cells to spread on FN-coated substrata (Fig.4.4) clearly demonstrates that the ganglioside-deficient variants M1, M3 and M7 had a similar ability to spread on FN to the

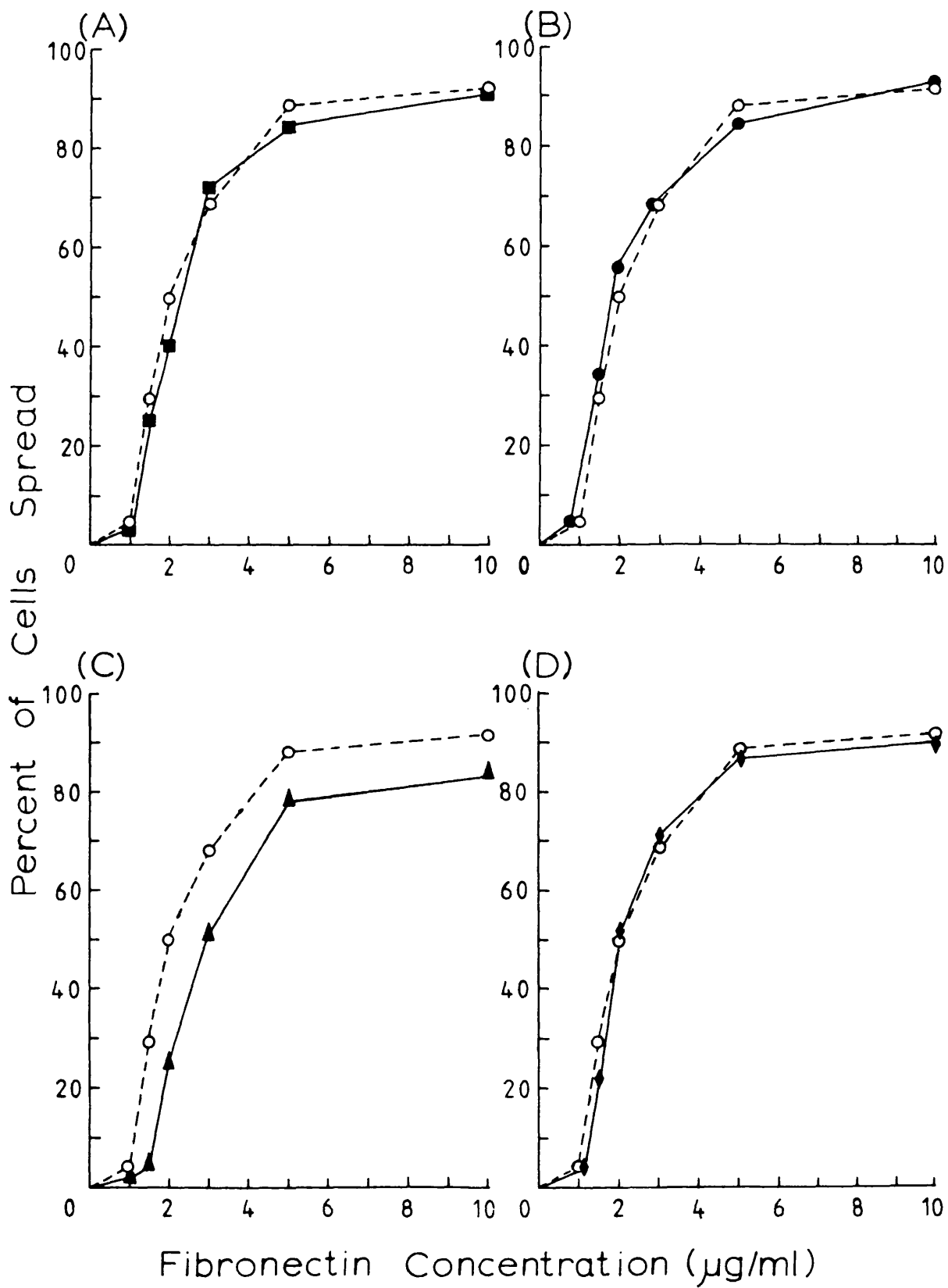


Fig. 4.4 - Quantitation of the ability of ganglioside-deficient variant cells to spread on FN-coated substrata

Essentially as described in Fig. 4.3. Cells were plated onto microtitre wells coated with varying concentrations of FN (0-10 µg/ml) and allowed to spread for 2 h , 37°C. Cell spreading was quantitated using fixed and stained cells as described in Materials and Methods. The ability of each variant to spread on varying concentrations of FN was compared to that of the parental S24 cell line (O ---- O)

A), M1 cells (■ — ■)

B), M3 cells (● — ●)

C), M6 cells (▲ — ▲)

D), M7 cells (◆ — ◆)

parental line S24 (50% cell spreading at approximately 2 $\mu\text{g/ml}$ FN). The M6 line as indicated above appeared to have a slightly reduced ability to spread on FN (50% cell spreading at approximately 3 $\mu\text{g/ml}$), although this difference probably reflects the more elongated, fusiform morphology of this cell line in normal culture (Streuli, 1982) rather than a reduced ability to interact with FN. This is supported by the finding that good attachment of M6 cells is evident at low FN concentration.

When the ability of these cells to spread on CT-coated substrata was investigated, little or no spreading of the variant cells was observed even after pretreatment of these cells with neuraminidase (Fig.4.5; E-L). However, S24 cells were able to spread on CT with the degree of spreading being significantly increased by neuraminidase treatment (Fig.4.5; C + D). Interestingly, an increase in the level of cell attachment to the CT-coated wells was observed with the M7 cell line following neuraminidase treatment. This might suggest that small amounts of higher gangliosides are present in this variant and can be converted to GM_1 following neuraminidase treatment. Alternatively, neuraminidase may in some way unmask small amounts of GM_1 not normally available for CT binding and not detectable by metabolic labelling. The presence of cryptic gangliosides in cell membranes has previously been demonstrated by Gahmberg and Hakomori, (1974).

A direct comparison of the ability of S24 and variant cells to spread on FN- and CT- coated substrata is given in Table 4.2. The percentage cell spreading of S24's at 3- and 10 $\mu\text{g/ml}$ FN is comparable with all the variant

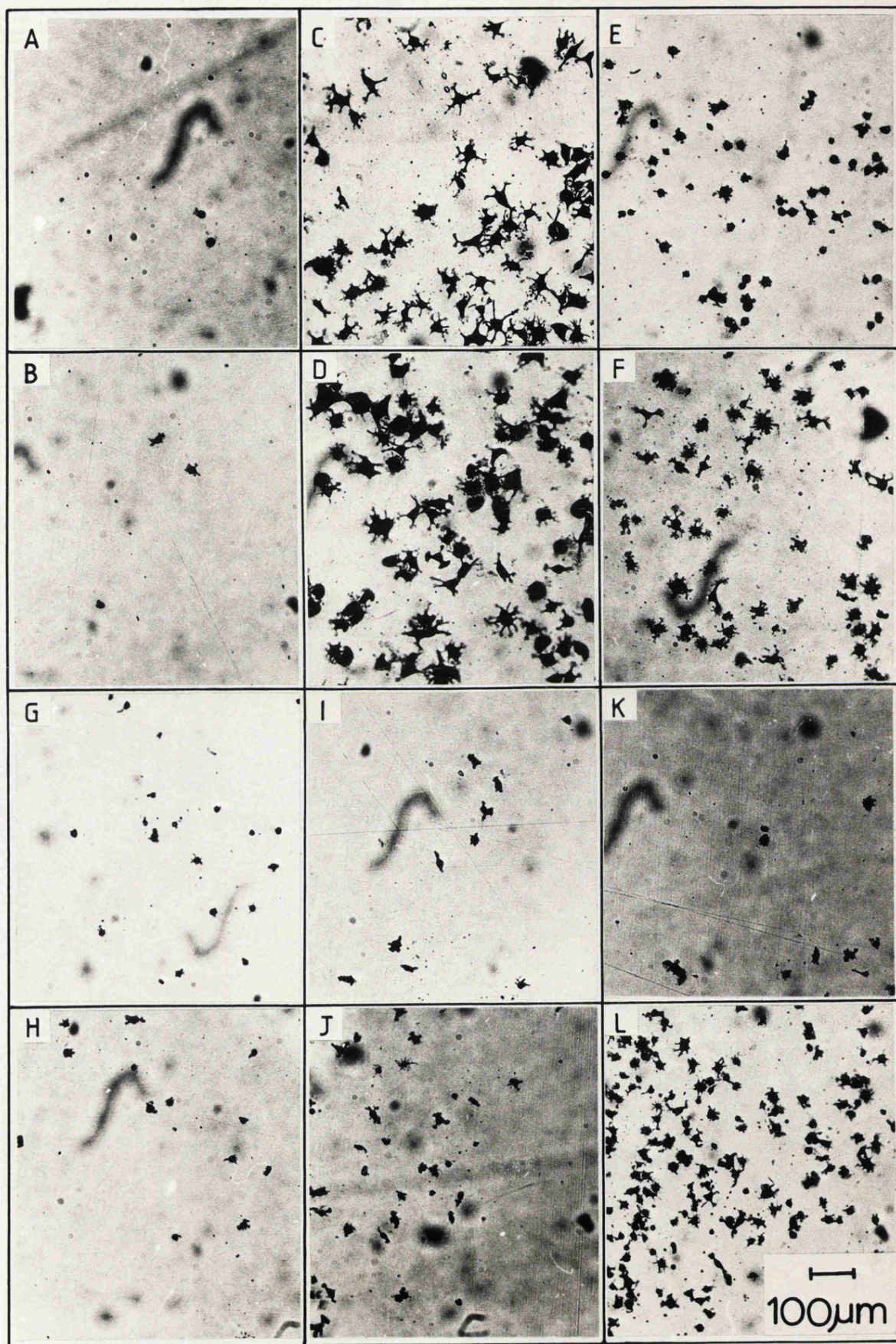


Fig. 4.5 - Ability of Balb/c 3T3 cells and ganglioside-deficient variant cells to spread on CT-coated substrata

Microtitre wells were coated with toxin B subunit (100 µg/ml PBS) for 2 h , 37°C and blocked with 10 mg/ml BSA/PBS for a further 1 h , 37°C. Blocking of wells with BSA prevented attachment of all cell lines in the absence of CT. Freshly trypsinised cells were washed twice in DMEM/1 mM PMSF and applied to each well in 100 µl of DMEM. After spreading for 2 h , 37°C cells were fixed and stained as described in Materials and Methods. Where indicated, cells were pretreated with *Vibrio cholerae* neuraminidase (0.04 U/ml DMEM) for 1 h , 37°C prior to trypsinisation.

A + B,	Balb/c 3T3 (S24) cells, minus CT
C + D,	Balb/c 3T3 (S24) cells, plus CT
E + F,	M1 cells, plus CT
G + H,	M3 cells, plus CT
I + J,	M6 cells, plus CT
K + L,	M7 cells, plus CT.

Wells B, D, F, H, J, and L show cells which had been pretreated with neuraminidase.

Percent Cell Spreading

Ligand	Balb/c 3T3	M1	M3	M6	M7
No additions	0	0	0	0	0
FN (3 µg/ml)	68.6 [±] 6.0	71.9 [±] 4.3	68.6 [±] 3.4	50.2 [±] 5.0	71.0 [±] 4.6
FN (10 µg/ml)	91.9 [±] 3.6	91.0 [±] 1.9	92.6 [±] 1.6	83.2 [±] 4.8	90.9 [±] 1.0
CT (B subunit)	40.4 [±] 8.9	0	0	0	0
	* (77.7 [±] 6.4)	(6.4 [±] 3.5)	(0)	(0)	(3.4 [±] 1.4)

* Cell spreading on CT following pretreatment of cells with neuraminidase.

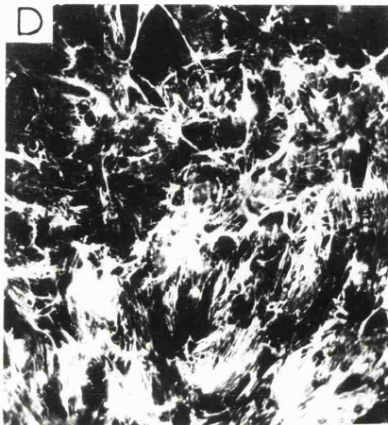
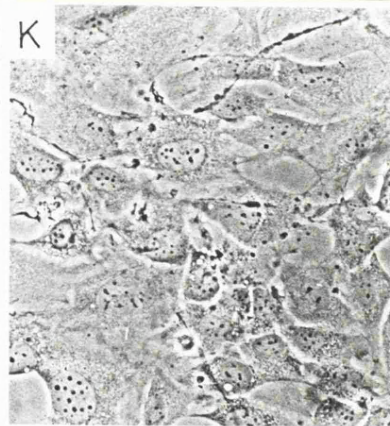
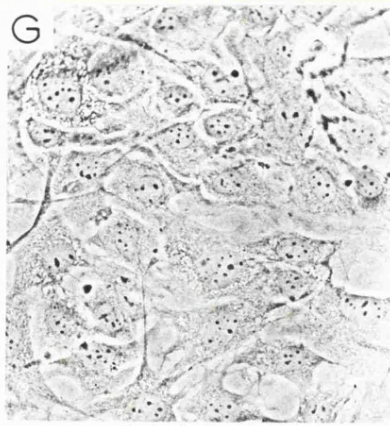
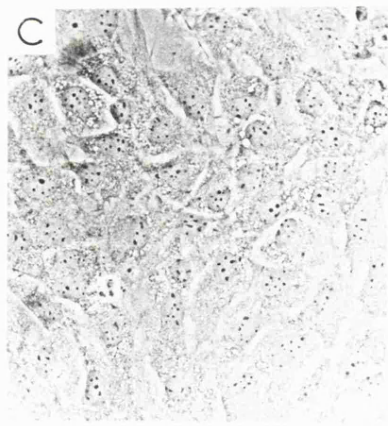
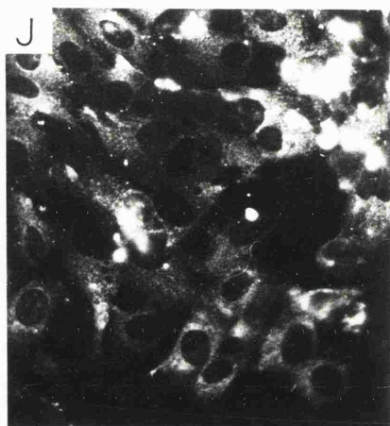
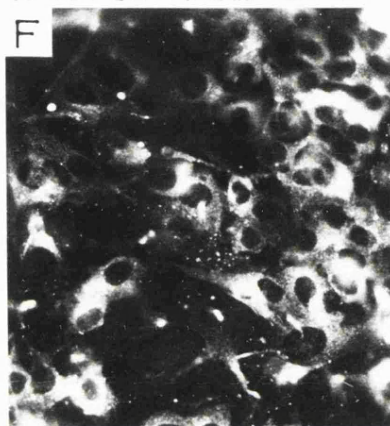
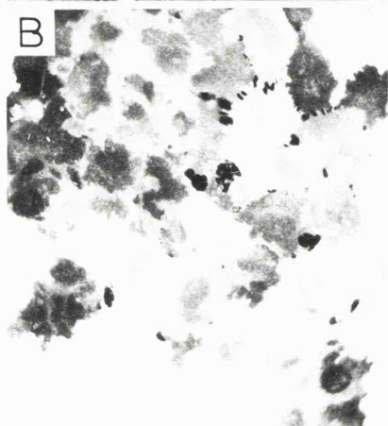
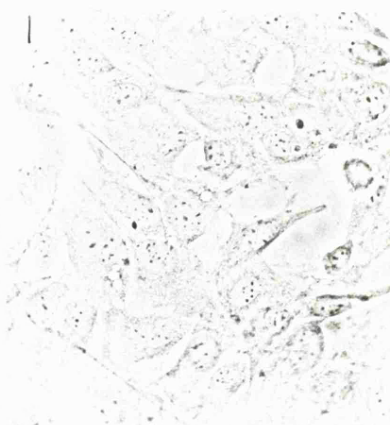
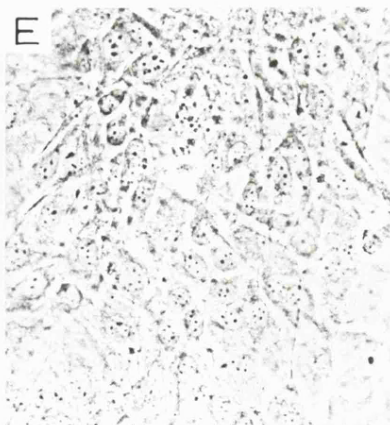
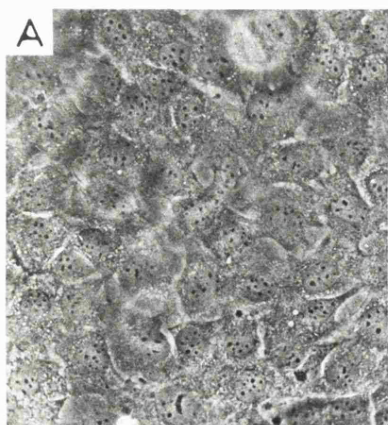
Table 4.2 - Comparison of the cell spreading ability of Balb/c 3T3 cells and ganglioside-deficient variants on FN- and CT- coated substrata

Microtitre wells were coated with either FN or CT-B subunit for 2 h , 37°C and subsequently blocked with 10 mg/ml BSA. Wells were coated twice with PBS and cells (1×10^4 in 100 ml DMEM) added to each well and allowed to spread for 2 h , 37°C in a CO₂ incubator. Cells were then fixed, stained and cell spreading quantitated. Where indicated, cells were pre-treated with neuraminidase (0.04 units/ml in DMEM) for 1 h , 37°C prior to trypsinisation and plating onto CT-coated wells.

cell lines (approximately 70% at 3 $\mu\text{g/ml}$ and 90% at 10 $\mu\text{g/ml}$). Little or no spreading of the variant cells was evident on CT-coated wells even following neuraminidase treatment, whereas spreading of S24 cells on CT-coated wells was increased from approximately 40% to 78% following similar enzyme treatment. It is thus apparent that ganglioside-deficient cells do not have an impaired capacity to spread on FN-coated substrata compared to the parental S24 cell lines.

The ability of a cell to spread on FN is generally assumed to reflect its ability to retain FN at the cell surface. This may not necessarily be the case in all instances. Hayman *et al.* (1980) demonstrated that transformed rat kidney cells contained little or no cell surface FN but secreted large amounts of it into the culture medium. However, these cells were able to spread normally on FN-coated-substrata. It was thus of interest to know if ganglioside-deficient cells are able to organise FN into a matrix form at the cell surface as well as being able to spread on FN-coated substrata.

When the level and organisation of FN at the cell surface of S24 and variant cell lines was analysed by indirect immunofluorescence, no gross differences in either its levels or organisation were evident (Fig.4.6; D, H, L, P, T). Binding of CT to variant cells grown under similar conditions clearly showed that under conditions where large amounts of well organised, fibrillar FN was present at the cell surface, little or no CT binding could be detected by immunofluorescence (Fig.4.6; F, J, N, R).



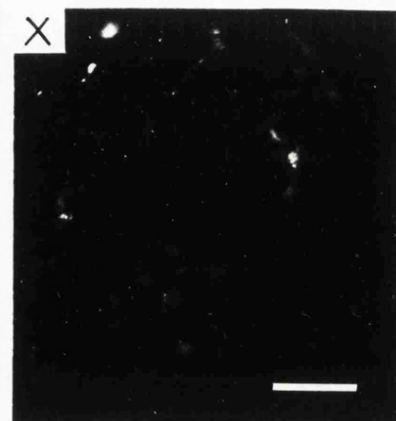
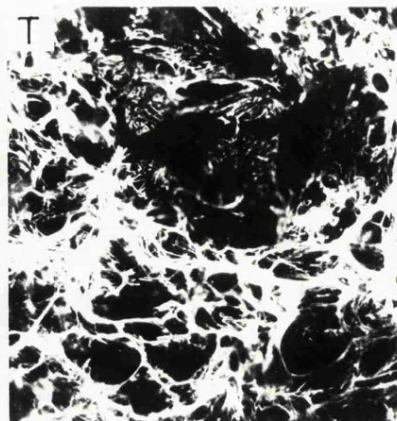
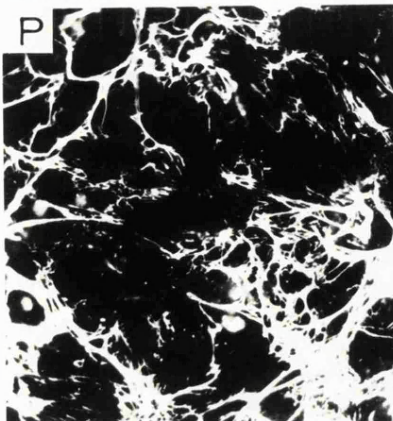
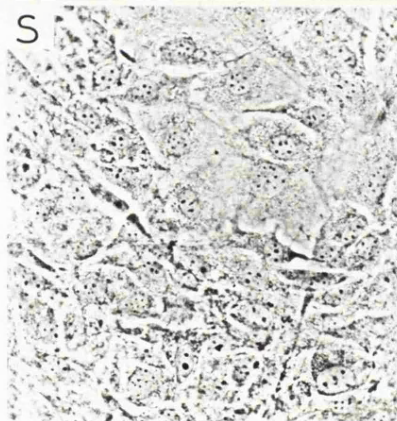
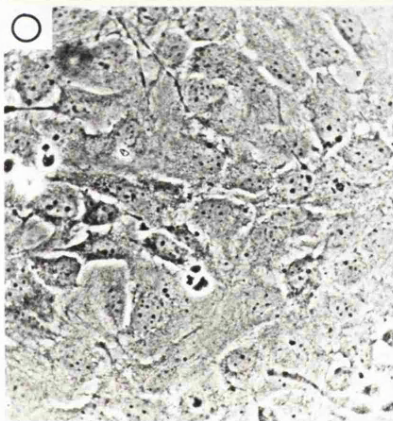
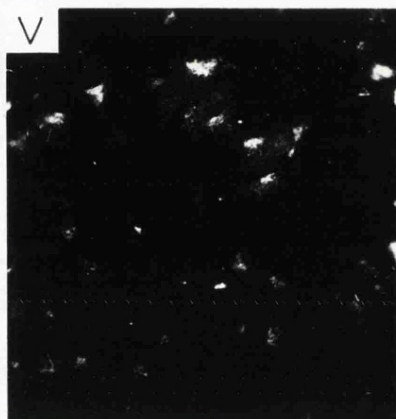
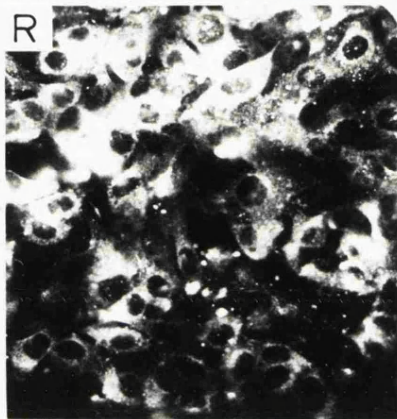
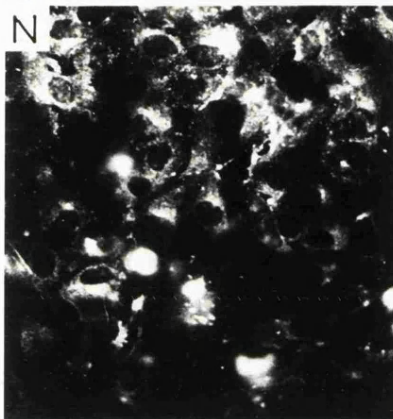
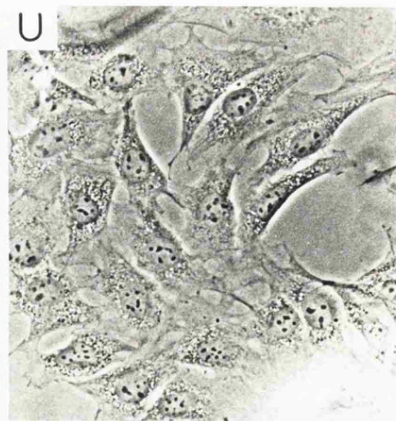
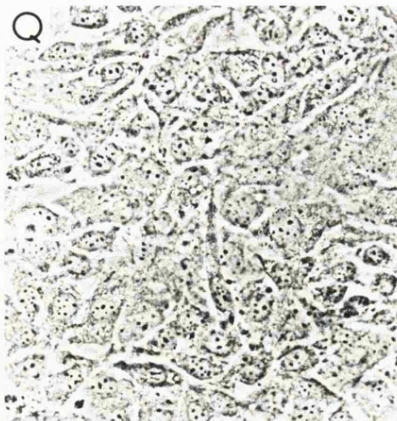
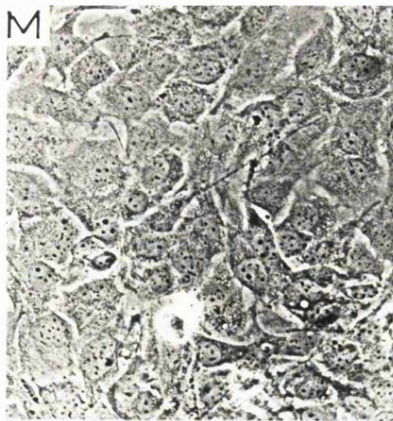


Fig. 4.6 - Immunofluorescent localisation of FN on Balb/c 3T3 cells and ganglioside-deficient variants. A comparison of their ability to bind CT.

Cells were plated onto sterile glass coverslips and cultured for 48 h , 37°C in DMEM containing 10% NCS. FN was then localised on formaldehyde-fixed cells using a rabbit anti-FN antiserum (diluted 1/100) and FITC-conjugated goat anti-rabbit IgG second antibody (diluted 1/50) as described in Materials and Methods. Where indicated, cell monolayers were incubated with CT (1 µg/ml in PBS/BSA) for 5', 37°C, washed twice with PBS and fixed with 3.8% formaldehyde for 40', RT°. Bound CT was visualised using a rabbit anti-CT antiserum (diluted 1/30) and an FITC-conjugated second antibody as for FN staining.

A - D, - Balb/c 3T3 (S24) cells

E - H, - M1 cells

I - L, - M3 cells

M - P, - M6 cells

Q - T, - M7 cells

B,F,J,N,R, - CT staining

D,H,L,P,T, - FN staining

U + V - Balb/c 3T3 (S24) cells; CT staining, minus CT control

W + X - Balb/c 3T3 (S24) cells; FN staining, preimmune serum.

Bar = 50 µm.

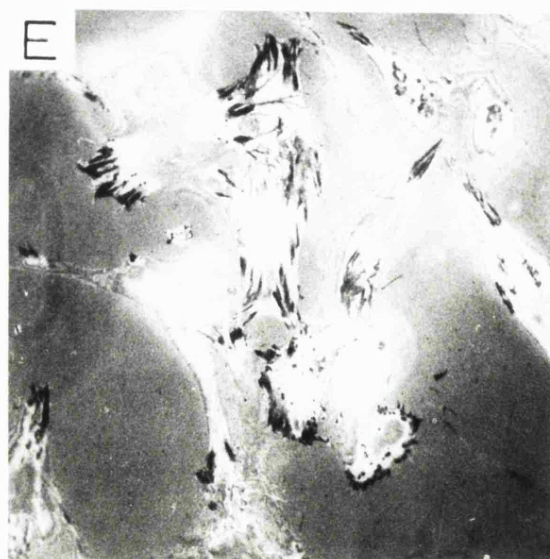
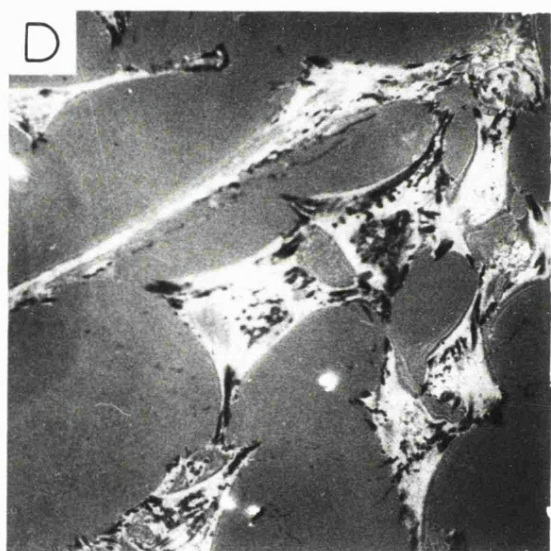
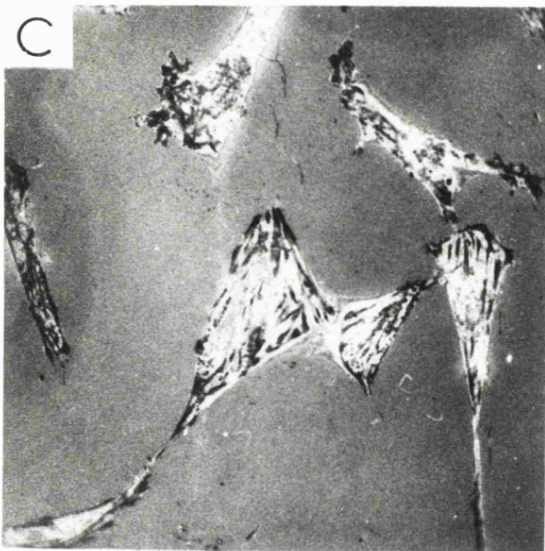
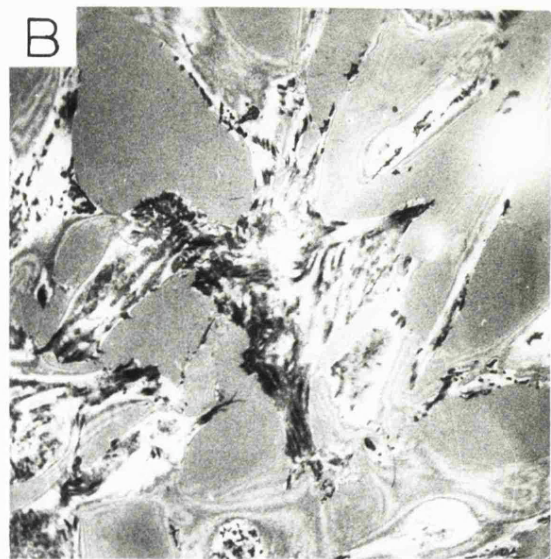
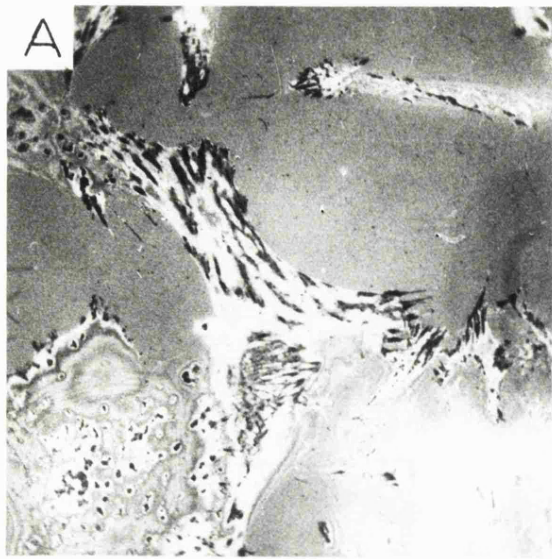
Strong CT immunofluorescence was evident, however, on the S24 cell line (Fig.4.6, B). Cells grown in parallel with the above were metabolically labelled with ^{14}C -palmitate and their ganglioside profiles analysed. They did not synthesise detectable levels of either GM_1 or GD1a under these conditions (Streuli, 1982). Recent quantitation of the levels of cell surface FN by lactoperoxidase-catalysed iodination and SDS-PAGE showed similar levels of labelled FN in all four variant cell lines to that in S24 cells (Griffiths et al. in preparation). Ganglioside-deficient variants of Balb/c 3T3 cells are thus able to spread on FN-coated substrata and to organise it into a matrix form to a similar degree as the parental 3T3 cell line.

Binding of FN to the cell surface triggers a variety of physiological responses. One such response is the reorganisation of intracellular actin-containing microfilaments which occurs during cell spreading on FN and following addition of exogenous FN to transformed cells. In the above I have shown that the ability of FN to trigger the cell spreading response is not impaired in ganglioside-deficient cells. It is important, however, to see if other responses which normally occur following FN binding to the cell surface are active in these cells. I have thus investigated whether the ganglioside-deficient variants are able to form focal contacts, structures which have been shown to develop following Balb/c 3T3 cell spreading on FN-coated substrata (see Chapter 3). Although FN is not unique in its ability to trigger focal contact formation (Couchman et al. 1983; Morgan and Garrod 1983), in Balb/c 3T3 cells at least, spreading on FN-coated

substrata is accompanied by the development of focal contacts. Examination of S24 and variant cells, grown on glass coverslips in serum-containing medium by IFM clearly showed the presence of focal contacts in both the S24 and the variant cell lines (Fig.4.7). Focal contacts were well developed and present at both the periphery and underneath the main body of the cells. Although not all cells contained focal contacts, the proportion that did was approximately equal for each cell type. The presence of focal contacts in these cells when grown in serum-containing medium was suggested previously (Streuli, 1982). It was demonstrated that under normal culture conditions the variant cells had well developed actin-containing microfilament bundles similar to S24 cells. These structures have previously been shown to terminate at focal adhesions. More recently, the ability of these cells to form focal contacts and microfilament bundles has been investigated following spreading on FN-coated substrata in serum-free medium (Griffiths et al. in preparation). S24 and variant cell lines were equally able to form focal contacts and microfilament bundles. As has previously been demonstrated with S24 cells, significantly more areas of close contact were present in cells spread on FN in serum-free medium compared to the same cells grown in serum-containing medium.

The effects of exogenous gangliosides on the ability of cells to interact with FN

Growth or preincubation of mammalian cells in the presence of exogenous gangliosides results in saturable binding of the gangliosides to the plasma membrane (Callies et al. 1977; Krishnaraj, Saat and Kemp, 1980). A



—
20 μ m

Fig. 4.7 - Ability of ganglioside-deficient variant cells to form focal contacts

Cells were plated onto 12 mm diameter glass coverslips in DMEM containing 10% NCS and cultured overnight, 37°C in a CO₂ incubator. Live cells were then examined for the presence of focal contacts by IRM as described in Materials and Methods.

- A) M1 cells
- B) M3 cells
- C) M6 cells
- D) M7 cells
- E) Balb/c 3T3 (S24) cells.

proportion of this membrane-associated ganglioside is believed to be functionally incorporated into the lipid bilayer via the ceramide moiety (Fishman et al. 1976). I have utilised this phenomenon to investigate the effect of reconstituting ganglioside-deficient cells on their ability to interact with FN.

The transformed murine fibroblast cell line, NCTC 2071A, is known to be deficient in the complex gangliosides GM₁, GM₃ and GD1a due to a lack of two biosynthetic enzymes, CMP-sialic acid: lactosylceramide sialyltransferase and UDP-galactose: GM₂ galactosyltransferase, required for their synthesis (Fishman et al. 1976). Growth of this cell line in serum-free medium containing exogenous GM₁ is able to convert them from being unable to bind CT to CT binding (Fishman et al. 1976). Under normal culture conditions it was found that these cells had very little cell-associated FN as detected by indirect immunofluorescence (Fig.4.8, A + B). However, they are able to synthesis FN and secrete it into the medium where a proportion subsequently adsorbed to the substratum (Fig.4.8 A + B). As these cells are normally grown in serum-free medium, the FN absorbed to the substratum is known to be cell-derived and not due to serum FN. Growth of these cells in serum-free medium containing 30 µg/ml bovine brain mixed gangliosides resulted in a dramatic increase in the amount of FN retained at the cell surface (Fig.4.8 C + D). The FN was in the form of numerous small stitches at the periphery of the cell. In addition, the levels of substratum-bound FN appeared to be reduced in cultures grown in ganglioside containing medium. As a positive control for ganglioside uptake the

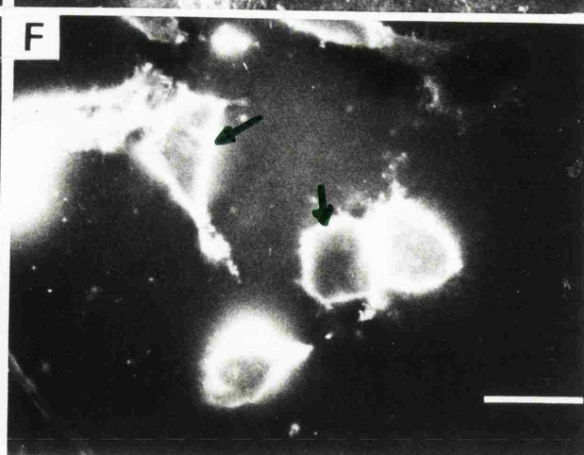
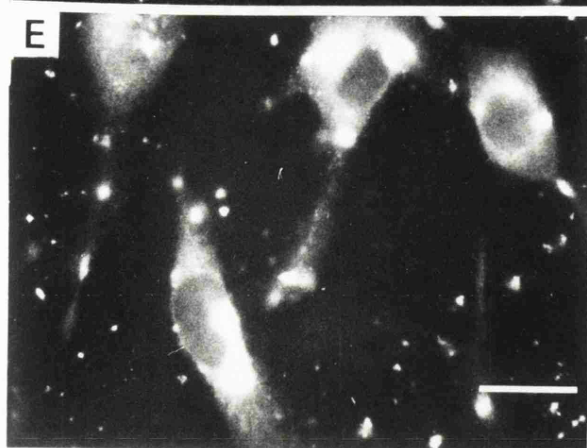
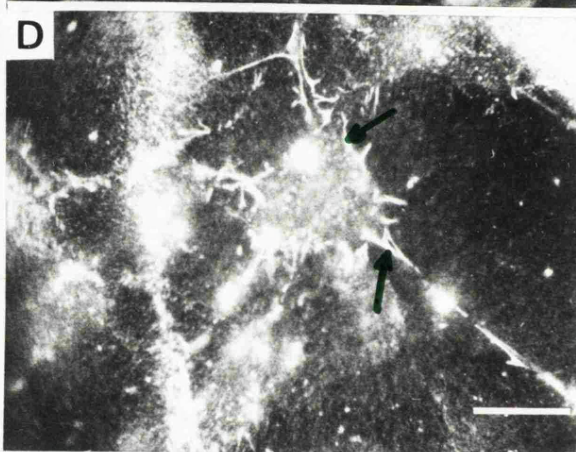
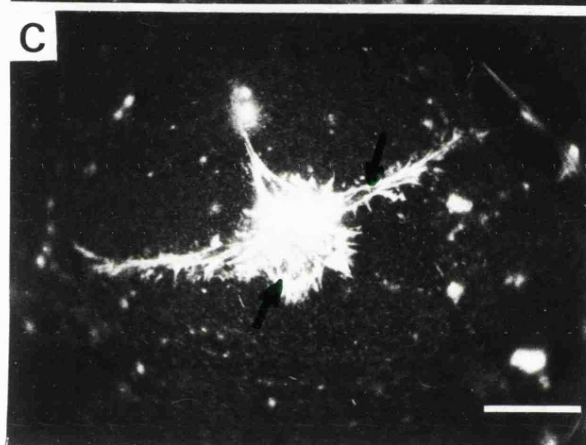
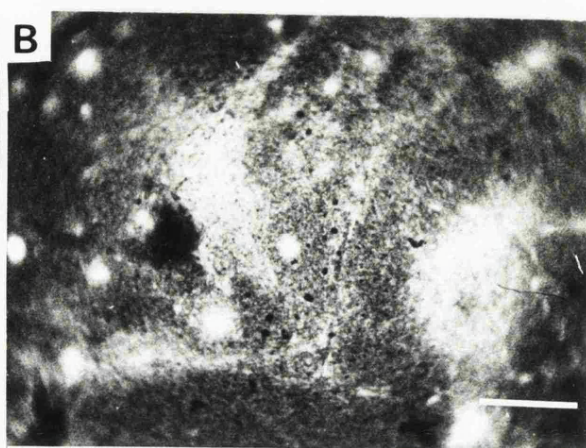
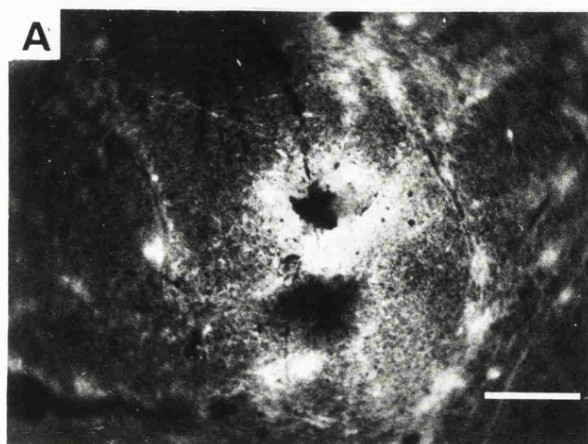


Fig. 4.8 - Effect of mixed gangliosides on the distribution of FN in
NCTC 2071A cells

NCTC 2071A cells (a cell line deficient in endogenous gangliosides) were plated onto glass coverslips and after 4 h , 37°C bovine brain mixed gangliosides were added to give a final concentration of 30 µg/ml. After incubating for 18 hours at 37°C the cells were fixed and FN localised by indirect immunofluorescence. As a positive control for ganglioside insertion, cells were incubated with cholera toxin and the binding detected using immunofluorescence as described in Fig. 4.6 legend. (Bar 20 µm).

- | | |
|-------|--|
| A + B | Cells not incubated with gangliosides and stained for FN. |
| C + D | Cells incubated with ganglioside and stained for FN.
Arrows indicate retention of FN at the cell surface. |
| E | Cells not incubated with ganglioside, cholera toxin bound,
and stained with rabbit anti-cholera toxin and FITC goat
anti-rabbit antiserum. |
| F | Cells incubated with gangliosides, cholera toxin bound
and stained as in E. |

Arrows indicate increased CT binding at the cell surface.

ability of cells to bind CT was monitored. Parallel coverslips to those taken through for FN immunofluorescence were incubated with 1 µg/ml CT for 15', RT⁰ and bound CT detected by indirect immunofluorescence. Cells grown in ganglioside-containing medium showed good CT immunofluorescence while cells grown in the absence of exogenous ganglioside showed only background levels of fluorescence (Fig.4.8 E + F). This clearly indicates that incorporation of gangliosides into the plasma membrane of otherwise ganglioside-deficient cells results in an increased retention of FN at the cell surface.

It was hoped to be able to test whether these cells were able to attach and spread on FN-coated substrata and whether the ganglioside specificity of the above effect on FN retention was similar to that shown for the inhibition of cell spreading. However, due to difficulties in maintaining these cells in culture, this line of research had to be discontinued. Yamada et al. (1983), using the same cell line as above, subsequently demonstrated the following order of ganglioside effectiveness in promoting FN retention to the cell surface, GT1b > GD1a > GM₁ > GM₃.¹ This is indeed similar to that demonstrated for ganglioside inhibition of cell-attachment and spreading (Kleinman et al. 1979; see Chapter 2). It is interesting to note that these cells were able to attach to the substratum under serum-free conditions and that the increased ability to retain cell surface FN following ganglioside treatment had no effect on cell morphology. Although it remains to be demonstrated what role, if any, FN has in mediating the adhesion and spreading of NCTC 2071A cells, it is

¹ The difference in FN retention seen in Fig 4.8 and that shown by Yamada et al (1984) probably reflects the different amounts of FN associated with the surface of sparse and confluent cells.

important to analyse the relative abilities of normal and ganglioside reconstituted cells to adhere to FN-coated substrata. It is also of interest to know if ganglioside pretreatment has any effect on the retention of other matrix components by these cells, e.g. collagens and proteoglycans.

BHK cells have low levels of higher gangliosides (Fig.4.1). Significant amounts of gangliosides more complex than GM₃ could not be detected by metabolic labelling although neuraminidase-treatment of these cells resulted in a 5-fold increase in CT binding suggesting that low levels of di- and tri-sialogangliosides are present. Given the low levels of higher gangliosides in these cells and the inverse relationship between receptor number and the ligand concentration required to trigger cell spreading (Aplin and Hughes, 1981a), it was of interest to determine if increasing the levels of complex gangliosides in these cells increased their ability to spread on FN. It was found that pre-incubation of BHK cells with mixed gangliosides under conditions which have previously been reported to result in their incorporation into the plasma membrane (Fishman, 1980; Beckner et al. 1981) had no effect on their ability to spread on FN-coated linbro wells (Fig.4.9). Both pre-incubated and unincubated cells gave 50% cell spreading at approximately 1 µg/ml FN with maximal cell spreading between 2-5 µg/ml. Additional experiments where BHK cells were cultured overnight in GD containing medium (5 µg/ml in DMEM plus 10% NCS) failed to detect any effect on the ability of cells to spread on FN (data not shown). However, it has previously been suggested that cells take up gangliosides only poorly from serum-containing medium (Fishman, Moss and Manganiella,

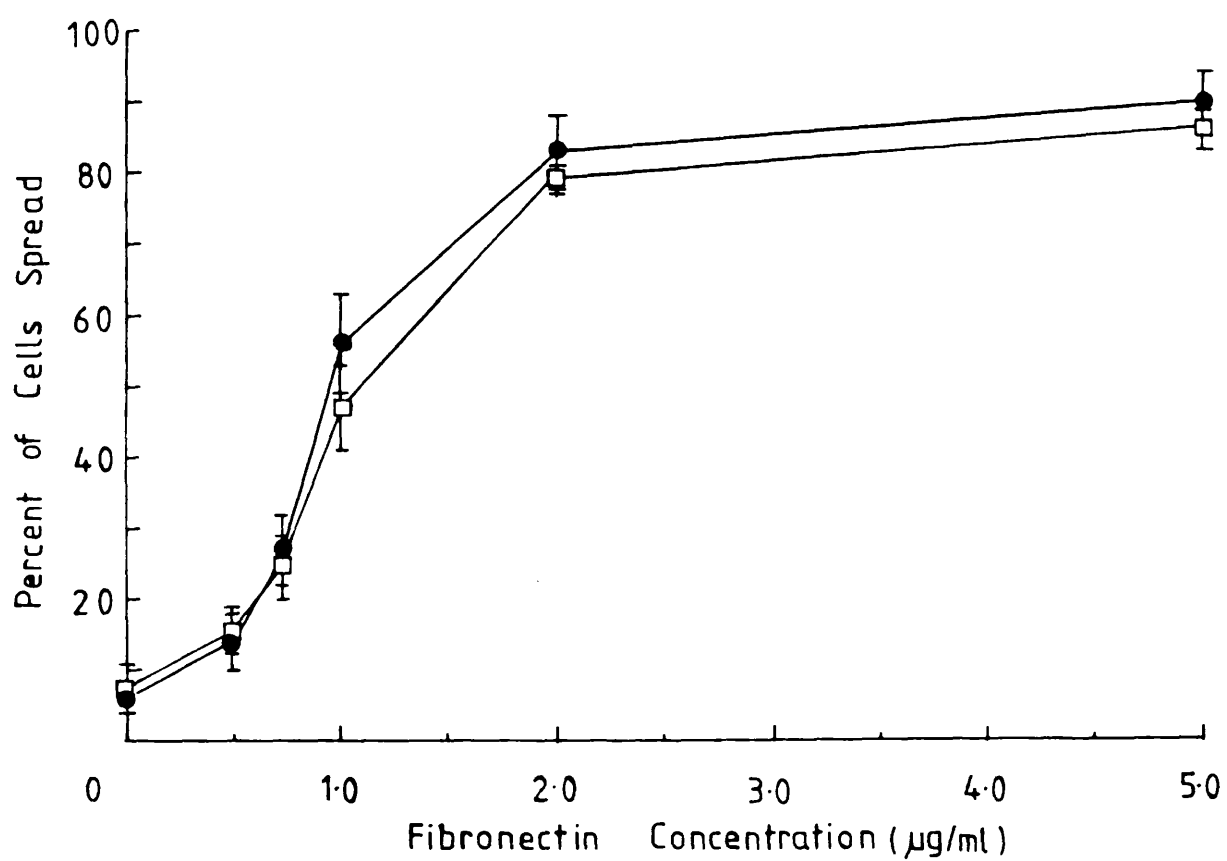


Fig. 4.9 - Effect of mixed gangliosides on the ability of BHK cells to spread on FN-coated substrata

BHK cell monolayers were incubated with 100 µg/ml bovine brain mixed gangliosides for 1 h , 37°C in DMEM (2 ml per 9 cm dish). After washing twice with DMEM, cells were trypsinised and plated into linbro wells (5 x 10⁴/well) coated with varying concentrations of FN. After being allowed to spread for 1 h , 37°C, cells were fixed with 3.8% formaldehyde/PBS and stained with crystal violet. Cell spreading was then quantified as described in Chapter 2, Materials and Methods.

□ — □ plus gangliosides

● — ● minus gangliosides

1977). It is therefore important to repeat such experiments in serum-free medium and to clearly demonstrate ganglioside uptake by the cells.

Attempts were also made to utilise the ricin-resistant BHK cell lines, Ric R17 and Ric R14. These cell lines, although able to synthesise FN, are unable to retain it at the cell surface and have an impaired ability to spread on FN-coated substrata (Meager et al. 1975; Edwards et al. 1976; Pena and Hughes, 1978). However, immunofluorescent staining of these cells in monolayer using antisera prepared in this laboratory (see Appendix II) and antisera provided by Dr. R.O. Hynes (prepared against hamster cell FN) detected sufficient matrix FN to make interpretation of add back experiments difficult. It is not known whether the ganglioside profile of ricin-resistant BHK cells is significantly different to parental BHK cells although as mentioned above, BHK cells contain relatively few complex gangliosides (Fig.4.1).

The effect of neuraminidase treatment of Balb/c 3T3 and BHK cells on their ability to spread on FN-coated substrata

Neuraminidase has been used to demonstrate a role for sialic acid-containing glycoconjugates in ligand binding (Haksar et al. 1974; Beckner et al. 1981). Here I have investigated the effect of such enzyme treatment on the ability of cells to spread on FN-coated substrata.

BHK cells have been shown to spread on FN in a concentration-dependent manner (see Chapter 2). Plating of cells onto linbro wells coated with

varying amounts of FN resulted in maximal cell spreading at a concentration between 2-5 $\mu\text{g/ml}$ within 1 hour of plating. Pretreatment of BHK cell monolayers with neuraminidase had no effect on their ability to spread on FN with maximal cell spreading again occurring between 2-5 $\mu\text{g/ml}$ FN (Fig.4.10). To demonstrate that such enzyme treatment resulted in significant alteration in ganglioside levels, the ability of these cells to bind ^{125}I -labelled CT was investigated before and after enzyme treatment (Table 4.3). In the absence of enzyme treatment, BHK cells were able to bind approximately 1.3×10^{-4} fmoles of CT per cell. This figure was increased 5-fold following treatment with neuraminidase (6.7×10^{-4} fmoles CT per cell). This increase in CT binding suggests that di- and tri-sialogangliosides are present in BHK cells and demonstrates that they are converted into GM_1 by neuraminidase. Examination of the ^{14}C -palmitate-labelled ganglioside profile of BHK cells following neuraminidase treatment shows little alteration due to the almost undetectable levels of GM_1 and disialogangliosides in these cells (Fig.4.1 A + B). The substantial reduction in the levels of di- and/or tri-sialogangliosides suggested in the above does not however, affect the ability of these cells to spread on FN-coated substrata.

Similar results to the above were obtained with Balb/c 3T3 cells. Neuraminidase treatment of these cells significantly increased their ability to bind CT (Table 4.1). Following such enzymatic treatment, no alteration in the ability of these cells to spread on FN-coated microtitre wells was observed (Fig.4.11). Maximal cell spreading was achieved with

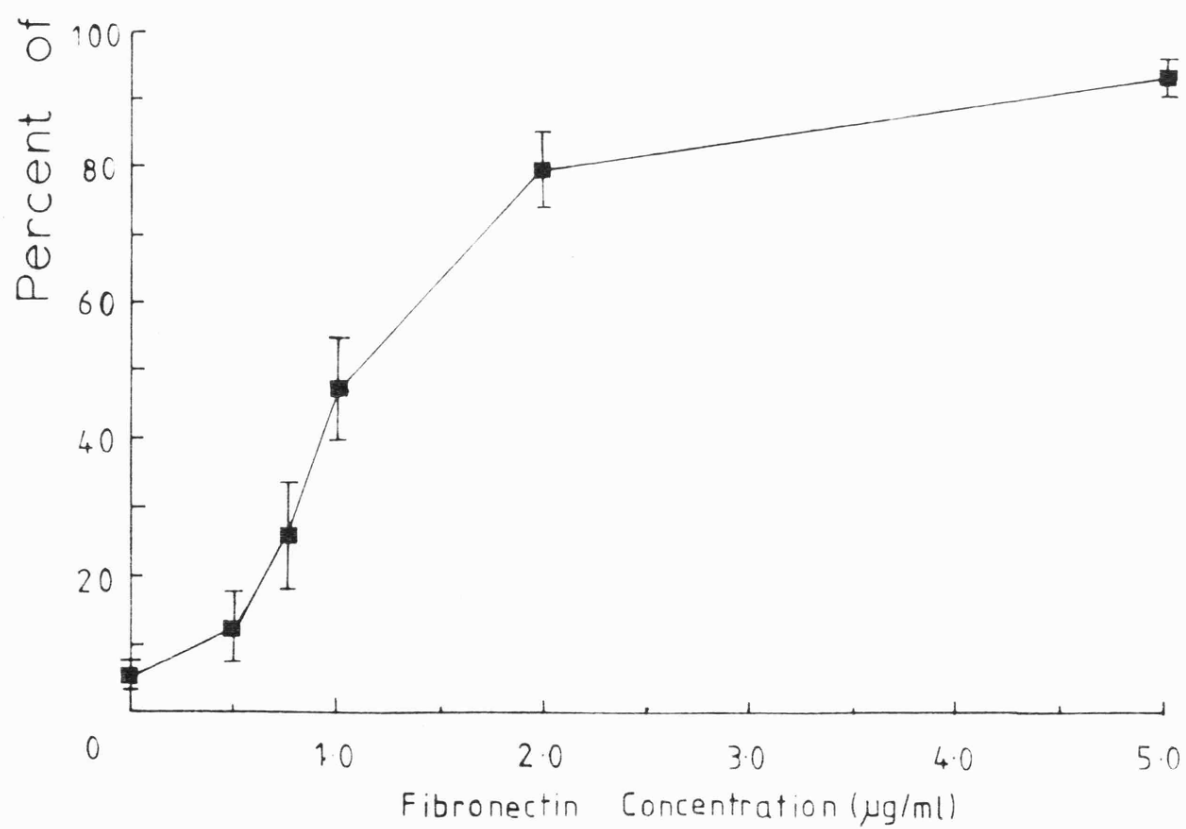
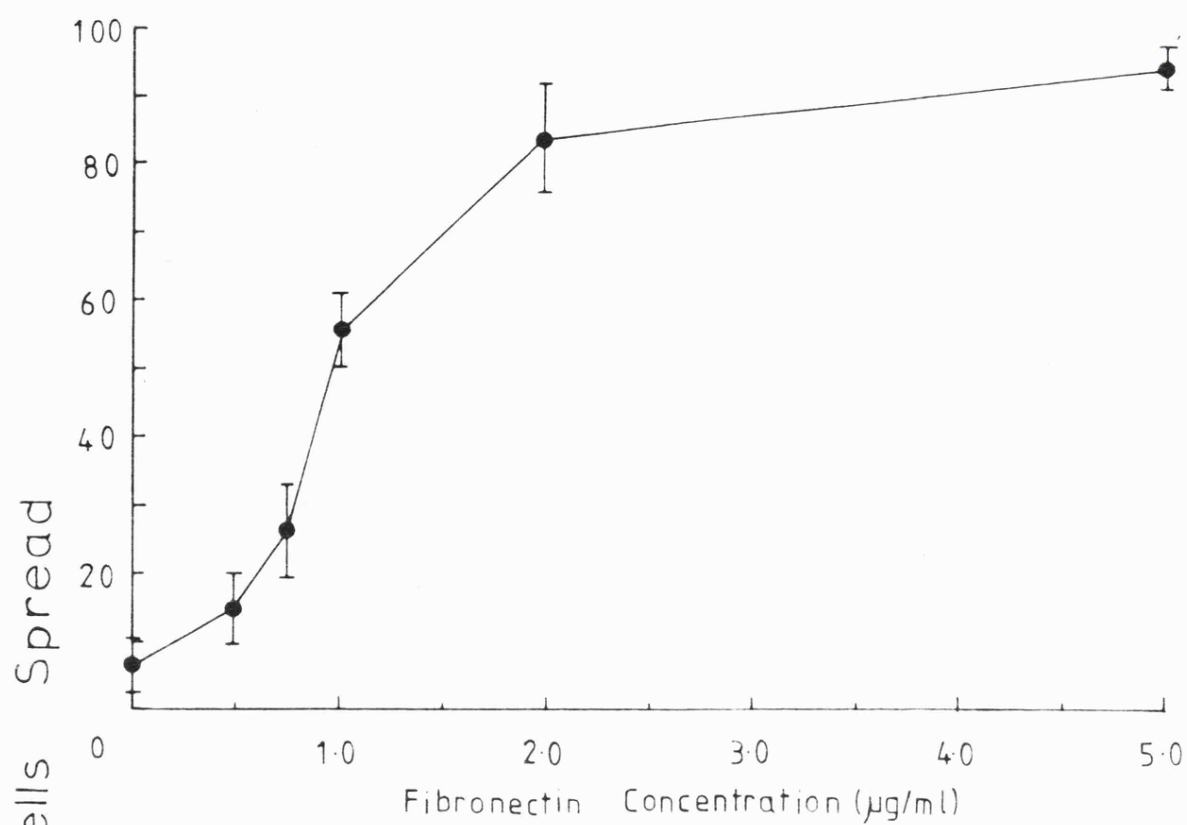


Fig. 4.10 - Effect of neuraminidase treatment on BHK cell spreading
FN-coated substrata

BHK cell monolayers were washed twice with DMEM and incubated with 2 ml of DMEM plus and minus 0.04 U/ml *Vibrio cholerae* neuraminidase for 1 h , 37°C. Cells were then trypsinised and plated into linbro wells (5 x 10⁴/well) and allowed to spread for 1 h , 37°C. Cell spreading was then quantitated using fixed and stained cells.

● — ● minus neuraminidase
■ — ■ plus neuraminidase

Cell Number	CPM bound	fmoles CT specifically bound
-------------	-----------	---------------------------------

A. Minus Neuraminidase

1	5×10^5	12780	
2	5×10^5	12120	71
3	5×10^5 + cold toxin	1086	
4	1×10^6	20496	
5	1×10^6	22044	126
6	1×10^6 + cold toxin	1122	

Average fmoles CT bound/cell = 1.34×10^{-4}

B. Plus Neuraminidase

1	1×10^5	12201	
2	1×10^5	10172	65
3	1×10^5 + cold toxin	720	
4	2.5×10^5	27660	
5	2.5×10^5	28548	169
6	2.5×10^5 + cold toxin	1092	
7	5×10^5	58962	
8	5×10^5	54078	342
9	5×10^5 + cold toxin	1866	

Average fmoles CT bound/cell = 6.67×10^{-4}

Table 4.3 - Effect of neuraminidase treatment of BHK cells on their ability to bind CT

BHK cell monolayers were washed with PBS and incubated with either PBS or PBS containing 0.04 units/ml *Vibrio cholerae* neuraminidase for 3 h., 37°C. Cells were then trypsinised and incubated with ¹²⁵I-labelled CT (3nM, 160 CPM/fmole) in Tris-buffered saline (TBS) 5 mg/ml BSA for 20 mins., RT°. Cells were then separated from unbound toxin by filtration under vacuum on cellulose acetate Millipore EAWP filters (1µm pore size) pre-soaked with TBS/BSA. Non-specific binding was determined in the presence of cold toxin (0.1µM).

Percent of Cells Spread

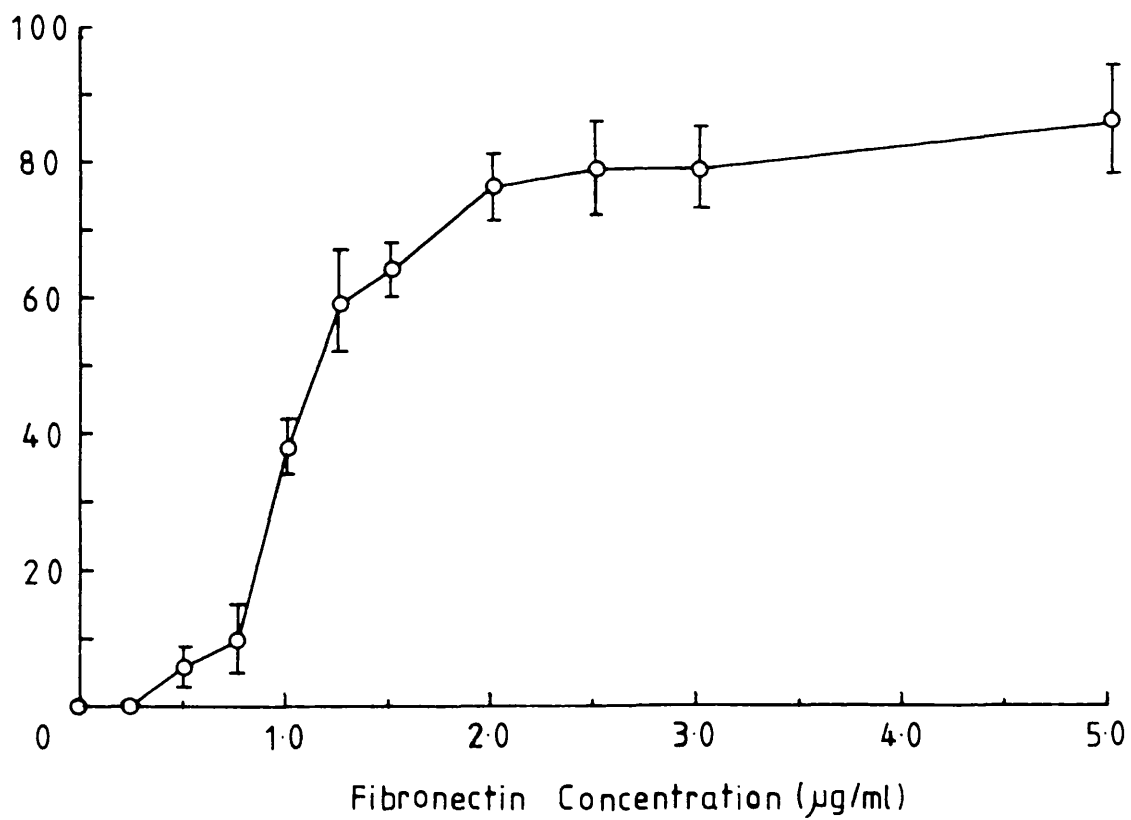
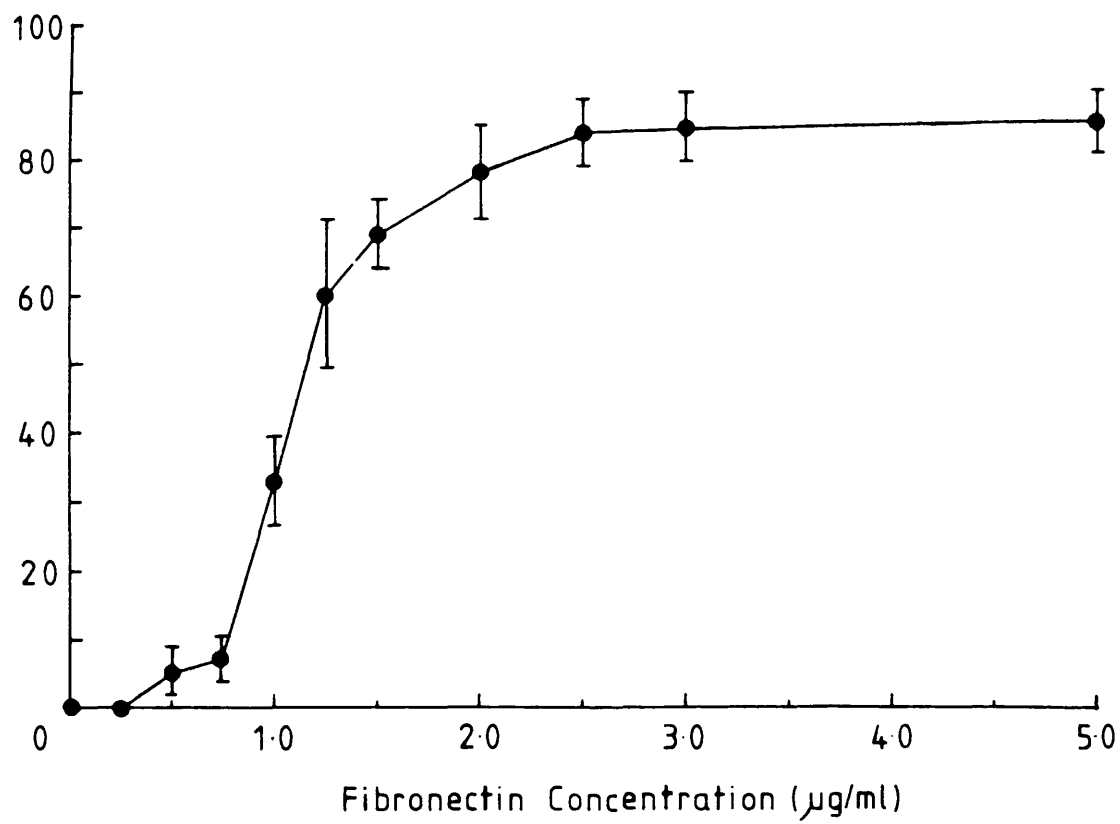


Fig. 4.11 - Effect of neuraminidase treatment of Balb/c 3T3 cells on their ability to spread on FN-coated substrata

Balb/c 3T3 monolayers were washed twice with DMEM and incubated with 2 ml of DMEM, plus and minus 0.04 U/ml *Vibrio cholerae* neuraminidase for 1 h , 37°C. Cells were then trypsinised, plated into microtitre wells (1×10^4 /well in 100 μ l DMEM), previously coated with varying concentrations of FN, and allowed to spread for 2 h , 37°C. Cell spreading was quantitated using fixed and stained cells as described in Materials and Methods.

●—● minus neuraminidase
○—○ plus neuraminidase

a coating concentration of 3 µg/ml FN. However, similar treatment of these cells significantly increased the ability of the cells to spread on CT-coated wells (Fig.4.12). Following neuraminidase treatment the coating concentration of CT required for 50% cell spreading was reduced from 70 µg/ml to 25 µg/ml. Again, this is evidence that neuraminidase has increased the levels of GM₁, presumably at the expense of di- and/or trisialogangliosides. This indicates that neuraminidase-sensitive sites on the surface of BHK and Balb/c 3T3 cells are not involved in FN-mediated cell spreading.

In conclusion, there does not appear to be any correlation between the levels of membrane gangliosides and the ability to interact with FN. Although one ganglioside-deficient cell line, NCTC 2071A, was unable to retain FN at the cell surface, a series of other ganglioside-deficient cell lines derived from Balb/c 3T3 cells, retained normal levels of FN at the cell surface and spread on FN-coated substrata with a concentration dependence similar to that of the parental cell line. Interestingly, growth of NCTC 2071A cells in ganglioside-containing medium resulted in an increased retention of FN showing that under certain conditions gangliosides can increase the organisation of FN into fibrillar structures at the cell surface. Whether this represents functional binding to a membrane receptor or a non-specific association with the cell surface has yet to be determined. Finally, alterations in the levels of cell surface gangliosides by neuraminidase-treatment failed to demonstrate a requirement for sialic-acid containing structures in FN-dependent cell spreading.

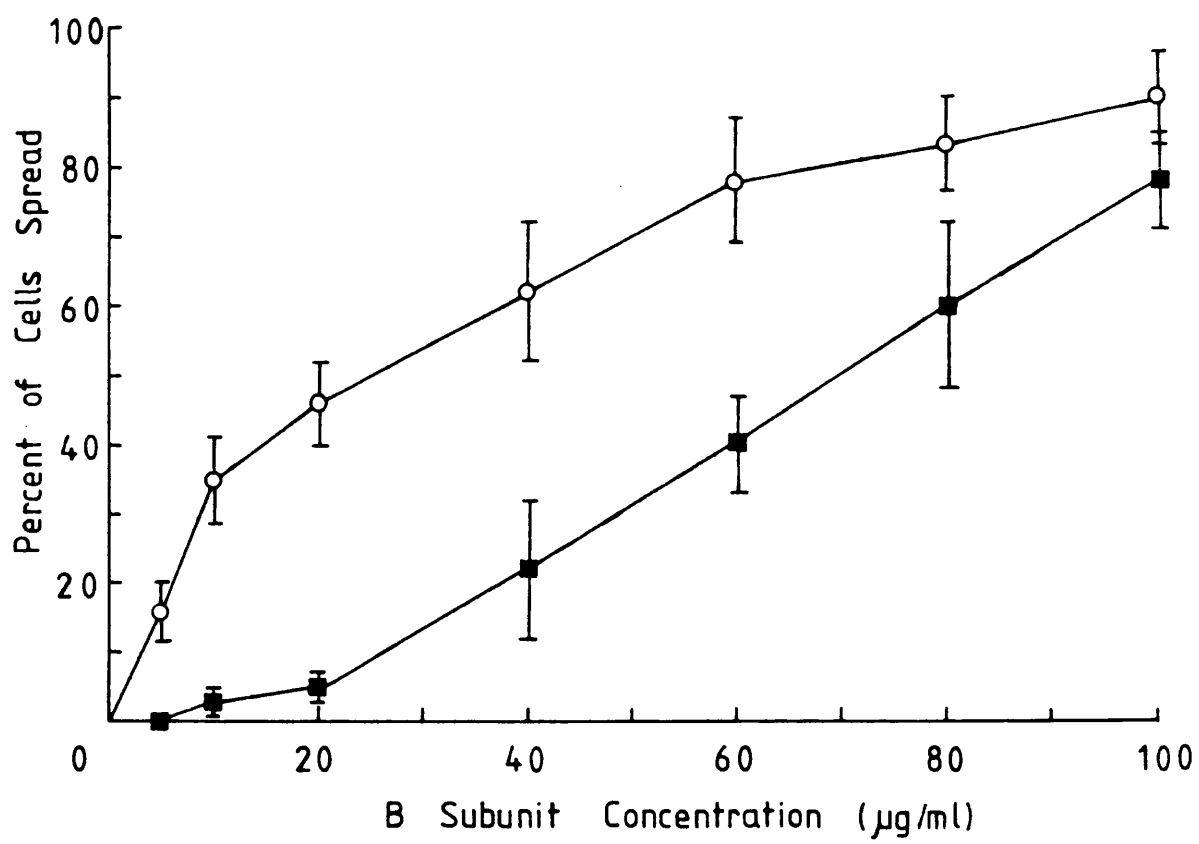


Fig 4.12 - Effect on neuraminidase treatment of Balb/c 3T3 cells on their ability to spread on CT-coated substrata

Balb/c 3T3 cells were treated with *Vibrio cholerae* neuraminidase as described in Fig. 4.11 legend. Cells (1×10^4) were then plated into microtitre wells, previously coated with varying concentrations of CT B subunit and allowed to spread for 2 h, 37°C . Cells were then fixed with 3.8% formaldehyde/PBS and cell spreading quantitated.

■ — ■	minus neuraminidase
○ — ○	plus neuraminidase

CHAPTER 5

CONCLUDING COMMENTS

The aim of this thesis was to make a critical evaluation of the proposed role of gangliosides as cell surface receptors for FN. It had previously been demonstrated that di- and tri-sialogangliosides, or their isolated oligosaccharide moities, were able to inhibit the attachment of CHO cells to a FN-coated substratum. These gangliosides, or glycoproteins with a similar carbohydrate structure (Tonegawa and Hakomori, 1977; Rauvala and Finne, 1979) were thus potential receptors for FN.

I present evidence which is generally not consistent with gangliosides acting as the sole cell surface receptor for FN. Thus :-

1) Although di- and tri-sialogangliosides were able to inhibit both FN-mediated CHO cell and BHK cell spreading with a similar specificity to that previously shown for FN-mediated CHO cell attachment, gangliosides were also able to inhibit ConA-mediated BHK cell spreading. This suggests that ganglioside inhibition of cell adhesion is a general phenomenon and not specific for FN-mediated adhesion.

2) The levels of gangliosides required to inhibit cell spreading on FN were approximately 200-fold higher than those required to inhibit cell spreading on a ganglioside specific ligand (CT).

3) Although direct binding of FN to gangliosides was demonstrated, the levels of binding were very low compared to binding of FN to gelatin or of CT to GM₁. A quantitative comparison of the affinity of FN for

gelatin and gangliosides was not possible due to the nature of the binding assay. This low level of binding may reflect the low affinity of FN for cell surfaces unless it has been activated by binding to a solid substratum or is associated with other extracellular matrix components. This is supported by the recent demonstration that fluorescent gangliosides bind tightly to the extracellular matrix of human fibroblasts and co-distribute with fibrillar FN. (Spiegel, Schlessinger and Fishman, 1984).

4) Cell surface gangliosides were shown to be able to support stable cell attachment and spreading by the use of CT-coated substrata. However, they could not support the formation of microfilament bundles or focal contacts seen following cell spreading on FN-coated substrata. It is unlikely, therefore, that an interaction between FN and gangliosides alone could account for the subsequent reorganisation of the cytoskeleton and the morphological characteristics of cells plated onto FN-coated substrata. Interestingly, cells spread on ConA did not develop these structures either, indicating that alone neither cell surface gangliosides or glycoproteins can support all of the physiological responses associated with cell spreading on FN. It has recently been suggested that cell surface GAG's might be co-operatively involved with some as yet unidentified membrane component in mediating Balb/c 3T3 cell adhesion to FN-coated substrata (Laterra et al. 1983a; 1983b). GAG's are also able to inhibit FN mediated cell attachment and the GAG-binding site in FN has been suggested to play a role in cell attachment (Rich, Pearlstein, Weissmann and Hoffstein, 1981; Klebe and Mock, 1982). Arguing against an involvement of GAG's in FN-mediated cell adhesion is the demonstration of cell attachment

and spreading on the cell-binding domain of FN in the absence of the GAG-binding domain. It remains important, however, to make a more detailed analysis of cell adhesion to the cell-binding domain of FN compared to the whole FN molecule.

5) No correlation could be demonstrated between the levels of cellular gangliosides and the ability to interact with FN. Thus ganglioside-deficient variant cell lines had a similar capacity to adhere to FN-coated substrata as the parental Balb/c 3T3 cell line. In addition, neuraminidase-treatment of Balb/c 3T3 and BHK cells did not affect their ability to spread on FN. However, incorporation of exogenous gangliosides into the plasma membrane of otherwise deficient cell line NCTC 2071 resulted in a significant increase in the level of cell-associated FN suggesting that under some conditions gangliosides may influence the organisation of FN at the cell surface.

Much of the evidence presented in this thesis has involved the use of cell adhesion assays to measure FN-receptor binding. Alternative assays involving the use of FN-coated latex beads have recently been developed (McAbee and Grimmell, 1983; Schwarz and Juliano, 1984). In contrast to cell adhesion assays, binding of FN-coated latex bead to CHO cells was shown to be independent of cytoskeletal and metabolic functions. It was found that GAG's (heparin, heparan sulphate and dermatan sulphate) gangliosides (GT₁ and GD_{1a}) and protease treatment (thermolysin, pronase, papain) all inhibited bead binding although neuraminidase-treatment of

cells prior to binding had no effect (Schwarz and Juliano, 1984). Thus although gangliosides inhibit bead binding, the lack of neuraminidase sensitivity again suggests that the inhibition may be non-specific.

APPENDIX I

PURIFICATION OF FN FROM HUMAN PLASMA

Protein estimation - Micro-Lowry (Lowry et al. 1951)

Aliquots for protein estimation (usually 10 μ l) were made up to 0.1 ml with distilled water and 0.1 ml of 1M sodium hydroxide added. After 10', RT⁰, 1 ml of 2% sodium carbonate, 1% sodium potassium tartrate, 0.5% copper sulphate (49:1:1) was added and left 10', RT⁰. 0.1 ml of 50% Folin reagent in water was then added while vortexing and left for a further 30', RT⁰. The optical density of 660 nm was then measured using a Cecil spectrophotometer and the protein concentration determined from a standard curve constructed using known concentrations of BSA.

Sodium dodecyl sulphate - polyacrylamide gel electrophoresis (SDS-PAGE)

A stock acrylamide solution was prepared containing 30% acrylamide/0.8% bis-acrylamide dissolved in distilled water and stored in the dark at 4°C. FN purification was routinely monitored by running samples on gels consisting of a 7.5% running gel and 4% stacking gel as described by Laemmli (1970).

Running gel (7.5%)

Acrylamide stock	7.5 ml.
1M Tris pH 8.8	11.2 ml.
20% SDS	0.15 ml.
Distilled water	11.2 ml.

The above reagents were mixed, degassed and 100 μ l of 10% freshly prepared

aqueous ammonium persulphate and 20 μ l of TEMED added.

Stacking gel (4%)

Acrylamide stock	1.33 ml.
1M Tris pH 6.8	1.25 ml.
20% SDS	0.05 ml.
Distilled water	7.36 ml.

As above, these reagents were mixed, degassed and 50 μ l of 10% ammonium persulphate and 10 μ l of TEMED added.

Running buffer

0.025 M Tris	
0.192 M Glycine	
0.1% SDS	pH 8.3

FN samples were precipitated with ethanol (75% final concentration) and taken up in a 0.1M Tris pH 6.8, 15% glycerol at a concentration of approximately 1 mg/ml. Samples were then boiled for 2' in the presence of 2% SDS, 0.1M DTT, 0.02% bromophenol blue and unsolubilised material removed by centrifugation (Beckman microfuge, 2'). After loading onto the gels, samples were run at 50V until the marker dye had entered the running gel and thereafter at 100V.

Gels were stained with 0.0125% Coomassie brilliant blue R in 52 ml H_2O /40 ml methanol/5.3 ml acetic acid and destained in the above without

the dye. They were then dried down onto Whatman 3MM paper and in some cases taken for autoradiography using Kodak X-Omat RP film plus an Ilford fast tungstate intensifying screen at -70°C .

Cyanogen bromide (CNBr) activation of Sepharose-4B

CNBr-activated Sepharose-4B was either purchased directly from Pharmacia or activated as follows. 100 ml of swollen Sepharose-4B was placed in a 400 ml beaker containing 100 ml of distilled water and the pH adjusted to 11.0 with 5M sodium hydroxide. 20 gms of ground CNBr was then added to the slurry in small aliquots while stirring. Care was taken to maintain the pH at 10.5 - 11.0 with 5M sodium hydroxide and the temperature was restricted to between 15 - 20°C by placing the slurry on ice when appropriate. Finally, the slurry was collected on a sintered filter and washed with 3.5 litres of ice cold 0.1M carbonate/bicarbonate buffer, pH 10.0. (180 ml 1M sodium bicarbonate plus 110 ml 2M sodium carbonate diluted to 4 litres.)

Coupling of gelatin to cyanogen bromide-activated Sepharose-4B

5 gms of CNBr-activated Sepharose-4B were soaked for 15' in 1mM hydrochloric acid (approximately 200 ml) and washed with a further 800 ml of the same solution on a sintered glass filter. 200 mg of gelatin (Sigma, type I) was dissolved in 25 ml of 0.1M sodium bicarbonate/0.5M sodium chloride (coupling buffer). After washing the swollen Sepharose with coupling buffer it was added to the gelatin/coupling buffer mix and gently

stirred overnight at 4°C. The Sepharose was washed with 1000 ml of coupling buffer on a sintered funnel and unreacted coupling sites blocked with 1M ethanolamine, pH 8.0 for 2 h, RT°. Three cycles of acid (0.1M sodium acetate/1M sodium chloride pH 4.0) and alkali (0.1M sodium tetraborate/1M sodium chloride, pH 8.0) were then used to free the column of non-covalently bound material. The column material was ready for use after further washing with water (500 ml) and PBS (500 ml).

Purification of human plasma fibronectin

FN was routinely purified from outdated citrated human plasma (Leicester Royal Infirmary). Cryoprecipitate (anti-haemophilic Factor VIII) prepared from fresh human plasma (also Leicester Royal Infirmary) was occasionally used as it was found to be a rich source of FN. However, it was only available in limited amounts. Approximately 50 ml of plasma was loaded (1 ml/min.) onto a 10 ml gelatin-Sepharose-4B affinity column which had been prewashed with 4M urea/PBS (20 ml) and PBS/1mM PMSF (40 ml). Unbound proteins were eluted with PBS/PMSF until the OD 280nm reached a steady baseline. Non-specifically bound material was eluted with 1M urea/0.05M Tris, pH 7.5 (1mM PMSF (20 ml). FN was then eluted with 4M urea/CAPS (10 mM cyclohexylamino propane sulphonic acid, 1mM calcium chloride, 0.15M sodium chloride) pH 11.0, containing 1mM PMSF.

Typical yields of FN were between 100 - 120µg per ml of plasma. Analysis of 4M urea-eluted material by SDS-PAGE (Fig. 6.1) showed the presence of a single band corresponding to a molecular weight of

220-225 Kd. Other minor bands were occasionally detected at high FN loadings although it is not known whether these proteins represent contaminating plasma proteins or result from limited proteolysis of FN during purification.

Passage of the plasma down a Sepharose-4B column prior to affinity chromatography in an attempt to remove Sepharose-binding proteins did not result in any improvement in the purity of the final FN preparation. In fact, there was evidence of limited degradation of the protein using this protocol presumably reflecting the longer time required for purification. The presence of the protease inhibitor PMSF in all column buffers was found to be essential to prevent degradation of FN during purification.

As mentioned above, FN was also purified from human cryoprecipitate when available. This gave a yield of approximately 850~~mg~~^{µg} FN per ml of cryoprecipitate and a similar level of purity to outdated plasma. FN from cryoprecipitate also had a similar molecular weight and biological activity (as determined by BHK cell spreading) to FN purified from outdated plasma.

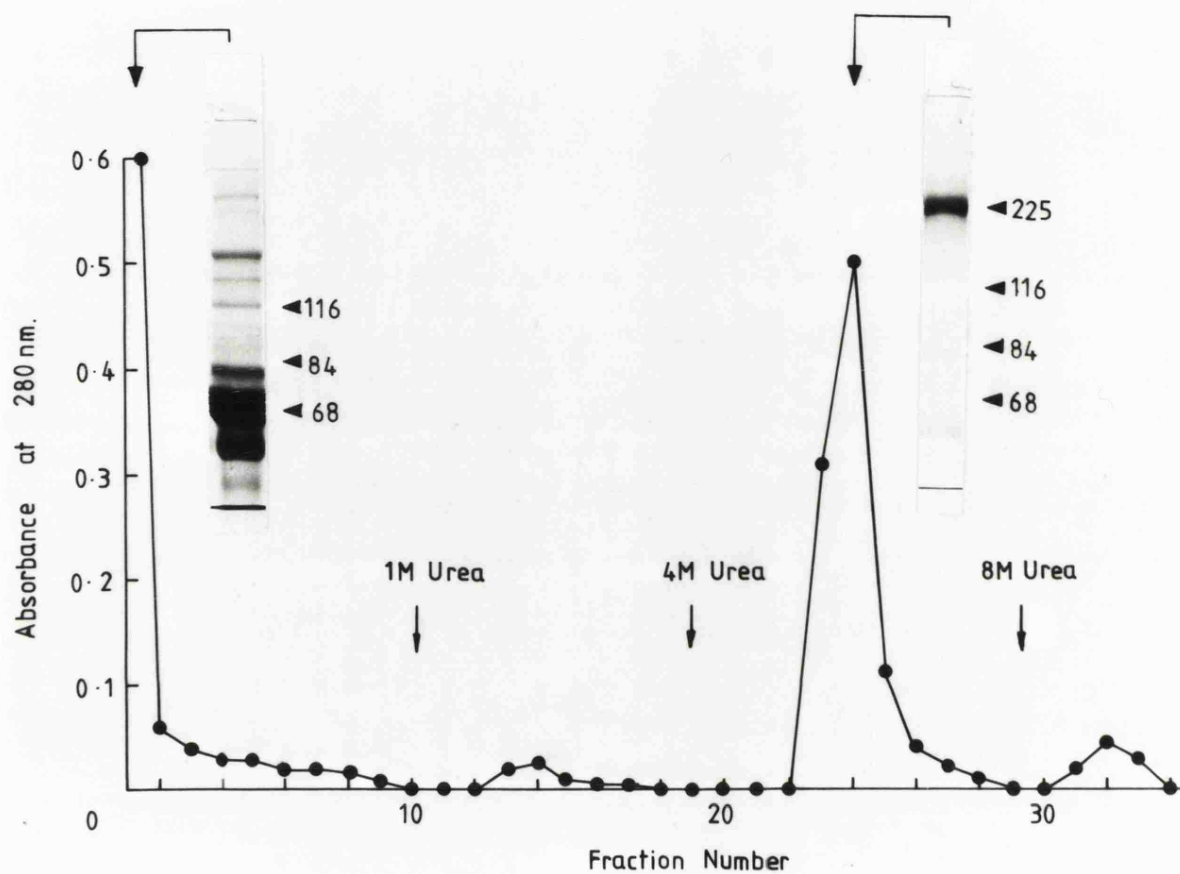


Fig. 6.1 - Purification of FN from human plasma

50 ml of outdated citrated human plasma was loaded onto a 10 ml gelatin-Sepharose column at approximately 1 ml per min. The column was washed with PBS/PMSF (40 ml) until a steady baseline was reached and then with 1M urea, 0.05 M Tris pH 7.5/1mM PMSF (20 ml) to remove non-specifically bound material. Bound FN was then eluted with 4M urea/CAPS, pH 11.0/1mM PMSF. Aliquots of human plasma (left hand insert) and 4M urea-eluted material (right hand insert) were run on a 7.5% SDS-polyacrylamide gel under reducing conditions to determine the level of purity of the FN preparation.

APPENDIX II

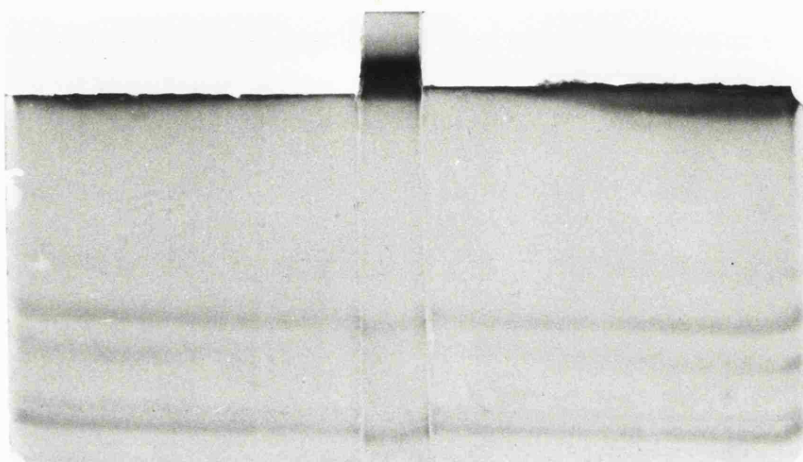
PRODUCTION AND CHARACTERISATION OF ANTI-FN ANTISERUM

Preparation of FN for immunisation (see Fig. 7.1)

Human plasma FN was prepared by gelatin-Sepharose affinity chromatography as described in Appendix I. 4M urea-eluted peak fractions were pooled, the protein precipitated with ethanol (75% final concentration), and boiled for 3' in SDS-PAGE sample buffer (1 mg/ml) as described previously. Unsolubilised material was removed by centrifugation (Beckman microfuge, 1') and 2 ml of the sample run on a preparative SDS-polyacrylamide gel (7.5% running gel, 4% stacking gel) for 15 h, at 50 mV. The position of the FN band was located by removing a vertical strip from the gel and staining it with Coomassie brilliant blue R while the rest of the gel was stored at 4°C (Fig. 7.1A). Using the stained gel as a marker, the FN-containing band was removed from the main body of the gel and the protein recovered by electroelution as described below.

A standard Bio-rad tube gel electrophoresis apparatus was modified to accommodate wide-bore glass tubes (15 x 95 mm). The tubes had a slight restriction at the lower end so as to prevent the gel from becoming dislodged. Tubes were sealed at the lower end with Nescofilm and 0.5 - 1.0 ml of standard SDS-PAGE 6% acrylamide added and allowed to polymerise for 20 - 30', RT°. The FN-containing band from the preparative gel was homogenised by passing it twice through a 10 ml disposable syringe containing 1 ml 10% glycerol in running buffer. It was found that any more than two passages through the syringe caused large numbers of air bubbles to accumulate in the gel. This appeared to impair efficient elution of the FN from

(A)
FN▶



(B)



Fig. 7.1 - Purification of FN by preparative SDS-PAGE

2 mg of gelatin-Sepharose purified FN was applied to a 7.5% preparative SDS-polyacrylamide gel in 2 ml of SDS-PAGE sample buffer (containing 0.1M dithiothreitol) and electrophoresed for 15 h. at 50 mV. A vertical strip was removed from the centre of the gel for staining so as to localise the FN band (A). This band was cut from the rest of the gel and the FN removed by electroelution. The material was then run on a 7.5% SDS-polyacrylamide gel to test its purity (B).

the gel. The homogenate was then transferred to the electroelution tubes and gently mixed with glycerol/running buffer to remove large air bubbles. The gel was overlayed with running buffer and a piece of dialysis tubing, containing 0.5 - 1 ml of running buffer, placed around the end of the tube and sealed with a rubber grommet. After transfer to the electrophoresis apparatus the FN was eluted overnight, RT⁰ (2-4 mA per tube) and dialysed extensively against 4M urea/CAPS buffer, pH 11.0. Between 30 - 40% of the material loaded onto the preparative gel was recovered by electroelution. Analysis of this FN by analytical SDS-PAGE showed the presence of a single band following Coomassie blue staining (molecular weight 220 - 225 Kd, Fig. 7.2B).

Immunisation protocol

1 mg of human plasma FN, prepared by preparative SDS-PAGE and electroelution was emulsified with Freund's complete adjuvant (FCA) (1:1) by passage through a narrow gauge needle. This was injected subcutaneously into a New Zealand white rabbit at several sites along its back at day 0. On day 14 and 28 a further 0.5 mg of FN in FCA was injected and antiserum collected by ear bleeding on day 38. The rabbit was further boosted once a month and antiserum sampled 10 days later. After 5 months the rabbit was sacrificed and blood collected by cardiac puncture.

Affinity purification of anti-FN antiserum

3 mg of FN were coupled to 3 gms of CNBr-activated Sepharose-4B as

described for the coupling of gelatin. The reaction was terminated with 0.1M triethylamine 1 h , RT^o and the slurry washed with 3 cycles of :-

- (i) 0.1M sodium acetate/1M sodium chloride, pH 4.0
- (ii) 0.1M Tris/1M sodium chloride, pH 8.0.

Coupling efficiency was monitored using tracer ¹²⁵I-labelled FN. 5 ml of antiserum was mixed with 5 ml of PBS and the immunoglobulin precipitated by the addition of an equal volume of saturated ammonium sulphate. The immunoglobulin was taken up in PBS, reprecipitated twice more and finally taken up in 3 ml of PBS. After dialysis overnight, 4^oC, the OD 280nm was measured and the yield of immunoglobulin calculated assuming 1 OD unit = 0.7 mg/ml. The recovered immunoglobulin (44 mg) was then applied to a 2 ml FN-Sepharose-4B column over a 2 h period and unbound material eluted as follows :-

- (i) 20 ml PBS
- (ii) 20 ml 0.1M sodium acetate/0.5M sodium chloride, pH 4.8
- and (iii) 20 ml 0.1 Tris/0.5M sodium chloride, pH 8.5

Bound immunoglobulin was then eluted with 0.2M acetic acid/0.5M sodium chloride pH 2.5 and each fraction neutralised with 1M NaOH. Peak fractions were combined, precipitated with 50% ammonium sulphate and dialysed against PBS overnight, 4^oC. Affinity purified material (2.1 mg) was then aliquoted and stored at -20^oC.

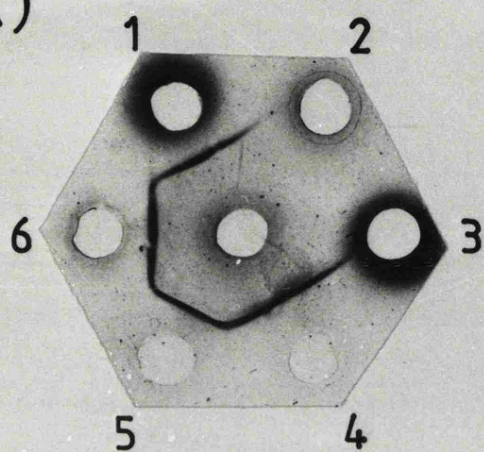
Characterisation of rabbit anti-FN antiserum

(i) Ouchterlony double diffusion test. Molten Difco agar (2% in PBS) was poured onto a glass microscope slide and left to set at RT⁰. Wells were then made in the agar and either antibody or antigen applied so that each well was full. Slides were placed in a humidified container at 37°C and left overnight to allow a precipitation line to form. For photography, Ouchterlony plates were stained with Coomassie brilliant blue R as follows. Plates were soaked with PBS overnight, 4°C and pressed under a lead weight for 20 - 30', RT⁰. The weight was separated from the agar by a piece of Whatman No.1 filter paper and absorbent paper towels. The plate was re-hydrated for 15' with distilled water, pressed as above, and dried down on a hot plate (30 - 40°C) until the agar became clear. To stain the precipitation bands the dried Ouchterlony plate was immersed in a solution of 50 ml of 0.115% Coomassie brilliant blue stain R in ethanol/16 ml glacial acetic acid/134 ml distilled water for 15 - 30', RT⁰ and washed in water.

It was found that the anti-FN antiserum recognised human FN together with a component of bovine, swine and horse serum. No precipitation band was found with human serum albumin or rabbit serum (Fig. 7.2A). With a sample of FN (625 mg/ml) in the centre well and dilutions of the antiserum in the outside wells, a precipitation band was observed at antiserum dilutions as low as 1/16 (Fig. 7.2B).

(ii) Immunofluorescence - The anti-FN antiserum was tested for its ability to detect FN present in cell monolayers using an indirect

(A)



(B)

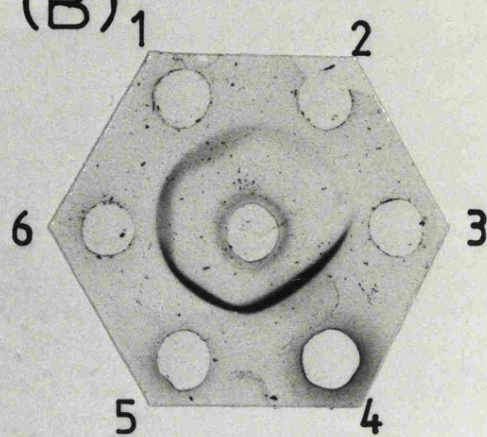


Fig. 7.2 - Analysis of anti-FN antiserum by the Ouchterlony double diffusion test

10 μ l of sample was placed in each well of a 2% agar plate and incubated overnight at 37°C in a humidified container. Plates were then stained as described above.

(A) - Centre well = rabbit anti-FN

Well 1 = human plasma FN (625 μ g/ml)

Well 2 = human serum albumin (1 mg/ml)

Well 3 = rabbit serum (neat)

Well 4 = Newborn calf serum (neat)

Well 5 = Swine serum (neat)

Well 6 = Horse serum (neat)

(B) - Centre well = human plasma FN (μ g/ml)

Well 1 = anti-FN antiserum diluted 1:8

Well 2 = anti-FN antiserum diluted 1:16

Well 3 = anti-serum diluted 1:32

Well 4 = anti-FN antiserum neat

Well 5 = anti-FN antiserum diluted 1:2

Well 6 = anti-FN antiserum diluted 1:4.

immunofluorescent technique (Fig. 7.3). Balb/c 3T3 cell monolayers were grown on glass coverslips for 48 h., under standard tissue culture conditions. The coverslips were washed twice with PBS containing 1mM PMSF and then fixed with 3.8% formaldehyde/PBS for 40', RT⁰. After further washing, the coverslips were incubated with the antiserum in PBS for 30', RT⁰ and bound antibody detected by a fluoresceine isothiocyanate (FITC) - conjugated goat anti-rabbit immunoglobulin diluted 1:50 with PBS. Fluorescence was viewed using a Zeiss standard 16 microscope equipped with epifluorescence.

Strong immunofluorescent staining of FN was produced by the antiserum at a dilution of 1:250 (Fig. 7.3 A + B). Further dilution of the antiserum resulted in reduced intensity of staining although fluorescence was still detectable at a dilution of 1:500. Little or no immunofluorescence was detected when preimmune rabbit serum was used (Fig. 7.3 E + F). Affinity purified antibody gave similar staining at a concentration of 50 µg/ml (Fig. 7.3 C + D) although staining was still apparent at a concentration as low as 10 µg/ml. When breakthrough material for the FN affinity column was substituted for the affinity purified antibody, no staining was observed (data not shown). The fibrillar distribution of FN produced by staining with this antiserum was similar to that observed when the same cells were stained with a rabbit anti-hamster FN, kindly provided by Dr. R.O. Hynes (Fig. 7.3 G + H).

(iii) Immunoelectrophoresis (IEF) - Preformed agarose plates were loaded with 5 µl samples and run at 15mA for 2 h. Anti-FN antisera was

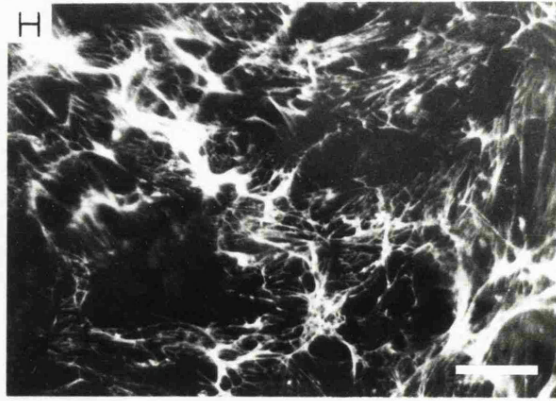
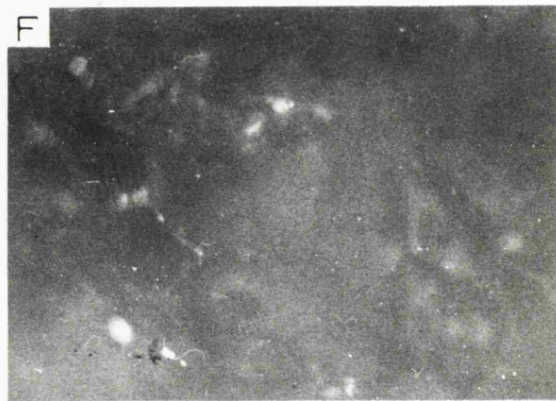
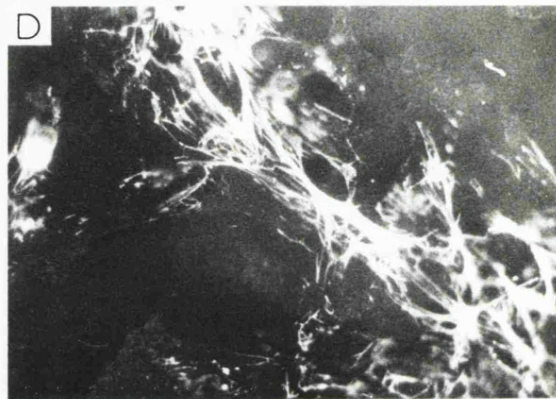
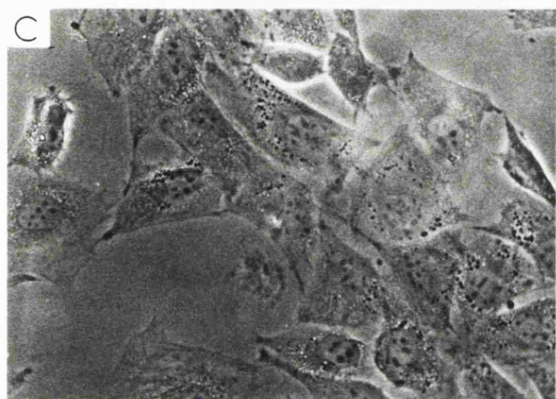
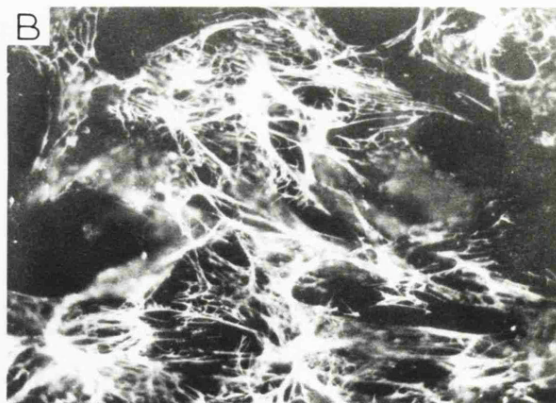
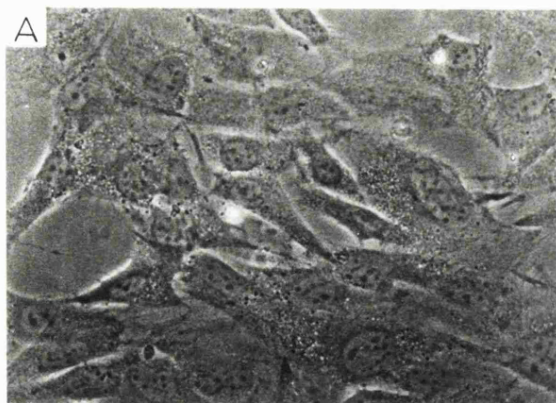


Fig 7.3 - Immunofluorescent localisation of FN on mouse fibroblasts using anti-FN antiserum

Balb/c 3T3 cells were grown on glass coverslips for 48 h and then fixed with 3.8% formaldehyde/PBS. Cells were then incubated with anti-serum for 30', RT⁰, and bound antibody detected with an FITC-conjugated goat anti-rabbit immunoglobulin second antibody.

- A + B = anti-FN antiserum diluted 1:250
- C + D = affinity purified antiserum 50 µg/ml
- E + F = preimmune rabbit serum diluted 1:250
- G + H = rabbit anti-hamster cell FN diluted
1:250 (kindly provided by Dr. R.O. Hynes).

A, C, E, and G are corresponding phase images of B, D, F, and H respectively.

Bar = 40 µm.

then loaded into the troughs and the plate incubated in a humidified atmosphere overnight RT⁰ to allow formation of precipitation arcs. After extensive washing with PBS plates were stained with Coomassie brilliant blue R as for Ouchterlony plates. A single precipitation arc was detected in human and swine serum which had a similar position to that of test FN (Fig. 7.4). This suggests that the anti-serum recognises a single serum component similar to FN with no cross-reactivity with other proteins. No reaction was observed with collagen.

(iv) Immune precipitation of FN from ³⁵S-methionine-labelled BHK cells - Logarithmically growing BHK cells (1 x 9 cm dish) were grown in 5 ml of methionine-free DMEM containing 10% foetal calf serum and 100 μ Ci ³⁵S-methionine (Radiochemical Centre, Amersham) for 18 h, 37°C in a humidified CO₂ incubator. The medium was removed, the monolayer washed twice with PBS/2mM PMSF and the cells scraped with a rubber policeman. The cell pellet was taken up in 0.5 ml of extraction buffer (0.2% SDS, 0.1M Tris pH 8.1, 2mM EDTA, 2 mM PMSF,) and aliquots taken for protein estimation. The ratio of SDS:protein was then adjusted to 3:2 with buffer and a further 1 mg of SDS was added per ml of buffer. The mix was boiled for 5' to solubilise the protein and spun for 15', 4°C in a Beckman microfuge to pellet any debris. The supernatant was used for immune precipitation as follows. In the following order, 100 μ l of 10 mg/ml ovalbumin, 100 μ l of 2% NP40/2% deoxycholate, 200 μ l of sample and 10 μ l of antiserum or pre-immune serum were added to a 1.5 ml glass conical tube and incubated for 1 h, 37°C with occasional mixing. 30 μ l of a 50% suspension of protein

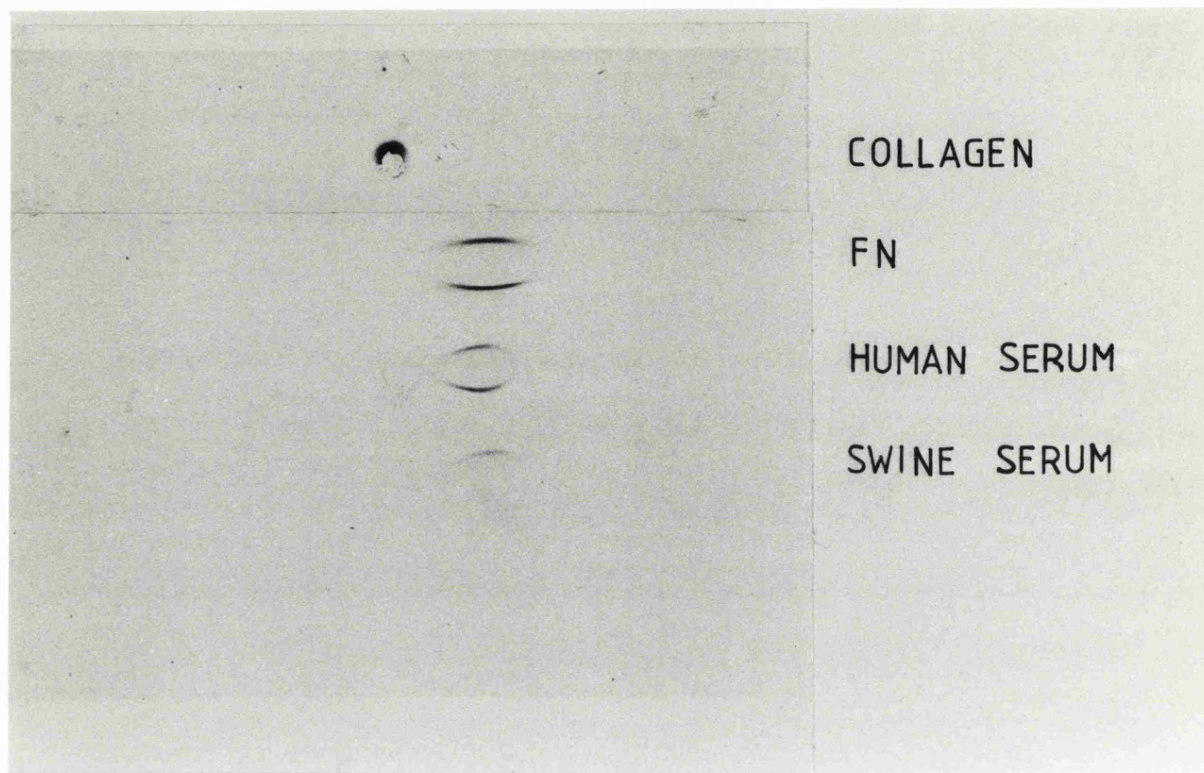


Fig. 7.4 - Analysis of anti-FN antiserum by immunoelectrophoresis (IEF)

5 μ l samples were loaded into the wells of preformed IEF plates and electrophoresed for 2 h at 15 mA. Troughs were then filled with anti-FN antiserum and the plate incubated overnight, RT^O in a humidified container. Precipitation arcs were then stained as for Ouchterlony plates.

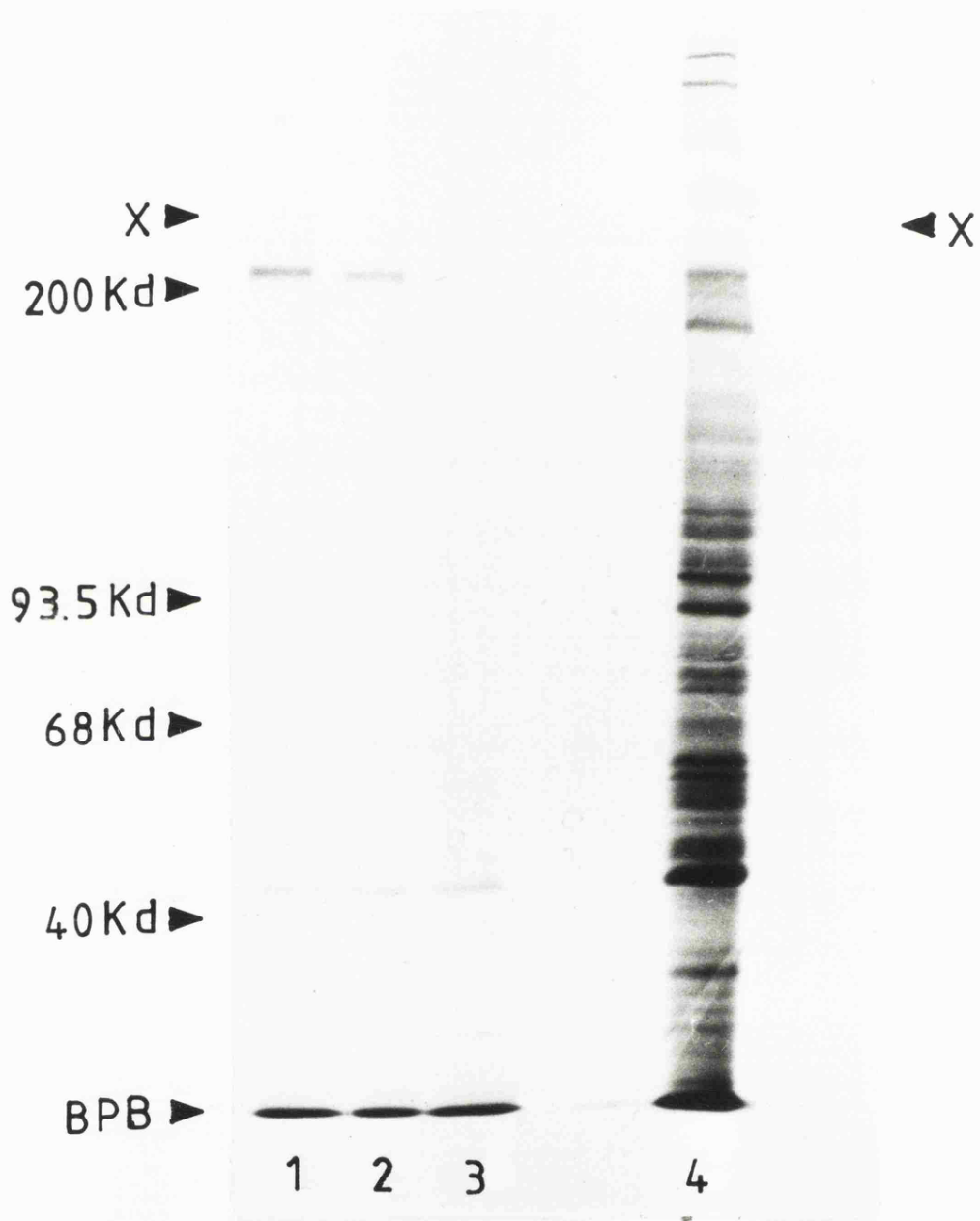


Fig. 7.5 - Analysis of the specificity of anti-FN antiserum by immune precipitation of BHK cell FN from whole cell protein

³⁵S-methionine-labelled BHK whole cell protein was solubilised in 0.2% SDS, 0.1M Tris pH 8.1, 2mM EDTA, 2mM PMSF and the ratio of SDS to protein adjusted to 3:2. An excess of 1 mg SDS per ml of sample was then added and the sample boiled for 5'. 200 µl of sample was incubated with 10 µl of antiserum for 1 h., 37°C and immune complexes precipitated with 30 µl of 50% protein A-Sepharose suspension (1 h., 37°C). After washing with 0.1% SDS, 0.5% NP40, 0.5% deoxycholate, 0.1M Tris pH 8.1, 2mM EDTA, 2mM PMSF, the protein A-Sepharose was extracted with SDS-PAGE sample buffer and run on a 6% polyacrylamide gel under reducing conditions.

Track 1 - BHK cell lysate plus anti-FN (7440 CPM)

Track 2 - BHK cell lysate plus anti-FN (5800 CPM)

Track 3 - BHK cell lysate plus preimmune rabbit serum (5260 CPM)

Track 4 - BHK cell lysate (10,000 CPM)

BPP = bromophenol blue marker

X-X = running gel/stacking gel interface.

A-Sepharose was then added to each tube and further incubated for 1 h at 37°C. The beads were pelleted, the supernatant removed and the pellet washed three times with 0.1% SDS, 0.5% NP40, 0.5% deoxycholate, 0.1M Tris pH 8.1, 2mM EDTA, 2mM PMSF. The pellet was finally extracted in SDS-PAGE sample buffer (100 µl), run on a 6% SDS-polyacrylamide gel and the labelled material visualised by fluorography using EN³HANCE (New England Nuclear). Figure 7.5 clearly demonstrates that a single component of BHK cells is specifically immune precipitated with this antiserum. This component has a similar molecular weight to FN (approximately 220 Kd by SDS-PAGE under reducing conditions). A minor species (molecular weight approximately 45 Kd) was also precipitated, however, this component was also precipitated by control serum.

(v) Immunoblotting - Samples of NIL-8 fibroblast whole cell proteins and human plasma FN were run on an SDS-polyacrylamide gel (7.5%) as described above. The separated proteins were then transferred to a nitrocellulose membrane (0.45 µm, Sartorius) by electroblotting essentially as described by Towbin et al. (1979). Transfer was performed at 80V for 1 h with chilled and degassed transfer buffer (25mM Tris pH 8.3, 192 mM glycine, 10% methanol). To evaluate the efficiency of the transfer, test nitrocellulose was stained with imido-black stain. After blocking with 3% BSA in 10mM Tris pH 7.2, 0.9% sodium chloride (Tris/saline) overnight at 4°C, the nitrocellulose was incubated with anti-FN antiserum (5 µg/ml in Tris/saline containing 5 mg/ml BSA) or control serum at a similar concentration for 1.5 h, 37°C. Unbound antibody was then removed by

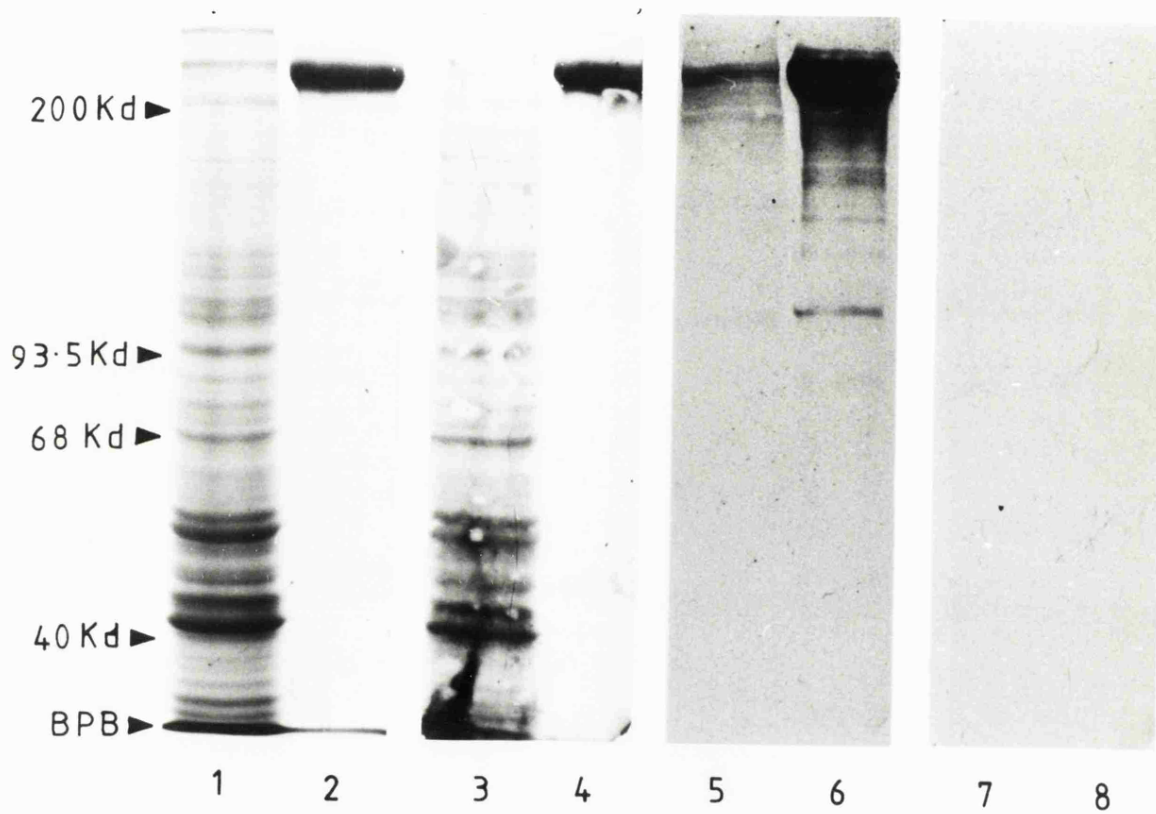


Fig. 7.6 - Analysis of anti-FN antiserum by immunoblotting

NIL-8 whole cell protein and gelatin-Sepharose purified human plasma FN were run on a 7.5% SDS-polyacrylamide gel under reducing conditions and transferred to a 0.45 μ m nitrocellulose membrane by electroblotting. The nitrocellulose was then incubated with anti-FN antibody (5 μ g/ml in Tris/saline containing 5 mg/ml BSA) or control serum at a similar concentration for 1.5 h , 37°C and specifically bound antibody detected by further incubation with 125 I-labelled protein A for 1 h , 37°C. Bound counts were visualised by autoradiography.

Track 1 + 2	Coomassie brilliant blue stained gel
Track 3 + 4	Imido-black stained nitrocellulose
Track 5 + 6	Anti-FN plus 125 I-labelled protein A
Track 7 + 8	Control antibody plus 125 I-labelled protein A

Track 1, 3, 5 and 7 = NIL 8 whole cell protein

Track 2, 4, 6 and 8 = human plasma FN.

washing three times with Tris/saline containing 1% sarcosyl/1% NP40 and once with Tris/saline alone. Bound antibody was detected using ^{125}I -labelled protein A prepared as described in Chapter 2. Nitrocellulose was incubated with ^{125}I -labelled protein A in Tris/saline containing 5 mg/ml BSA (0.5×10^6 CMP/track) for 1 h. 37°C . Unbound material was removed by extensive washing with Tris/saline containing 1% sarcosyl/1% NP40 and the nitrocellulose dried and bound counts detected by autoradiography using Kodak X-Omat RP film.

The anti-FN antiserum bound to a 220 Kd protein in BHK cells and also to human plasma FN (Fig. 7.6). A series of BHK cell proteins between 200 - 220 Kd were also recognised by the antiserum and may represent limited degradation of FN during the preparation of the sample. This may explain the series of bands in the FN track which are also recognised by the anti-serum. In the presence of control antibody, no counts were bound to either BHK cell proteins or the FN marker.

Affinity purified anti-FN antiserum was used in a collaborative study with the Department of Pathology, University of Leicester investigating the relationship between the metastatic potential of human rectal carcinomas and the levels of stromal FN (Niemczuk, Perkins, Talbot and Critchley, 1982).

BIBLIOGRAPHY

- Abercrombie, M. and Dunn, G.A. (1975) : *Exp. Cell Res.* 92, 57-62.
- Adams, S.L., Sobel, M.E., Howard, B.H., Olden, K., Yamada, K.M., de Crombrugghe, B. and Pastan, I. (1977) : *Proc. Natl. Acad. Sci. (USA)* 74, 3399-3403.
- Akiyama, S.K., Yamada, K.M. and Hayashi, M. (1981) : *J. Supramolec. Struct.* 16, 345-358.
- Alexander, S.S., Colonna, G. and Edelhoch, H. (1979) : *J. Biol. Chem.* 254, 1501-1505.
- Alexander, S.S., Colonna, G., Yamada, K.M., Pastan, I. and Edelhoch, H. (1978) : *J. Biol. Chem.* 253, 5820-5824.
- Ali, I.U. and Hynes, R.O. (1978) : *Cell* 14, 439-446.
- Ali, I.U., Mautner, V.M., Lanza, R.P. and Hynes, R.O. (1977) : *Cell* 11, 115-126.
- Allen, C., Saba, T.M. and Molnar, J. (1973) : *J. Reticuloendothelial Sci.* 13, 410-423.
- Amstein, C.F. and Hartman, P.A. (1975) : *J. Clin. Microbiol.* 2, 46-51.
- Aplin, J.D. and Hughes, R.C. (1981a) : *J. Cell Sci.* 50, 89-103.
- Aplin, J.D. and Hughes, R.C. (1981b) : *Anal. Biochem.* 113, 144-148.
- Aplin, J.D., Hughes, R.C., Jaffe, C.L. and Sharon, N. (1981c) : *Exp. Cell Res.* 134, 488-494.
- Atherton, B.T. and Hynes, R.O. (1981) : *Cell* 25, 133-141.
- Aznur, Z. and Geiger, B. (1981) : *Cell* 25, 121-132.
- Barak, L.S., Yocum, R.R., Nothnagel, E.A. and Webb, W.W. (1980) : *Proc. Natl. Acad. Sci. (USA)* 77, 980-984.
- Barnes, D.W., Silnutzen, J., See, C. and Shaffer, M. (1983) : *Proc. Natl. Acad. Sci. (USA)* 80, 1362-1366.

- Barnhart, B.J., Cox, S.H. and Kraemer, P.M. (1979) : Exp. Cell Res. 119, 327-332.
- Beckner, S.K., Brady, R.O. and Fishman, P.H. (1981) : Proc. Natl. Acad. Sci. (USA) 78, 4848-4852.
- Bevilacqua, M.P., Amrani, D., Mosesson, M.W. and Bianco, C. (1981) : J. Exp. Med. 153, 42-60.
- Bing, D.H., Almeda, S., Isliker, M., Lahav, J. and Hynes, R.O. (1982) : Proc. Natl. Acad. Sci. (USA) 79, 4198-4201.
- Birchmeier, C., Kreis, T.E., Eppenberger, H.M., Winterhalter, K.H. and Birchmeier, W. (1980) : Proc. Natl. Acad. Sci. (USA) 77, 4108-4112.
- Birdwell, C.R., Brasier, A.R. and Taylor, L.A. (1980) : Biochem. Biophys. Res. Comm. 97, 574-581.
- Birdwell, C.R., Gospodarowicz, D. and Nicolson, G.L. (1978) : Proc. Natl. Acad. Sci. (USA) 75, 3723-3727.
- Blumenstock, F.A., Saba, T.M., Weber, P. and Laffin, R. (1978) : J. Biol. Chem. 253, 4287-4291.
- Brady, R.O. and Mora, P.T. (1970) : Biochim. Biophys. Acta 218, 308-319.
- Bremer, E.G., Hakomori, S., Bowen-Pope, D.F., Raines, E. and Ross, R. (1984) : J. Biol. Chem. 259, 6818-6825.
- Bretscher, M. (1976) : Nature 260, 21-23.
- Bretscher, M. (1984) : Science 224, 681-686.
- Briles, E.B. (1982) : Int. Rev. Cytol. 75, 101-165.
- Burridge, K. (1978) : Methods in Enzymol. 50, 54-64.
- Burridge, K. and Feramisco, J. (1980) : Cell 19, 587-595.
- Butters, T.D., Devalia, V., Apolin, J.D. and Hughes, R.C. (1980) : J. Cell Sci. 44, 33-58.

- Callies, R., Schwarzmann, G., Radsak, K., Siegert, R. and Wiegandt, H.
(1977) : Euro. J. Biochem. 80, 425-432.
- Caputto, R., Maccioni, A.H.R. and Caputto, B.L. (1977) : Biochem. Biophys.
Res. Comm. 74, 1046-1052.
- Carter, W.G. (1982) : J. Biol. Chem. 257, 3249-3257.
- Carter, W.G. and Hakomori, S. (1981) : J. Biol. Chem. 256, 6953-6960.
- Carter, W.G., Rauvala, H. and Hakomori, S. (1981) : J. Cell Biol. 88,
138-148.
- Chen, L.B., Gallimore, P.H. and McDougall, J.K. (1976) : Proc. Natl. Acad.
Sci. (USA) 73, 3570-3574.
- Chen, L.B., Maitland, N., Gallimore, P.H. and McDougall, J.K. (1977) : Exp.
Cell Res. 106, 39-46.
- Chen, W.T. and Singer, S.J. (1980) : Proc. Natl. Acad. Sci. (USA) 77,
7318-7322.
- Couchman, J.R., Hook, M., Rees, D.A. and Timpl, R. (1983) : J. Cell Biol.
96, 177-183.
- Critchley, D.R., Ansell, S., Perkins, R., Dilks, S. and Ingram, J. (1979):
J. Supramol. Struct. 12, 273-291.
- Critchley, D.R., England, M.A., Wakely, J. and Hynes, R.O. (1979) : Nature
280, 498-500.
- Critchley, D.R., Magnani, J.L. and Fishman, P.H. (1981) : J. Biol. Chem.
256, 8724-8731.
- Critchley, D.R., Streuli, C.H., Kellie, S., Ansell, S. and Patel, B. (1982)
: Biochem. J. 204, 209-219.

- Critchley, D.R. and Vicker, M.G. (1977) : G. Poste and G.L. Nicolson
(eds.) Dynamic Aspects of Cell Surface Organisation Elsevier/North-
Holland Biomedical Press pp 307-370.
- Cuatrecasas, P.(1973) : Biochemistry 12, 3547-3557.
- Culp, L.A., Murray, B.A. and Rollins, B.J. (1979) : J. Supramol. Struct.
11, 401-427.
- Curtis, A.S.G. (1964) : J. Cell Biol. 20, 199-215.
- De Petro, G., Barlati, S., Vartio, T. and Vaheri, A. (1981) : Proc. Natl.
Acad. Sci. (USA) 78, 4965-4969.
- Der, C.J. and Stanbridge, E.J. (1978) : Cell 15, 1241-1251.
- Dessau, W., Adelmann, B.C., Timpl, R. and Martin, G.R. (1978) : Biochem.
J. 169, 55-59.
- Edwards, J.G., Dysart, J.M.K. and Hughes, R.C. (1976) : Nature 264,
66-68.
- Ehrishmann, R., Roth, D.E., Eppenberger, H.M. and Turner, D.C. (1982) :
J. Biol. Chem. 257, 7381-7387.
- Engvall, E. and Ruoslahti, E. (1977) : Int. J. Cancer 20, 1-5.
- Eriksen, H.O., Clemmensen, I., Hansen, M.S. and Ibsen, K.K. (1982) :
Scand. J. Clin. Invest. 42, 291-295.
- Fagan, J.B., Sobel, M.E., Yamada, K.M., de Crombrughe, B. and Pastan, I.
(1981) : J. Biol. Chem. 256, 520-525.
- Finne, J., Burger, M.M. and Prieels, J.P. (1982) : J. Cell Biol. 92, 277-
282.
- Fishman, P.H. (1980) : J. Membrane Biol. 54, 61-72.

- Fishman, P.H. and Brady, R.O. (1976) : Science 194, 906-915.
- Fishman, P.H., Moss, J. and Manganiello, V.C. (1977) : Biochemistry 16, 1871-1875.
- Fishman, P.H., Moss, J. and Vaughan, M. (1976) : J. Biol. Chem. 251, 4490-4494.
- Folkman, J. and Moscona, A. (1978) : Nature 273, 345-349.
- Formisano, S., Johnson, M.L., Lee, G., Aloj, S.M. and Edelhoch, H. (1979) : Biochemistry 18, 1119-1124.
- Fredman, P., Nilsson, O., Tayot, J. and Svernerholm, L. (1980) : Biochim. Biophys. Acta 618, 42-52.
- Fukuda, M. and Hakomori, S. (1979) : J. Biol. Chem. 254, 6746-6751.
- Furie, M.B., Frey, A.B. and Rifkin, D.B. (1980) : J. Biol. Chem. 255, 4391-4394.
- Gahmberg, C.G., Kiehn, D. and Hakomori, S. (1974) : Nature 248, 413-415.
- Gahmberg, C.G. and Hakomori, S. (1974) : Biochem. Biophys. Res. Comm. 59, 283-291.
- Geiger, B. (1979) : Cell 18, 193-205.
- Geiger, B., Tokuyasu, K.T., Dutton, A.H. and Singer, S.J. (1980) : Proc. Natl. Acad. Sci. (USA) 77, 4127-4131.
- Gingell, D. (1981) : J. Cell Sci. 49, 237-247.
- Gingell, D. and Vince, S. (1982) : J. Cell Sci. 54, 255-285.
- Ginsberg, M.H., Pointer, R.G., Forsyth, J., Birdwell, C. and Plow, E.F. (1980) : Proc. Natl. Acad. Sci. (USA) 77, 1049-1053.
- Gold, L.I. and Pearlstein, E. (1979) : Biochim. Biophys. Acta 581, 237-245.

- Goldman, R.D., Schloss, J.A. and Starger, J.M. (1976) : Cold Spring Harbour Conf. Cell Proliferation 3, 217-245.
- Gottlieb, C. and Kornfeld, S. (1976) : J. Biol. Chem. 251, 7761-7768.
- Griffin, J.H. (1978) : Proc. Natl. Acad. Sci. (USA) 75, 1998-2002.
- Griffiths, S.L., Perkins, R.M., Streuli, C.H. and Critchley, D.R. :
in preparation.
- Grinnell, F. (1974) : Arch. Biochem. Biophys. 165, 524-530.
- Grinnell, F. (1976) : Exp. Cell Res. 97, 265-274.
- Grinnell, F. (1978) : Int. Rev. Cytol. 53, 65-144.
- Grinnell, F. (1980a): J. Cell Biol. 86, 104-112.
- Grinnell, F. (1980b): Cell Biol. Int. Rep. 4, 1031-1036.
- Grinnell, F., Feld, M. and Minter, D. (1980) : Cell 19, 517-525.
- Grinnell, F. and Hays, D.G. (1978) : Exp. Cell Res. 116, 275-284.
- Grinnell, F., Hays, D. and Minter, D. (1977) : Exp. Cell Res. 110, 175-190.
- Grinnell, F. and Minter, D. (1978) : Proc. Natl. Acad. Sci. (USA) 75,
4408-4412.
- Hagman, J. and Fishman, P.H. (1982) : Biochim. Biophys. Acta 720, 181-187.
- Hahn, L.H.E. and Yamada, K.M. (1979) : Proc. Natl. Acad. Sci. (USA) 76,
1160-1163.
- Hakomori, S. (1981) : Ann. Rev. Biochem. 50, 733-764.
- Haksar, A., Maudsley, D.V. and Peron, F.G. (1974) : Nature 251, 514-515.
- Harper, P.A. and Juliano R.L. (1981) : Nature 290. 136-138.
- Hassell, J.R., Pennypacker, J.P., Kleinman, H.K., Pratt, R.M. and Yamada,
K.M. (1979) : Cell 17, 821-826.

- Hayashi, M., Schlesinger, D.F., Kennedy, D.W. and Yamada, K.M. (1980) :
J. Biol. Chem. 255, 10017-10020.
- Hayashi, M. and Yamada, K.M. (1981) : J. Biol. Chem. 256, 11292-11300.
- Hayashi, M. and Yamada, K.M. (1982) : J. Biol. Chem. 257, 5263-5267.
- Hayman, E.G., Engvall, E., A'Hearn, E., Barnes, D., Pierschbacher, M. and
Ruoslahti, E. (1982) : J. Cell Biol. 95, 20-23.
- Hayman, E.G., Engvall E. and Ruoslahti, E. (1980) : J. Cell Biol. 88,
352-357.
- Hayman, E.G., Pierschbacher, M.D. Ögren, Y. and Ruoslahti, E. (1983) :
Proc. Natl. Acad. Sci. (USA) 80, 4003-3007.
- Heasman, J., Hynes, R.O., Swan, A.P., Thomas, V. and Wylie, C.C. (1981) :
Cell 27, 437-447.
- Hedman, K., Johansson, S., Vartio, T., Kjellman, L., Vaheri, A. and Höök, M.
(1982) : Cell 28, 663-671.
- Hedman, K., Vaheri, A. and Wartiovaara, J. (1978) : J. Cell Biol. 76, 748-
760.
- Hewitt, A.T., Kleinman, H.K., Pennypacker, J.P. and Martin, G.R. (1980) :
Proc. Natl. Acad. Sci. (USA) 77, 385-388.
- Hewitt, A.T., Varner, H.H., Silver, M.H., Dessau, W., Wilkes, C.M. and
Martin, G.R. (1982) : J. Biol. Chem. 257, 2330-2334.
- Hirano, H., Yamada, Y., Sullivan, M., de Crombrughe, B., Pastan, I. and
Yamada, K.M. (1983) : Proc. Natl. Acad. Sci. (USA) 80, 46-50.
- Hoch, S.O. (1982) : Biochem. Biophys. Res. Comm. 106, 1353-1361.
- Holmgren, J., Elwing, H., Fredman, P. and Svennerholm, L. (1980) : Euro.
J. Biochem. 106, 371-379.

- Hsieh, P. and Sueoka, N. (1980) : J. Cell Biol. 86, 866-873.
- Hughes, R.C., Butters, T.D. and Aplin, J.D. (1981) : Euro. J. Cell Biol. 26, 198-207.
- Hughes, R.C., Pena, S.D.J., Clark, J. and Dourmashkin, R.R. (1979) : Exp. Cell Res. 121, 307-314.
- Hynes, R.O. (1973) : Proc. Natl. Acad. Sci. (USA) 70, 3170-3174.
- Hynes, R.O. (1976) : Biochim. Biophys. Acta 458, 73-107.
- Hynes, R.O. (1981) : In Cell Surface Reviews Vol. 7 Cytoskeletal Elements and Plasma Membrane Organisation. G. Poste and G.L. Nicolson, editors. pg. 100-107; Elsevier/North Holland Biomedical Press Amsterdam, New York.
- Hynes, R.O., Ali, I.U., Destree, A.T., Mautner, V., Perkins, M.E., Senger, D.R., Wagner, D.D. and Smith, K.K. (1978) : Ann. N.Y. Acad. Sci. 312, 317-342.
- Hynes, R.O. and Bye, J.M. (1974) : Cell 3, 113-120.
- Hynes, R.O. and Destree, A.T. (1977) : Proc. Natl. Acad. Sci. (USA) 74, 2855-2859.
- Hynes, R.O. and Destree, A. (1978) : Cell 15, 875-886.
- Hynes, R.O., Destree, A.T. and Wagner, D.D. (1982) : Cold Spring Harbour Symp. Vol. XLVI, 659-670.
- Hynes, R.O. and Yamada, K.M. (1982) : J. Cell Biol. 95, 369-377.
- Iwanaga, S., Suzuki, K. and Hashimoto, S. (1978) : Ann. N.Y. Acad. Sci. 312, 56-73.
- Izzard, C.S. and Lochner, L.R. (1976) : J. Cell Sci. 21, 129-159.

- Johansson, S. and Höök, M. (1984) : J. Cell Biol. 98, 810-817.
- Jone, C. and Hager, L.P. (1976) : Biochem. Biophys. Res. Comm. 68, 16-20.
- Kanda, S., Inone, K., Nojima, S., Utsumi, H. and Wiegandt, H. (1982) :
J. Biochem. 91, 1707-1718.
- Karnovsky, M.J. (1965) : J. Cell Biol. 27, 137A-138A.
- Keenan, T.W., Morre, D.J. and Basu, S. (1974) : J. Biol. Chem. 249,
310-315.
- Keenan, T.W., Schmid, E., Franke, W.W. and Wiegandt, H. (1975) : Exp. Cell
Res. 92, 259-270.
- Kellie, S., Patel, B., Pierce, E.J. and Critchley, D.R. (1983) : J. Cell
Biol. 97, 447-454.
- Keski-Oja, J. and Yamada, K.M. (1981) : Biochem. J. 193, 615-620.
- Kjellen, L., Pettersson, L., and Höök, M. (1981) : Proc. Natl. Acad. Sci.
(USA) 78, 5371-5375.
- Klebe, R.J. (1974) : Nature 250, 248-251.
- Klebe, R.J., Bentley, K.L. and Schoen, R.C. (1981) : J. Cell Physiol. 109,
481-488.
- Klebe, R.J. and Mock, P.J. (1982) : J. Cell Physiol. 112, 5-9.
- Kleinman, H.K., Klebe, R.J. and Martin, G.R. : J. Cell Biol. 88, 473-485.
- Kleinman, H.K., Hewitt, A.T., Murray, J.C., Liotta, L.A., Rennard, S.I.,
Pennypacker, J.P., McGoodwin, E.B., Martin, G.R. and Fishman, P.H.
(1979b) : J. Supramolec. Struct. 11, 69-78.
- Kleinman, H.K., Martin, G.R. and Fishman, P.H. (1979a) : Proc. Natl. Acad.
Sci. (USA) 76, 3367-3371.

- Kleinman, H.K., McGoodwin, E.B., Martin, G.R., Klebe, R.J., Fietzek, P.P. and Wolley, D.E. (1978) : J. Biol. Chem. 253, 5642-5646.
- Kleinman, H.K., McGoodwin, E.B., Rennard, S.I. and Martin, G.R. (1979c) : Anal. Biochem. 94, 308-312.
- Knudsen, K.A., Rao, P., Damsky, C.H. and Buck, C.A. (1981). : Proc. Natl. Acad. Sci. (USA) 78, 6071-6075.
- Kolega, J., Shure, M.S., Chen, W.T. and Young, N.D. (1982) : J. Cell Sci. 54, 23-34.
- Krishnaraj, R., Saat, Y.A. and Kemp, R.G. (1980) : Cancer Res. 40, 2808-2813.
- Kurkinen, M., Vartio, T. and Vaheri, A. (1980) : Biochim. Biophys. Acta 624, 490-498.
- Kurkinen, M., Wartiovaara, J. and Vaheri, A. (1978) : Exp. Cell Res. 111, 127-
- Labat-Robert, J., Potazman, J.P., Derouette, J.C. and Robert, L. (1981) : Cell Biol. Int. Reports 5, 969-973.
- Laemmli, U.K. (1970) : Nature 227, 680-685.
- Lahav, J., Schwartz, M.A. and Hynes, R.O. (1982) : Cell 31, 253-262.
- Laine, R.A. and Hakomori, S. (1973) : Biochem. Biophys. Res. Comm. 54, 1039-1045.
- Laterra, J., Ansbacher, R. and Culp, L.A. (1980) : Proc. Natl. Acad. Sci. (USA) 77, 6662-6666.
- Laterra, J. and Culp, L.A. (1982) : J. Biol. Chem. 257, 719-726.
- Laterra, J., Norton, E.K., Izzard, C.S. and Culp, L.A. (1983a) : Exp. Cell Res. 146, 15-27.

- Laterra, J., Silbert, J.E. and Culp, L.A. (1983b) : J. Cell Biol. 96, 112-123.
- Lehto, V.P., Vartio, T. and Virtanen, I. (1980) : Biochem. Biophys. Res. Comm. 95, 909-916.
- Lindahl, U. and Höök, M. (1978) : Ann. Rev. Biochem. 47, 385-417.
- Linder, E., Stenman, S., Lehto, V.P. and Vaheri, A. (1978) : Ann. N.Y. Acad. Sci. 312, 151-159.
- Linder, E., Vaheri, A., Ruoslahti, E. and Wartiovaara, J. (1975) : J. Exp. Med. 142, 41-49.
- Lingwood, C.A. and Hakomori, S. (1977) : Exp. Cell Res. 108, 385-391.
- Lingwood, C.A., Hakomori, S. and Ji, T.H. (1980) : FEBS Lett. 112, 265-268.
- Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) : J. Biol. Chem. 193, 265-275.
- Luiza, M., Barbosa, F. and de Silva, P.P. (1983) : Cell 33, 959-966.
- Magnani, J.L., Smith, D.F. and Ginsburg, V. (1980) : Anal. Biochem. 109, 399-402.
- Marquette, D., Molnar, J., Yamada, K.M., Schlesinger, D., Darby, S. and Van Alten, P. (1981) : Mol. Cell. Biochem. 36, 147-155.
- Martin, G.R. and Rubin, H. (1974) : Exp. Cell Res. 85, 319-333.
- McAbee, D.D. and Grinnell, F. (1983) : J. Cell Biol. 97, 1515-1523.
- McMaster, G. and Zardi, L. (1982) : Biochem. Biophys. Res. Comm. 107, 609-615.
- Meager, A., Ungkitchanukit, A., Nairn, R. and Hughes, R.C. (1975) : Nature 257, 137-139.

- Milhaud, P., Yamada, K.M. and Gottesman, M.M. (1980) : J. Cell Physiol. 104, 163-170.
- Mora, P.T., Brady, R.O., Bradley, R.M. and McFarland, V.W. (1969) : Proc. Natl. Acad. Sci. (USA) 63, 1290-1296.
- Morgan, J. and Garrod, D. (1984) : J. Cell Sci. 66, 133-145.
- Morrison, P., Edsall, R. and Miller, S.G. (1948) : J. Am. Chem. Soc. 70, 3103-3108.
- Mosesson, M.W. and Amrani, D.L. (1980) : Blood 56, 145-158.
- Mosesson, M.W., Chen, A.B. and Huseby, R.M. (1975) : Biochim. Biophys. Acta 386, 509-524.
- Mosher, D.F. and Proctor, R.A. (1980) : Science 209, 927-929.
- Mosher, D.F., Schad, P.E. and Kleinman, H.K. (1979) : J. Supramolec. Struct. 11, 227-235.
- Mosher, D.F. and Williams, E.M. (1978) : J. Lab. Clin. Med. 91, 729-735.
- Moss, J., Fishman, P.H., Manganiello, V.C., Vaughan, M. and Brady, R.O. (1976) : Proc. Natl. Acad. Sci. (USA) 73, 1034-1037.
- Muir, L.W., Bornstein, P. and Ross, R. (1976) : Eur. J. Biochem. 64, 105-114.
- Murray, B.A., Ansbacher, R. and Culp, L.A. (1980) : J. Cell Physiol. 104, 335-348.
- Nath, K. and Srere, P.A. (1977) : J. Cell Physiol. 92, 33-42.
- Niemczuk, P., Perkins, R.M., Talbot, I.C. and Critchley, D.R. (1982) : Br. J. Cancer 45, 500-505.
- Odermatt, E. and Engel, J. (1982) : J. Mol. Biol. 159, 109-123.
- O'Keefe, E. and Cuatrecasas, P. (1977) : Life Sci. 21, 1649-1654.

- Oh, E., Pierschbacher, M. and Ruoslahti, E. (1981) : Proc. Natl. Acad. Sci. (USA) 78, 3218-3221.
- Oldberg, A., Linney, E. and Ruoslahti, E. (1983) : J. Biol. Chem. 258, 10193-10196.
- Olden, K., Pratt, R.M. and Yamada, K.M. (1979) : Proc. Natl. Acad. Sci. (USA) 76, 3343-3349.
- Orly, J. and Sato, G. (1979) : Cell 17, 295-305.
- Pande, H. and Shively, J.E. (1982) : Arch. Biochem. Biophys. 213, 258-265.
- Pearlstein, E. (1976) : Nature 262, 497-499.
- Pearlstein, E. and Gold, L.I. (1978) : Ann. N.Y. Acad. Sci. 312, 278-292.
- Pearlstein, E., Gold, L.I. and Garcia-Pardo, A. (1980) : Molec. and Cell Biochem. 29, 103-127.
- Pena, S.D.J. and Hughes, R.C. (1978) : Nature 276, 80-83.
- Perkins, M.E., Ji, T.H. and Hynes, R.O. (1979) : Cell 16, 941-952.
- Perkins, R.M., Kellie, S., Patel, B. and Critchley, D.R. (1982) : Exp. Cell Res. 141, 232-243.
- Petersen, T.E., Thøgersen, H.C., Skorstengaard, K., Vibe-Pedersen, K., Sahl, P., Sottrup-Jensen, L. and Magnusson, S. (1983) : Proc. Natl. Acad. Sci. (USA) 80, 137-141.
- Pierschbacher, M.D., Hayman, E.G. and Ruoslahti, E. (1981) : Cell 26, 259-267.
- Pierschbacher, M.D., Ruoslahti, E., Sundelin, J., Lind, P. and Petersen, P.A. (1982) : J. Biol. Chem. 257, 9593-9597.
- Plow, E.F. and Ginsberg, M.H. (1981) : J. Biol. Chem. 256, 9477-9482.
- Pouyssegur, J. and Pastan, I. (1976) : Proc. Natl. Acad. Sci. (USA) 73, 544-548.

- Pouyssegur, J., Willingham, M. and Pastan, I. (1977) : Proc. Natl. Acad. Sci. (USA) 74, 243-247.
- Quaroni, A., Isselbacher, K.J. and Ruoslahti, E. (1978) : Proc. Natl. Acad. Sci. (USA) 75, 5548-5552.
- Radsak, K., Schwarzmann, G. and Wiegandt, H. (1982) : Hoppe-Seyler's Z. Physiol. Chem. 363, 263-272.
- Rajaraman, R., Rounds, D.E., Yen, S.P.S. and Rembaum, A. (1974) : Exp. Cell Res. 88, 327-339.
- Rauvala, H. (1984) : J. Cell Biol. 98, 1010-1016.
- Rauvala, H., Carter, W.G. and Hakomori, S. (1981) : J. Cell Biol. 88, 127-137.
- Rauvala, H. and Finne, J. (1979) : FEBS lett. 97, 1-8.
- Rees, D.A., Badley, R.A., Lloyd, C.W., Thom, D. and Smith, C.G. (1978) : Soc. Exp. Biol. Symp. 32, 241-260.
- Rennard, S.I., Wind, M.L., Hewitt, A.T. and Kleinman, H.K. (1981) : Arch. Biochem. Biophys. 206, 205-212.
- Révész, T. and Greaves, M.F. (1975) : Nature 257, 103-106.
- Rich, A.M., Pearlstein, E., Weissmann, G. and Hoffstein, S.T. (1981) : Nature 293, 224-226.
- Richter, H., Seidl, M. and Hormann, H. (1981) : Hoppe-Seyler's Z. Physiol. Chem. 362, 399-408.
- Ruoslahti, E. and Engvall, E. (1978) : Ann. N.Y. Acad. Sci. 312, 178-191.
- Ruoslahti, E. and Engvall, E. (1980) : Biochim. Biophys. Acta 631, 350-358.
- Ruoslahti, E., Engvall, E. and Hayman, E.G. (1981) : Coll. Res. 1, 95-128.

- Ruoslahti, E., Engvall, E., Hayman, E.G. and Spiro, R. (1981) : Biochem. J. 193, 295-299.
- Ruoslahti, E., Hayman, E.G., Pierschbacher, M. and Engvall, E. (1982) : Methods in Enzymol. 82, 803-831.
- Ruoslahti, E., Vaheri, A., Kuusela, P. and Linder, E. (1973) : Biochim. Biophys. Acta 322, 352-358.
- Saba, T.M., Blumenstock, F.A., Scovill, W.A. and Bernard, H. (1978) : Science 201, 622-624.
- Saba, T.M. and Jaffe, E. (1980) : Am. J. Med. 68, 577-594.
- Sahyoun, N., Shatila, T., Levine, H. and Cuatrecasas, P. (1981) : Biochem. Biophys. Res. Comm. 102, 1216-1222.
- Sato, C., Duff, R., Rapp, F. and Davidson, E.A. (1973) : Proc. Natl. Acad. Sci. (USA) 70, 54-56.
- Schachner, M., Schoonmaker, G. and Hynes, R.O. (1978) : Brain Res. 158, 149-158.
- Schwarz, M.A. and Juliano, R.L. (1984) : Exp. Cell Res. 152, 302-312.
- Schwarzbauer, J.E., Tamkun, J.W., Lemischka, I.R. and Hynes, R.O. (1983) : Cell 35, 421-431.
- Sear, C.H.J., Grant, M.E. and Jackson, D.S. (1977) : Biochem. J. 168, 91-103.
- Sekiguchi, K., Fukuda, M. and Hakomori, S. (1981) : J. Biol. Chem. 256, 6452-6462.
- Sekiguchi, K. and Hakomori, S. (1980) : Proc. Natl. Acad. Sci. (USA) 77, 2661-2665.
- Sekiguchi, K. and Hakomori, S. (1983) : Biochem. 22, 1415-1422.

- Sela, B.-A., Raz, A. and Geiger, B. (1978) : Euro. J. Immunol. 8, 268-274.
- Seppä, H.E.J., Yamada, K.M., Seppä, S.T., Silver, M.H., Kleinman, H.K. and Schiffmann, E. (1981) : Cell Biol. Int. Reports 5, 813-819.
- Sharom, F.J. and Grant, C.W.M. (1978) : Biochim. Biophys. Acta 507, 280-293.
- Singer, I.I. (1979) : Cell 16, 675-685.
- Singer, I.I. (1982) : J. Cell Biol. 92, 398-408.
- Singer, I.I. and Paradiso, P.R. (1981) : Cell 24, 481-492.
- Skipski, V.P., Gitterman, C.O., Prendergast, J.S., Betit-Yen, K., Lee, G., Luell, R. and Stock, C.C. (1980) : J. Natl. Cancer Inst. 65, 249-256.
- Skorstengaard, K., Thøgersen, H.C., Vibe-Pedersen, K., Peterson, T.E. and Magnusson, S. (1982) : Euro. J. Biochem. 128, 605-623.
- Smith, D.E. and Furcht, L.T. (1982) : J. Biol. Chem. 257, 6518-6523.
- Smith, D.E., Mosher, D.F., Johnson, R.B. and Furcht, L.T. (1982) : J. Biol. Chem. 257, 5831-5838.
- Smith, H.S., Riggs, J.L. and Mosesson, M.W. (1979) : Cancer Res. 39, 4138-4144.
- Spiegel, S., Schlessinger, J. and Fishman, P.H. (1984) : J. Cell Biol. 99, 699-704.
- Stathakis, N.E. and Mosesson, M.W. (1977) : J. Clin. Invest. 60, 855-865.
- Stemberger, A. and Hörmann, H. (1976) : Hoppe-Seyler's Z. Physiol. Chem. 357, 1003-1005.
- Streuli, C.H. (1982) : Ph.D. thesis, Leicester University.
- Streuli, C.H., Griffiths, S.L. and Critchley, D.R. (1984) : Exp. Cell Res. in press.

- Streuli, C.H., Patel, B. and Critchley, D.R. (1981) : Exp. Cell Res. 136, 247-254.
- Svennerholm, L. (1957) : Biochim. Biophys. Acta 24, 604-611.
- Svennerholm, L. (1970). In M. Florkin and E.H. Stotz (eds.) : Comprehensive Biochem. Vol. 18, 201-225 (Elsevier, Amsterdam).
- Svennerholm, L. and Fredman, P. (1980) : Biochim. Biophys. Acta 617, 97-109.
- Svennerholm, L., Mandel, P., Dreyfus, H. and Urban, P.F. (1980) : Adv. Exp. Med. Biol. 125 (Plenum press).
- Tarone, G., Galetto, G., Pratt, M. and Comoglio, P.M. (1982) : J. Cell Biol. 94, 179-186.
- Taylor, A.C. (1961) : Exp. Cell Res. Suppl. 8, 154-159.
- Thom, D., Powell, A.J. and Rees, D.A. (1979) : J. Cell Sci. 35, 281-305.
- Timpl, R., Rodhe, H., Robey, P.G., Rennard, S.I., Foidart, J.M. and Martin, G.R. (1979) : J. Biol. Chem. 254, 9933-9937.
- Tonegawa, Y. and Hakomori, S.I. (1977) : Biochem. Biophys. Res. Comm. 76, 9-17.
- Towbin, H., Stachelin, T. and Gordon, J. (1979) : Proc. Natl. Acad. Sci. (USA) 76, 4350-4354.
- Van Heyningen, S. (1974) : Science 183, 656-657.
- Van Heyningen, S. (1982) : Bioscience Reps. 2, 135-146.
- Vaheri, A., Kurkinen, M., Lehto, V.-P. Linder, E. and Timpl, R. (1978) : Proc. Natl. Acad. Sci. (USA) 75, 4944-4948.
- Vaheri, A. and Mosher, D.F. (1978) : Biochim. Biophys. Acta 516, 1-25.

- Vaheri, A., Ruoslahti, E., Westermark, B. and Ponten, J. (1976) : J. Exp. Med. 143, 64-72.
- Virtanen, I., Vartio, T., Badley, R.A. and Lehto, V.-P. (1982) : Nature 298, 660-663.
- Wagner, D.D. and Hynes, R.O. (1979) : J. Biol. Chem. 254, 6746-6754.
- Wagner, D.D. and Hynes, R.O. (1980) : J. Biol. Chem. 255, 4304-4312.
- Wagner, D.D., Ivatt, R., Destree, A.T. and Hynes, R.O. (1981) : J. Biol. Chem. 256, 11708-11715.
- Wallace, D.G., Donovan, J.W., Schneider, P.M., Meunier, A.M. and Lundblad, J.L. (1981) : Arch. Biochem. Biophys, 212, 515-524.
- Wartiovaara, J., Leivo, I., Virtanen, I., Vaheri, A. and Graham, C.F. (1978) : Nature 272, 355-356.
- Wartiovaara, J., Stenman, S. and Vaheri, A. (1976) : Differentiation 5, 85-89.
- Watanabe, K., Hakomori, S., Powell, M.E. and Yokota, M. (1980) : Biochem. Biophys. Res. Comm. 92, 638-646.
- Whateley, J.G. and Knox, P. (1980) : Biochem. J. 185, 349-354.
- Wiegandt, H. (1982) : Adv. Neurochem. 4, 149-223.
- Wolff, I., Timpl, R. Pecker, I. and Steffen, C. (1967) : VOX Sang. 12, 443-456.
- Wylie, D.E., Damsky, C.H. and Buck, C.A. (1979) : J. Cell Biol. 80, 385-402.
- Yamada, K.M. (1983) : Ann. Rev. Biochem. 52, 761-799.
- Yamada, K.M., Critchley, D.R., Fishman, P.H. and Moss, J. (1983) : Exp. Cell Res. 143, 295-302.

- Yamada, K.M. and Kennedy, D.W. (1979) : J. Cell Biol. 80, 492-498.
- Yamada, K.M., Kennedy, D.W., Grotendorst, G.R. and Momoi, T. (1981) :
J. Cell Physiol. 109, 343-351.
- Yamada, K.M., Kennedy, D.W., Kimata, K. and Pratt, R.M. (1980) : J. Biol.
Chem. 255, 6055-6063.
- Yamada, K.M., Schlesinger, D.H., Kennedy, D.W. and Pastan, I. (1977) :
Biochemistry 16, 5552-5559.
- Yamada, K.M. and Weston, J.A. (1974) : Proc. Natl. Acad. Sci. (USA) 71,
3492-3496.
- Yamada, K.M., Yamada, S.S. and Pastan, I. (1976) : Proc. Natl. Acad. Sci.
(USA) 73, 1217-1221.
- Yogeeswaran, G. (1981) : J. Natl. Cancer Inst. 66, 303-310.
- Yogeeswaran, G. and Hakomori, S. (1975) : Biochemistry 14, 2151-2156.
- Zardi, L., Siri, A., Carnemolla, B., Santi, L., Gardner, W.D. and Hoch,
S.O. (1979) : Cell 18, 649-657.
- Zucker, M.B., Mosesson, M.W., Broekman, M.J. and Kaplan, K.L. (1979) :
Blood, 54, 8-14.

GANGLIOSIDES AS RECEPTORS FOR FIBRONECTIN?

Comparison of Cell Spreading on a Ganglioside-specific Ligand with that on Fibronectin

R. M. PERKINS, S. KELLIE, B. PATEL and D. R. CRITCHLEY¹

Department of Biochemistry, University of Leicester, Leicester LE1 7RH, UK

SUMMARY

We have examined the possibility that gangliosides act as the cell surface receptor for fibronectin, as previously suggested by the data of Kleinman et al. (Proc natl acad sci US 76 (1979) 3367), using three different approaches.

1. Gangliosides inhibited the spreading of both CHO and BHK cells on fibronectin-coated substrates. 50% inhibition of cell spreading was produced by 1.0 and 3.5×10^{-5} M di- and tri-sialogangliosides respectively, although monosialogangliosides were less effective inhibitors. The inhibition was apparently due to an interaction of gangliosides with fibronectin and not due to a direct effect of gangliosides on the cells.

2. Using anti-fibronectin antibodies, 125 I-labelled protein A, and gangliosides adsorbed to polystyrene tubes, we have provided direct evidence that fibronectin will bind to gangliosides. However, the interaction is apparently of low affinity compared with binding of cholera toxin to gangliosides.

3. We have compared the ability of BALB/c 3T3 cells to spread on fibronectin with that on substrates coated with a ganglioside-specific ligand, cholera toxin B-subunit. Cells plated onto fibronectin-coated substrates rapidly (within 60 min) adopted a well spread morphology, whereas spreading on substrates coated with the toxin B-subunit was less extensive even after 2 h. In addition, the organization of F-actin within cells spread on the two types of substrate was also quite different.

We conclude that the interaction of cells with fibronectin may well be influenced by membrane-bound gangliosides. It is unlikely, however, that binding of fibronectin to such gangliosides can lead to the cytoskeletal reorganization which is characteristic of cells spread on fibronectin.

Fibronectin is a high molecular weight (MW) glycoprotein found in loose connective tissue and basement membranes throughout the body, as well as in the blood [1, 2]. In vitro experiments suggest that fibronectin plays an important role in cell adhesion, and the purified protein will support both cell attachment and spreading of a variety of cell lines on tissue culture plastic [3, 4]. Analysis of the structure of the protein shows it to be made up of a variety of distinct domains and using limited proteolysis, fragments have been isolated

which contain binding sites for gelatin (collagen) [5], heparin [6], fibrin [7], actin [8], as well as the cell surface [9, 10]. Cellular interaction with fibronectin is presumed to involve a surface receptor, but the evidence for the existence of such a receptor is weak, mainly because of the difficulties of carrying out classical ligand-binding experiments with fibronectin [11, 12].

One approach to identify the receptor has been to conduct cross-linking studies using

¹ To whom offprint requests should be sent.

fibronectin derivatised with a photoactivable group. Initial experiments showed that addition of fibronectin to cell monolayers resulted in cross-linking to proteoglycans, but this may well reflect interaction of fibronectin with other components of the extracellular matrix [13]. More recently, by allowing cells to spread on derivatised fibronectin, evidence has been presented to suggest that fibronectin interacts with a glycoprotein with a MW of 48 000 [14]. However, the same glycoprotein was also isolated when cells were allowed to spread on photoactivatable derivatives of conA and ricin. In an alternative approach Kleinman et al. [15] found that attachment of CHO cells to a fibronectin–collagen matrix was inhibited if the matrix was first preincubated with gangliosides, although the gangliosides did not inhibit binding of fibronectin to the collagen. The inhibitory activity of the gangliosides was partially retained in the isolated oligosaccharide moiety and a role for gangliosides as the cell surface receptor for fibronectin was therefore proposed.

In the present study we have extended the original observations of Kleinman et al. [15] by examining the ability of gangliosides to inhibit fibronectin-dependent BHK cell spreading, an assay commonly used to measure the biological activity of fibronectin. In addition we have also investigated the ability of fibronectin to bind to gangliosides, using an assay previously used to study the binding of cholera and tetanus toxins to gangliosides [16]. Since the interaction of cells with fibronectin-coated substrates triggers cell spreading, a process thought to involve reorganization of the cytoskeleton, some form of transmembrane signalling must occur [17]. We have therefore examined the extent to which cells can spread on a ligand specific for the carbohydrate sequence in a ganglioside, given

that such molecules are not thought to span the lipid bilayer. More specifically, we have studied the ability of BALB/c 3T3 cells to spread on the binding subunit of cholera toxin because (1) the current evidence suggests that the toxin receptor in BALB/c 3T3 cells is exclusively contained in the carbohydrate sequence of ganglioside GM₁; (2) these cells contain a substantial number of cell surface receptors for the toxin [18].

MATERIALS AND METHODS

Cell culture

Baby hamster kidney (BHK21), Chinese hamster ovary (CHO), and BALB/c 3T3 cells were all grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% newborn calf serum (Gibco Bio-cult, Paisley, Scotland).

Fibronectin-mediated cell spreading assay

Confluent monolayers of BHK or CHO cells were trypsinized (0.25% trypsin; Gibco), and the cells washed twice with phosphate-buffered saline (PBS), 1 mM phenylmethylsulphonylfluoride (PMSF) before resuspension in DMEM (2×10^5 cells/ml). Approx. 5×10^4 cells were added to Linbro wells (Flow Labs) coated with fibronectin, and after 1 h at 37°C the cells were fixed in 3.8% formaldehyde in PBS and subsequently stained with crystal violet (0.02% w/v). Cell spreading was quantitated by counting the number of spread cells in 3–6 fields of view (80–100 cells/field) and expressing this as a percentage of the total number of attached cells. Duplicate wells were used for each experimental condition. Where appropriate, gangliosides (1 mg/ml stock solution in DMEM) were added to fibronectin-coated wells and incubated for 1 h at 37°C before the direct addition of cells.

Fibronectin purification and production of anti-fibronectin

Fibronectin was purified from outdated human plasma (citrated) by affinity chromatography on gelatin-Sepharose, as described by Engvall & Ruoslahti [19]. Fibronectin was further purified by preparative SDS-polyacrylamide gel electrophoresis, and antisera were produced and characterised as described elsewhere [20].

Purification of gangliosides

Mixed gangliosides were extracted from human brain by the method of Svennerholm & Fredman [21] and separated into mono-, di- and tri-sialogangliosides using a DEAE-Sepharose CL6B column eluted step-

wise with potassium acetate [22]. Fractions were analysed by thin-layer chromatography on silica gel G plates (Merck) using the solvent chloroform:methanol:water 60:35:8 (v/v) and quantitated by the resorcinol assay of Svennerholm [23]. Ganglioside standards were purchased from Supelco (Bellefonte, Pa).

Binding of fibronectin to plastic-adsorbed gangliosides

Gangliosides dissolved in PBS were adsorbed to 10×55 mm polystyrene tubes (AB Heger-Plast, Stallarholm, Sweden) for 18 h, as described by Holmgren et al. [16]. The tubes were washed with PBS and incubated for 1 h at 37°C with 500 µl of 10 mg/ml BSA in PBS. ¹²⁵I-labelled cholera toxin or fibronectin was then added in 200 µl of PBS containing 5 mg/ml BSA (2 h, 20°C) and unbound material removed by washing the tubes three times with 0.05% Tween 20 in PBS. The tubes were counted in a Beckman 5500 gamma counter. Binding of fibronectin and cholera toxin to ganglioside-coated tubes was also determined indirectly using specific antisera and ¹²⁵I-labelled protein A. Tubes were incubated with 100 µl of toxin or fibronectin in PBS containing 5 mg/ml BSA, washed three times with PBS, and then incubated for 1 h at 37°C with 100 µl of rabbit antisera diluted 1:30 in PBS/BSA. Unbound antibody was removed by washing three times with PBS, and the tubes were incubated a further 1 h with ¹²⁵I-labelled protein A in PBS/BSA. They were then washed three times with PBS/0.05% Tween 20 before counting.

Iodination procedures

Protein A was iodinated using a modification of the chloramine T procedure. Five µl of 1 mM chloramine T was incubated with 20 µl of 0.1 M H₂SO₄ for 1 min, and for further 2 min after addition of 5 µl of ¹²⁵I- (500 µCi). Thirty µl of potassium phosphate buffer pH 7.0 was added followed by 20 µl of protein A (1 mg/ml), and the incubation continued for a further 10 min at 20°C with occasional mixing. Protein iodination was terminated by adding 30 µl of a saturated tyrosine solution, and the mix diluted with 100 µl of NET/gelatin buffer (150 mM NaCl, 5 mM EDTA, 50 mM Tris, pH 7.4, 0.05% NP40, 0.25% gelatin) plus 20 µl of β-mercaptoethanol. ¹²⁵I-Labelled protein A was separated from ¹²⁵I-labelled tyrosine and free ¹²⁵I- on a 10 ml Sephadex G25 column packed and eluted with NET/gelatin buffer. The specific activity of the ¹²⁵I-labelled protein A was 12 µCi/µg and >65% was able to bind to rabbit IgG. Fibronectin was labelled by lactoperoxidase-catalysed iodination using Enzyme-beads (Biorad Labs) as prescribed by the manufacturer. The ¹²⁵I-labelled fibronectin was repurified by gelatin-Sepharose chromatography before use. Analysis of the labelled fibronectin by SDS-polyacrylamide gel electrophoresis showed that >90% of the radioactivity was associated with a single band which comigrated with unlabelled fibronectin. The specific activity of the labelled fibronectin was 8 µCi/µg. ConA was iodinated by the chloramine T method, as described by Burridge [24]. The specific activity of the

labelled protein was 4 µCi/µg. Cholera toxin was iodinated either by the chloramine T method, as previously described [25], or using iodogen (Pierce, Rockford, Ill.) [26]. Specific activities between 15–20 µCi/µg were obtained and the specific binding activity was >60%.

Attachment and spreading of BALB/c 3T3 cells on substrates coated with cholera toxin B-subunit, conA and fibronectin

Dynatech microelisa wells (M129B) were incubated for 2 h with 100 µl of cholera toxin, toxin B-subunit (Schwarz Mann), conA (Miles Yeda) or fibronectin in PBS, followed by BSA (10 mg/ml in PBS) to block the subsequent non-specific attachment of cells. To quantitate attachment 2–4×10⁵ BALB/c 3T3 cells, labelled with [³⁵S]methionine (0.3 µCi/ml for 18 h in methionine-free medium) were added to each well in 100 µl of DMEM. After incubation for 2 h at 37°C in a humidified CO₂ incubator, unattached cells were removed by gently washing each well once with PBS. Attached cells were solubilized in 1 M NaOH and aliquots taken for counting using a toluene-based scintillant. The ability of cells to spread on the various ligands was examined in a similar manner, except unlabelled cells were used which were then fixed and stained after the 2 h incubation period.

Actin staining of BALB/c 3T3 cells

Cells were plated onto glass coverslips derivatized with various proteins, as described by Aplin & Hughes [27]. After 2 h at 37°C the coverslips were washed with warmed PBS, and attached cells fixed with 3.8% formaldehyde in PBS for 30 min at 20°C. Cells were permeabilized with 0.25% Triton X-100 in PBS for 5 min, and after washing, the F-actin organisation within the cell was visualized by staining (30 min) with 0.1 µM NBD phalloidin (7-nitrobenz-2-oxa-1,3 diazole phalloidin; Molecular Probes Inc., Plano, Tex.) as previously described by Barak et al. [28]. Coverslips were washed with three changes of PBS (30 min) and mounted in 50% v/v glycerol in PBS. Stained cells were examined on a Standard 16 Zeiss microscope equipped with epifluorescence, using the filter set 9. Photographs were taken on Ilford HP5 35 mm film (400 ASA) uprated to 800 ASA.

RESULTS

Inhibition of fibronectin-dependent cell spreading by gangliosides

It has previously been shown by Kleinman et al. [15] that the fibronectin-dependent attachment of CHO cells to collagen-coated surfaces is inhibited by gangliosides and their oligosaccharide moieties. An important extension of this work would be to ex-

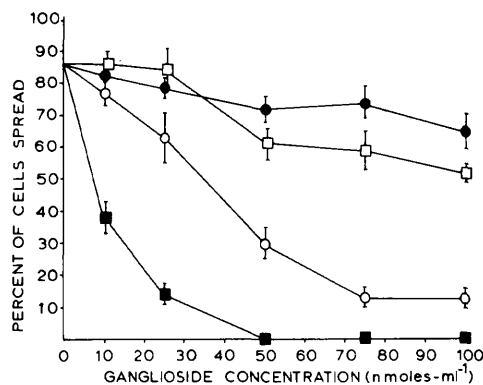


Fig. 1. Inhibition of fibronectin-mediated BHK cell spreading by gangliosides. Concentration dependence and ganglioside specificity. Linbro wells coated with 2 μ g/ml fibronectin (a concentration which just supports maximal cell spreading) were incubated with specific gangliosides (in 250 μ l DMEM) at various concentrations for 1 h, prior to the direct addition of 5×10^4 BHK cells. Cell spreading after 1 h at 37°C was quantitated as described in Methods. ●—●, GM₃; □—□, GM₁; ■—■, GD; ○—○, GT.

amine whether gangliosides are inhibitory in other assays commonly used to monitor the biological activity of fibronectin. We have therefore studied the ability of various gangliosides to inhibit spreading of BHK cells on fibronectin-coated tissue culture plastic. Gangliosides GD_{1a} and GT were consistently the most effective inhibitors of BHK cell spreading (fig. 1). The concentration of gangliosides GD_{1a} and GT needed to produce 50% inhibition of spreading on substrates coated with 2 μ g/ml fibronectin were approx. 10 and 35 μ M respectively. However, in some experiments the difference between the activity of GD_{1a} and GT was less apparent. Similar variability in the inhibition of fibronectin-dependent CHO cell attachment by gangliosides GD_{1a} and GT has previously been reported [15]. Monosialogangliosides GM₃ and GM₁ were consistently less effective inhibitors of BHK cell spreading, and the inhibition was incomplete (73%) even at high ganglioside concentration (200 μ M). Essentially similar

results were obtained when the ability of gangliosides to inhibit spreading of CHO cells on fibronectin was investigated (data not shown).

Because cells were added directly to ganglioside-containing medium in the above experiments, the basis for the inhibitory effects of gangliosides on fibronectin-dependent cell spreading is unclear. In an attempt to establish whether the inhibition is due to binding of gangliosides to fibronectin, or to a direct effect of gangliosides on the cells, we performed the following experiments. Firstly, pre-incubation of fibronectin-coated substrates with gangliosides, followed by removal of unbound ganglioside prior to the addition of cells, only partially relieved the inhibition (table 1A). Secondly, the inhibitory effect of gangliosides on cell spreading could be overcome by increasing the concentration of fibronectin used to coat the substrate (table 1B). These results show that gangliosides do not act directly on cells to inhibit spreading, and suggest (but do not prove) that it is binding of gangliosides to fibronectin which is the basis of this inhibition.

Studies on binding of fibronectin to plastic-adsorbed gangliosides

Gangliosides adsorbed to plastic have recently been used to investigate the chemical nature of both the cholera and tetanus toxin receptor [16]. We therefore attempted to demonstrate binding of ¹²⁵I-labelled fibronectin to various gangliosides using this technique. Binding of ¹²⁵I-labelled cholera toxin to tubes coated with ganglioside GM₁ was used to establish the methodology (data not shown). However, we were only able to demonstrate trace levels of ¹²⁵I-labelled fibronectin binding when tubes were pre-coated with very high concentrations of trisialogangliosides. In contrast, binding of

Table 1. *Inhibition of fibronectin-dependent cell spreading by gangliosides*

(A) Effect of removing unbound gangliosides; (B) effect of increasing fibronectin concentration

Incubation protocol	% Cell spreading	% Inhibition
A		
1 FN/wash/DMEM/CHO cells	74±10.9	0
2 FN/wash/DMEM+gangliosides/CHO cells	28±10.9	62.2
3 FN/wash/DMEM+gangliosides/wash/CHO cells	39±11.5	47.3
4 DMEM+gangliosides/wash/FN/wash/CHO cells	77±2.4	0
B		
1 1 µg/ml FN/wash/DMEM/BHK cells	82.6±3.3	0
2 1 µg/ml FN/wash/DMEM+gangliosides/BHK cells	7.4±2.5	91
3 5 µg/ml FN/wash/DMEM/BHK cells	90.9±2.8	0
4 5 µg/ml FN/wash/DMEM+gangliosides/BHK cells	74.5±4.9	18

Linbro wells were incubated for 1 h at 37°C with each of the above additions (250 µl/well) in the order shown. Where indicated wells were washed twice with DMEM prior to the next addition. 5×10^4 cells were then added to each well in DMEM and the percentage cell spreading determined after 1 h at 37°C, as described in Materials and Methods.

Fibronectin (FN, 3 µg/ml) was added in PBS.

Mixed gangliosides (bovine brain) were added in DMEM to a final concentration of 500 µg/ml.

fibronectin to gangliosides could be clearly demonstrated using a fibronectin antiserum and ^{125}I -labelled protein A (table 2). Interestingly, binding of fibronectin to di- and tri-sialogangliosides was generally greater than to monosialogangliosides GM_3 and GM_1 , reminiscent of the relative effectiveness of these molecules in inhibiting fibronectin-dependent cell spreading. As with previous experiments using ^{125}I -labelled fibronectin, binding could only be detected when tubes were coated at high ganglioside concentration. On the other hand, binding of cholera toxin to ganglioside GM_1 was readily detectable even at low ganglioside concentration.

Investigation of attachment and spreading of BALB/c 3T3 cells on cholera toxin

If the cell surface receptor for fibronectin were to be exclusively ganglioside in nature, one might expect cells to be able to spread on other ligands which interact with cell surface gangliosides. To test this hypo-

Table 2. *Demonstration of the binding of fibronectin to gangliosides using a solid phase radioimmune assay*

Immobilized ganglioside	Binding assay	^{125}I -labelled protein A bound (cpm)	
		A, 2 µM ganglioside	B, 200 µM ganglioside
GM_3	Fibronectin	0	392
GM_1	Fibronectin	0	935
GD	Fibronectin	0	3 004
GT	Fibronectin	0	1 367
GM_1	Cholera toxin	97 039	38 732

Binding of fibronectin (114 nM) to polystyrene tubes coated with various gangliosides (2 and 200 µM) was determined using a rabbit anti-fibronectin antiserum (diluted 1:30 in PBS) and ^{125}I -labelled protein A (sp. act. 350 cpm/fmole, 140 000 cpm/tube in expt A; 216 cpm/fmole, 110 000 cpm/tube in expt B). As a positive control for binding, tubes were coated with 1 mg/ml of gelatin, and fibronectin binding assayed as above. The cpm of ^{125}I -labelled protein A bound were 73 693 cpm (expt A) and 39 610 (expt B). To establish that gangliosides were adsorbed efficiently to the tubes, cholera toxin (40 nM) binding was assayed using a rabbit anti-cholera toxin antiserum and ^{125}I -labelled protein A.

In all cases, non-specific binding of ^{125}I -labelled protein A to tubes not coated with ganglioside has been subtracted (1010 cpm) from the total counts bound. The results are expressed as the mean of duplicate determinations differing from the mean by <10%.

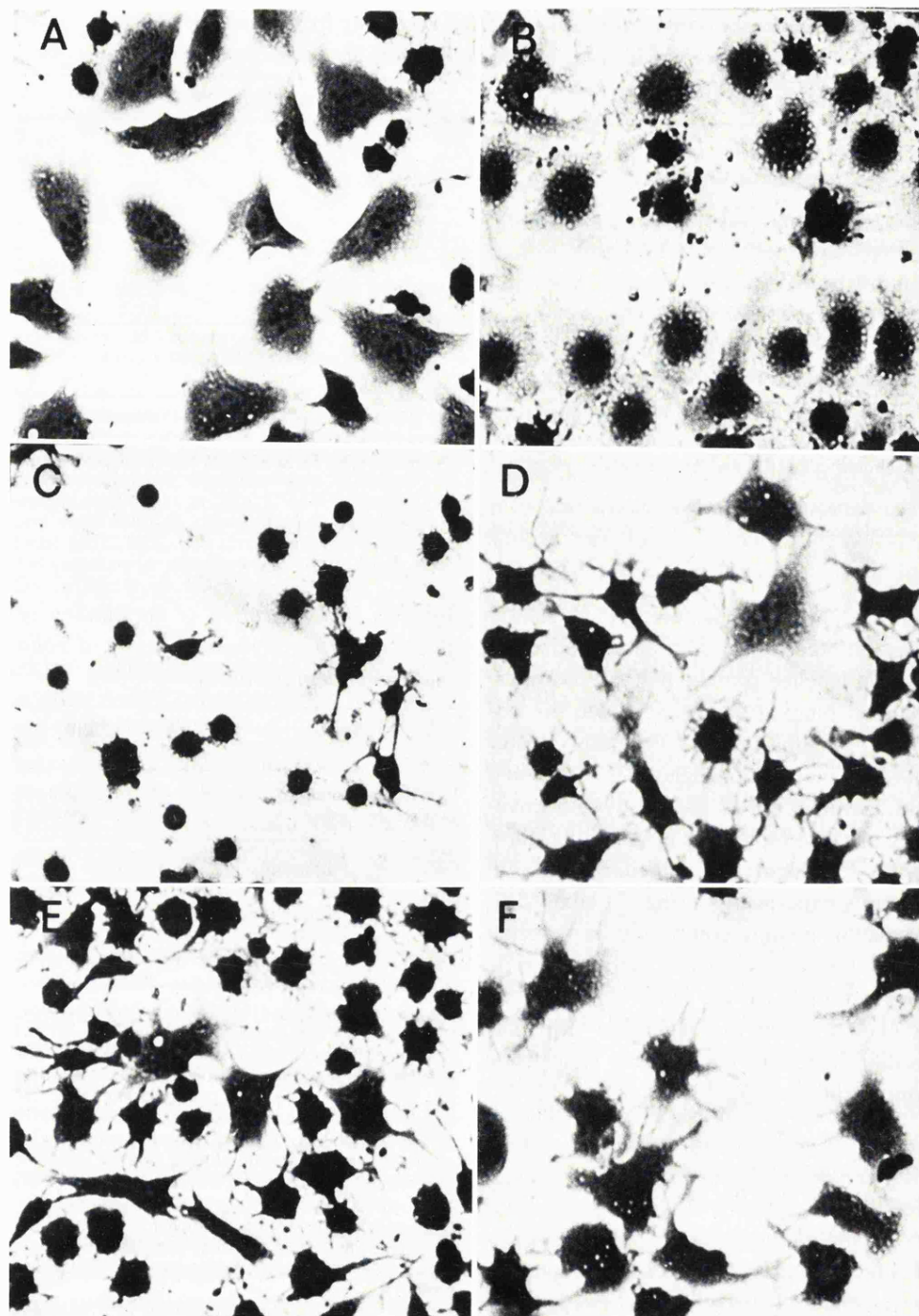


Fig. 2. Spreading of BALB/c 3T3 cells on fibronectin, conA and cholera toxin B-subunit-coated substrates. Cells were plated onto Dynatech microelisa wells coated with the above ligands, and incubated for 2 h at 37°C before fixing and staining. (A) 10 μ g/ml (22.5 nM) fibronectin; (B) 100 μ g/ml (0.91 μ M) conA; (C, D) 10 μ g/ml (0.182 μ M) cholera toxin B-subunit; (E,

F) 100 μ g/ml (1.82 μ M) of toxin B-subunit. In (D) and (F), cells were pretreated with 0.04 units/ml of neuraminidase (Behringwerke) for 1 h at 37°C, prior to trypsinization and plating onto wells coated with cholera toxin B-subunit. (Molarities expressed assuming the following MWs: Fibronectin, 440 000; conA 110 000; cholera toxin B-subunit, 55 000). $\times 200$.

thesis we chose to investigate the ability of BALB/c 3T3 cells to spread on substrates coated with cholera toxin and its binding subunit (toxin B subunit), because we have recently established that the toxin receptor in these cells is exclusively contained on the carbohydrate moiety of ganglioside GM₁ [18]. BALB/c 3T3 cells were able to attach to substrates coated with both cholera toxin and toxin B subunit, as well as conA and fibronectin (table 3). However, as addition of cholera toxin to BALB/c 3T3 cells is known to cause cell rounding, due to an increase in cAMP levels in response to activation of adenylate cyclase by the toxin A-subunit [29, 30], the ability of cells to spread on the toxin B-subunit was investigated. BALB/c 3T3 cells were able to spread on substrates coated with fibronectin (10 µg/ml, 22.5 nM) and conA (100 µg/ml, 0.91 µM), although the morphology of the spread cells was distinctly different (fig. 2A, B). In contrast, cells plated onto substrates coated with toxin B-subunit (100 µg/ml, 1.82 µM) were in general unable to spread (fig. 2E). However, the cells did show evidence of extensive process formation, as though they were attempting to spread, and the occasional fully spread cell could be found.

One possible trivial explanation which could account for failure of the cells to spread on toxin B-subunit is that the B-subunit preparation used might contain substantial amounts of intact toxin or toxin A-subunit. The following experiments provide definitive evidence against such an explanation. Firstly, addition of as little as 0.1 ng/ml (1.2 fmoles/ml) of cholera toxin to BALB/c 3T3 cells in monolayer causes a distinctive change in cell morphology within 1 h at 37°C. The change can be mimicked by db-cAMP, by phosphodiesterase inhibitors, and by prostaglandin E1 which ac-

Table 3. Attachment of BALB/c 3T3 cells to substrates coated with fibronectin, conA and cholera toxin

Ligand	No. of cells attached per well
None	0.22×10^4
Fibronectin (10 µg/ml)	2.3×10^4
ConA (100 µg/ml)	3.2×10^4
Cholera toxin (100 µg/ml)	3.0×10^4
Cholera toxin B-subunit (100 µg/ml)	2.4×10^4

3.75×10^4 cells (labelled with [³⁵S]methionine, 1400 cpm/ 10^5 cells) were added to Dynatech microelisa wells coated with various ligands, and the number of cells attached after a 2 h incubation at 37°C determined as described in Methods. The results represent the mean of triplicate points, not differing from the mean by >10%.

tivates adenylate cyclase in these cells [30]. In contrast, incubation of BALB/c 3T3 cells with 100 µg/ml (1.82 nmoles/ml) of the toxin B-subunit preparation produced no such morphology change. Calculation shows that contamination of the toxin B-subunit by intact toxin or toxin A-subunit accounts for $<6.6 \times 10^{-5}\%$ by weight. Secondly, iodination of the B-subunit preparation and autoradiography of the iodinated polypeptides separated in SDS-polyacrylamide gels showed that there was no detectable A-subunit present. Failure of cells to spread on toxin B-subunit might be explained if the efficiency with which the protein adsorbs to plastic is much less than that for conA and fibronectin. Quantitation of the relative binding efficiencies of conA and cholera toxin to plastic showed that 4.03 pmoles of conA were bound/cm² at a concentration of 0.5 µM (55 µg/ml), and 2.74 pmoles of cholera toxin were bound at a concentration of 1 µM (84 µg/ml). Standard iodination procedures inactivate toxin B-subunit and it is therefore difficult to obtain meaningful binding data. On the assumption that cholera toxin adsorbs to plastic with a similar

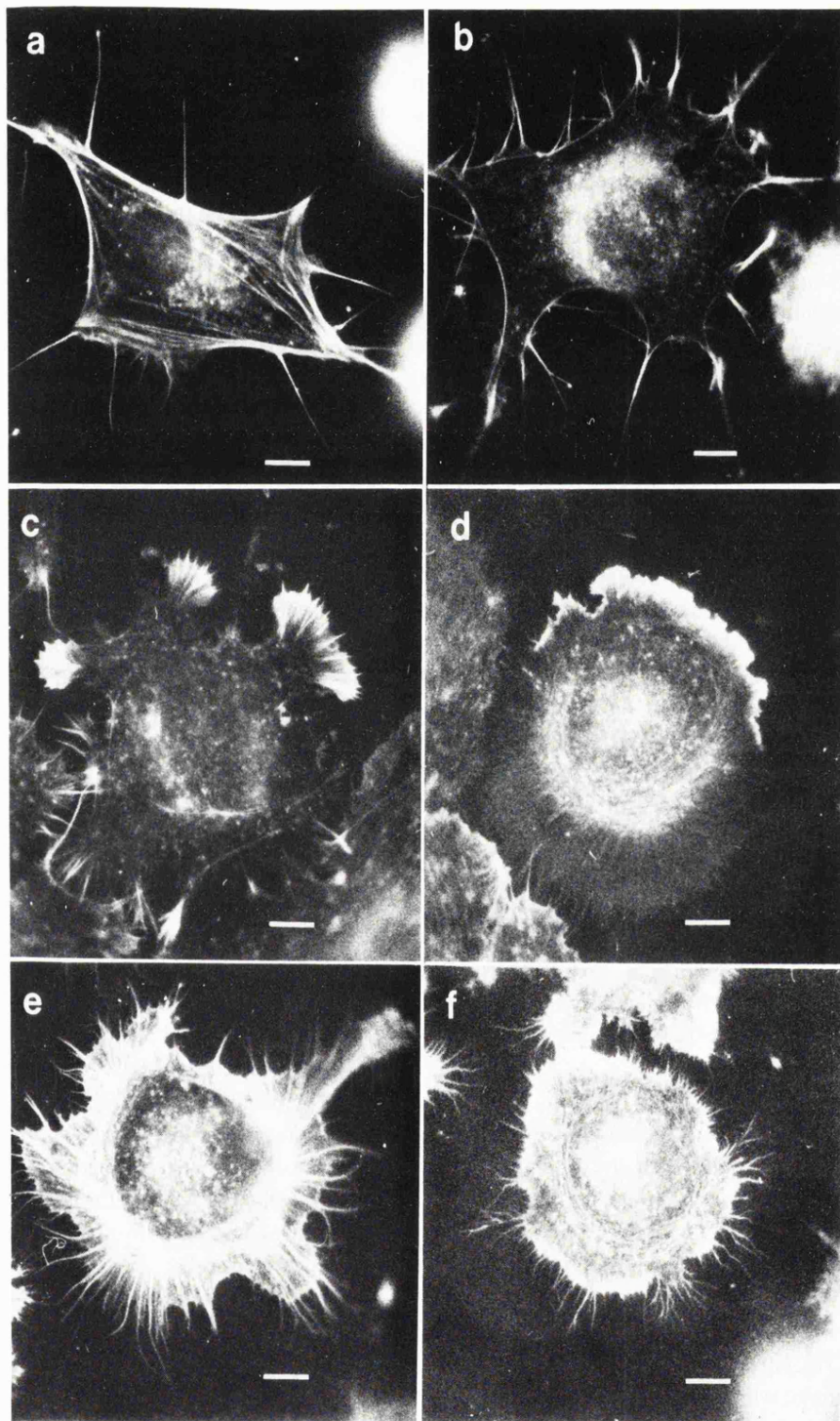
binding efficiency to toxin B-subunit, it seems unlikely that the small difference in the amount of cholera toxin bound compared to conA could account for the inability of BALB/c 3T3 cells to spread on toxin B-subunit.

In addition to the number of molecules of ligand adsorbed to the plastic, the density of cell surface receptors may also be an important determinant of spreading. We have previously shown that the toxin-binding capacity of BALB/c 3T3 cells ($0.8\text{--}1.6 \times 10^6$ molecules/cell) can be increased approx. 2-fold by pretreatment of cell monolayers with neuraminidase [31]. A comparison of the ability of neuraminidase-treated and untreated cells to spread on substrates coated at both a low (10 $\mu\text{g/ml}$) and high (100 $\mu\text{g/ml}$) concentration of toxin B-subunit is shown in fig. 2C–F. Interestingly, neuraminidase treatment markedly increased the ability of cells to spread at both the concentrations of toxin B-subunit tested (cf fig. 2C, D and E, F). However, even at the highest concentration of toxin B-subunit used (100 $\mu\text{g/ml}$) spreading appeared to be incomplete, particularly when compared with that supported by fibronectin. In addition, the periphery of cells plated onto toxin B-subunit showed evidence of extensive process formation, a feature not found to the same extent in cells plated onto conA or fibronectin.

Because the cytoskeleton is thought to play a major role in the spreading process, we examined the organization of F-actin in cells spreading on the three different substrates using fluorescent phalloidin. Two hours after plating onto fibronectin-derivatized glass coverslips, most of the cells were well spread with a morphology resembling that seen under normal culture conditions. The majority of the F-actin was localized at the periphery of the cell forming

a continuous fine sheet just under the cell membrane (fig. 3a, b). The staining also extended into thin processes projecting from the main body of the cell. Microfilament bundles were a major feature of cells spread on fibronectin, running in the direction of the long axis of the cell. There was very little peri-nuclear staining, although small fluorescently stained aggregates were common throughout the cytoplasm. In contrast to the morphology of cells spreading on fibronectin-coated substrates, cells plated onto conA were generally less well spread and had a rounded, more symmetrical appearance (fig. 3c, d). Many of the cells showed short intensely stained actin bundles associated with fan-shaped structures similar to lamellipodia (fig. 3c). Between these bundles were areas of diffuse but distinct staining, giving the periphery of the cell in these regions a webbed appearance. In some cells the submembranous actin staining was less well defined (fig. 3d). These latter cells frequently contained actin bundles which ran concentrically around the nucleus. The morphology of neuraminidase-treated cells plated on toxin B-subunit was distinct from either that adopted on fibronectin or conA (fig. 3E, f). The cells showed intense staining of actin bundles running concentrically around the nucleus, and actin bundles extended radially to protrude beyond the general cell outline. The cell periphery in between these filaments

Fig. 3. Comparison of F-actin organisation in BALB/c 3T3 cells spreading on fibronectin, conA and cholera toxin B-subunit. Cells plated onto glass coverslips derivatized with the various ligands, and incubated for 2 h at 37°C, were fixed and stained for F-actin using NBD-phalloidin as described in Methods. Coverslips were derivatized with (a, b) fibronectin, 0.5 mg/ml (1.1 μM); (c, d) conA, 1 mg/ml (9 μM); (e, f) cholera toxin B-subunit 0.1 mg/ml (1.82 μM). All cells were pretreated with neuraminidase. Two micrographs of cells spreading on each substrate are presented to demonstrate the different morphologies and staining profiles within each population. Bar, 10 μm .



also stained for actin again giving rise to a webbed appearance.

The possibility that spreading of cells on toxin B-subunit is due to secretion of newly synthesized fibronectin in the period following attachment is considered unlikely, based on the following experiments. BALB/c 3T3 cells were able to spread on toxin B-subunit in the presence of 20 $\mu\text{g/ml}$ of affinity-purified rabbit anti-fibronectin IgG, a concentration which completely inhibited cell spreading on fibronectin. As expected, cell attachment on toxin B-subunit was quantitatively inhibited by mixed brain gangliosides, and the IgG fraction of rabbit anti-cholera toxin completely blocked spreading on toxin B-subunit. In addition, using indirect immunofluorescence, we found no evidence for the presence of fibronectin on the dorsal or ventral surface of these cells up to 2 h after plating on a substrate coated with toxin B-subunit.

Spreading of BALB/c 3T3 cells on toxin B-subunit was completely inhibited by preincubation of cells with either cytochalasin B (5 $\mu\text{g/ml}$) or sodium azide (5 mM), as was spreading on fibronectin and conA. The result suggests that the cytoskeleton plays a key role in the spreading of cells on a ganglioside-specific ligand, as well as on fibronectin and conA.

DISCUSSION

There has been considerable recent interest in the possibility that glycosphingolipids act as cell surface receptors for a variety of different agents including cholera and tetanus toxin [32, 33], the glycoprotein hormones [34, 35], interferon [36], macrophage migration inhibition factor [37] and Sendai virus [38]. However, except in the case of cholera toxin, where the receptor has been isolated and characterized as a ganglioside (GM₁)

[18, 30], the evidence for such a role for glycosphingolipids is almost exclusively indirect, including that which suggests a role for gangliosides as the cell surface receptor for fibronectin [15]. In the present study we have extended the earlier work of Kleinman *et al.* [15] to show that gangliosides inhibit the spreading of both CHO and BHK cells on a fibronectin-coated substratum. Di- and tri-sialogangliosides were the best inhibitors of spreading in agreement with previous data using the CHO cell attachment assay [15], but the concentrations required to give 50% inhibition of spreading (10 and 35 μM respectively) were less than that required to produce a similar inhibition of CHO cell attachment (250 μM). The inhibition was only partially relieved by removing unbound ganglioside from the fibronectin-coated substrate prior to addition of cells, suggesting that it was not due to a direct effect of gangliosides on cells. This conclusion is also supported by the observation that inhibition of cell spreading by gangliosides can be overcome by increasing the concentration of fibronectin used to coat the substrate. The results suggest that the inhibition is due to binding of gangliosides to fibronectin, and provide indirect evidence that the cell surface receptor for fibronectin might be ganglioside in nature, as originally proposed by Kleinman *et al.* [15]. A similar conclusion has recently been drawn by Yamada *et al.* [39], based on the observations that gangliosides inhibit fibronectin-mediated hemagglutination and BHK cell spreading, as well as the ability of fibronectin to restore a normal morphology to transformed cells. A variety of other carbohydrate-containing macromolecules, including negatively charged glycosaminoglycans (e.g., hyaluronic acid and heparan sulphate), glycoproteins, and BHK cell surface glycopeptides were largely ineffective.

Negatively charged phospholipids did inhibit fibronectin-mediated hemagglutination, although they were an order of magnitude less effective than gangliosides. However, no direct evidence that gangliosides bind to fibronectin was presented.

Using a sensitive solid phase radioimmune assay we have now clearly demonstrated that fibronectin can bind to gangliosides. Interestingly, binding of fibronectin to di- and tri-sialogangliosides was consistently greater than to monosialogangliosides, in agreement with the relative effectiveness of these gangliosides in inhibiting cell spreading. Because of the qualitative nature of the radioimmune assay used, the results do not allow us to compare the relative binding affinities of fibronectin and cholera toxin for gangliosides. However, the fact that we were unable to convincingly demonstrate binding of ^{125}I -labelled fibronectin to ganglioside-coated tubes under conditions where we could readily detect binding of ^{125}I -labelled cholera toxin, suggests that the ligands bind to gangliosides with widely differing affinities. Relatively low affinity binding between gangliosides and fibronectin could account for the variable nature of the inhibition of cell spreading seen when unbound gangliosides were removed from fibronectin-coated substrates prior to addition of cells. It would also explain the observation that substantially lower levels of gangliosides are needed to inhibit cell attachment to a sialic acid-specific lectin than to fibronectin [40]. Interestingly, binding of fibronectin in solution to cells in suspension is also difficult to demonstrate [41], and it is possible that the binding affinity of fibronectin in matrix form for gangliosides at the cell surface might be increased by some form of cooperative interaction.

A role for gangliosides in binding fibro-

nectin at the cell surface is supported by the recent observation that fibronectin is synthesized, but not retained at the surface of a cell line (NCTC 2071), which is unable to synthesize the more complex gangliosides [42]. Growth of the cell line in medium supplemented with gangliosides led to a dramatic increase in the amount of fibronectin localized at the cell surface. Although this result clearly establishes the potential importance of gangliosides in influencing the organization of fibronectin at the cell surface, it does not prove that gangliosides are involved in the process of cell attachment and spreading on a fibronectin matrix. The mechanism by which the interaction of a cell with fibronectin leads to reorganization of the cytoskeleton and subsequent cell spreading is unclear [17, 43]. In the red blood cell there is good evidence that at least one of the transmembrane proteins, band 3 protein, is linked directly to a component of the cytoskeleton, ankyrin [44]. In other cell types, the evidence for membrane protein-cytoskeleton interaction is indirect [45, 46]. Nevertheless, it is relatively easy to conceive how the interaction of fibronectin with such a system might lead to reorganization of the cytoskeleton, and a change in cell shape. It is less obvious how the interaction of fibronectin with a lipid could exert such control. However, the binding of a variety of ligands to glycosphingolipids [47, 48] and phospholipids [49] at the lymphocyte cell surface has been shown to result in the redistribution of the bound ligand into caps, a process thought to involve the cytoskeleton. It may also be relevant that a substantial amount of total cellular ganglioside remains associated with cytoskeletons made by extracting cells at 0°C with Triton X-100 [25]. In the present study we have therefore attempted to establish whether the interaction of a cell

(BALB/c 3T3 cells) with a substrate coated with a ligand specific for the carbohydrate sequence in a ganglioside (cholera toxin B-subunit) can subsequently lead to cell spreading. Our results show that the toxin B-subunit is able to support limited reorganization of the microfilament system and partial cell spreading, provided the cell surface receptor density is increased by pre-treating the cells with neuraminidase. However, the morphology of cells spread on toxin B-subunit was clearly different from that of cells spread on fibronectin, as was the organization of F-actin within the cell. On this basis, we conclude that any interaction that might occur between fibronectin and cell surface gangliosides is unlikely to be sufficient to explain all of the morphological features of cells spread on fibronectin.

The idea that gangliosides alone act as the receptor for fibronectin is also difficult to reconcile with the observation that many cells which have little di- and tri-sialoganglioside (e.g., CHO and BHK cells [31]) spread perfectly well on fibronectin. Similarly treatment of BALB/c 3T3 cells with neuraminidase which converts much of the cell surface GD_{1a} to GM_1 [31] did not affect the ability of the cells to spread on fibronectin. However, fibronectin can clearly bind to a number of different gangliosides in contrast to cholera toxin which binds to a single ganglioside species, GM_1 . It is therefore possible that if gangliosides do play a role in binding fibronectin at the cell surface, the structure of the gangliosides involved might vary depending on the pattern of glycosphingolipid biosynthesis in a given cell type. In addition, the possibility that membrane glycoproteins contain carbohydrate sequences similar to those found in gangliosides and therefore bind fibronectin must also be considered [50].

We are grateful to Dr J. Holmgren for supplying us with tubes suitable for immobilising gangliosides, and Dr R. C. Hughes for helpful discussions on spreading of cells on cholera toxin.

REFERENCES

1. Ruoslahti, E, Engvall, E & Hayman, E G, *Coll res* 1 (1981) 95.
2. Pearlstein, E, Gold, L & Garcia-Pardo, A, *Mol cell biochem* 29 (1980) 103.
3. Grinnell, F & Minter, D, *Proc natl acad sci US* 75 (1978) 4408.
4. Carter, W G, Rauvala, H & Hakomori, S, *J cell biol* 88 (1981) 138.
5. Ruoslahti, E, Hayman, E G, Kuusela, P, Shively, J E & Engvall, E, *J biol chem* 254 (1979) 6054.
6. Hayashi, M, Schlesinger, D H, Kennedy, D W & Yamada, K M, *J biol chem* 255 (1980) 10017.
7. Sekiguchi, K & Hakomori, S, *Biochem biophys res commun* 97 (1980) 709.
8. Keski-Oja, J & Yamada, K M, *Biochem j* 193 (1981) 615.
9. Hahn, L E & Yamada, K M, *Cell* 18 (1979) 1043.
10. Pierschbacher, M D, Hayman, E G & Ruoslahti, E, *Cell* 26 (1981) 259.
11. Wagner, D, Ivatt, R, Destree, A & Hynes, R O, *J biol chem* 256 (1981) 11708.
12. Plow, E F & Ginsberg, M H, *J biol chem* 256 (1981) 9477.
13. Perkins, M E, Tae, H J & Hynes, R O, *Cell* 16 (1979) 941.
14. Aplin, J D, Hughes, R C, Jaffe, C L & Sharon, N, *Exp cell res* 134 (1981) 488.
15. Kleinman, H K, Martin, G R & Fishman, P H, *Proc natl acad sci US* 76 (1979) 3367.
16. Holmgren, J, Elwing, H, Fredman, P & Svennerholm, L, *Eur j biochem* 106 (1980) 371.
17. Hynes, R O & Destree, A T, *Cell* 15 (1978) 875.
18. Critchley, D R, Streuli, C H, Kellie, S, Patel, B & Ansell, S, *Biochem j* 204 (1982) 209.
19. Engvall, E & Ruoslahti, E, *Int j cancer* 20 (1977) 1.
20. Niemczuk, P, Perkins, R M, Talbot, I & Critchley, D R, *Brit j cancer* 45 (1982) 500.
21. Svennerholm, L & Fredman, P, *Biochim biophys acta* 617 (1980) 97.
22. Fredman, P, Nilsson, O, Tayot, J L & Svennerholm, L, *Biochim biophys acta* 618 (1980) 42.
23. Svennerholm, L, *Biochim biophys acta* 24 (1957) 604.
24. Burridge, K, *Methods in enzymol* 50 (1978) 54.
25. Streuli, C H, Patel, B & Critchley, D R, *Exp cell res* 136 (1981) 247.
26. Salacinski, P R P, McLean, C, Sykes, J E C, Clement-Jones, V V & Lowry, P J, *Anal biochem* 117 (1981) 136.
27. Aplin, J D & Hughes, R C, *Anal biochem* 113 (1981) 144.
28. Barak, L S, Nothnagel, E A & Webb, W W, *Proc natl acad sci US* 77 (1980) 980.
29. Lai, C Y, *CRC crit rev biochem* 9 (1980) 171.
30. Critchley, D R, Ansell, S, Perkins, R, Dilks, S & Ingram, J, *J supramol struct* 12 (1979) 273.

31. Critchley, D R & Vicker, M, Cell surface reviews (ed G Poste & G Nicolson) vol. 3, pp. 307-370. North-Holland, Amsterdam (1977).
32. Moss, J, Manganiello, V C & Fishman, P H, Biochemistry 16 (1977) 1876.
33. Rogers, T & Snyder, S H, J biol chem 256 (1981) 2402.
34. Kohn, L D, Receptors and recognition ser A (ed P Cuatrecasas & M Greaves) vol. 5, p. 135. Chapman Hall, London (1978).
35. Pacuska, T, Osborne, J C, Brady, R O & Fishman, P H, Proc natl acad sci US 75 (1978) 764.
36. Besancon, F & Ankel, H, Nature 252 (1974) 478.
37. Higgins, T J, Liu, D Y, Remold, H G & David, J R, Biochem biophys res commun 93 (1980) 1259.
38. Markwell, M A, Svennerholm, L & Paulson, J C, Proc natl acad sci US 78 (1981) 5406.
39. Yamada, K M, Kennedy, D W, Grotendorst, G R & Marnoi, T, J cell physiol 109 (1981) 343.
40. Rauvala, H, Carter, W G & Hakomori, S, J cell biol 88 (1981) 127.
41. Rennard, S I, Wind, M L, Hewitt, A T & Kleinman, H K, Arch biochem biophys 206 (1981) 205.
42. Fishman, P H, Critchley, D R, Moss, J, Homm, B E & Yamada, K, J supramolec struct and cellular biochem, suppl. 5 (1981) 302.
43. Hynes, R O, Cell surface reviews (ed G Poste & G Nicolson) vol. 7, pp 97. Elsevier/North-Holland, Amsterdam (1981).
44. Bennett, V & Stenbruck, P J, Nature 280 (1979) 468.
45. Ben-Ze'ev, A, Duerr, A, Solomon, F R & Penman, S, Cell 17 (1979) 859.
46. Flanagan, J & Koch, G L E, Nature 273 (1978) 278.
47. Revesz, T & Greaves, M, Nature 257 (1975) 103.
48. Sela, B A, Raz, A & Geiger, B, Eur j immunol 8 (1978) 268.
49. Schroit, A J & Pagano, R E, Cell 23 (1981) 105.
50. Rauvala, H & Finne, J, FEBS lett 97 (1979) 1.

Received December 30, 1981

Revised version received April 13, 1982

Accepted April 20, 1982

An Evaluation of the Role of Gangliosides as Receptors for Fibronectin

by Robert M. Perkins

The demonstration that exogenous gangliosides, in particular the di- and tri-sialogangliosides can inhibit fibronectin-mediated cell adhesion led to the proposal that these molecules may act as cell surface receptors for fibronectin.

I have attempted to make a detailed evaluation of this proposal by taking the following approaches.

1. Making a more extensive study of the ability of gangliosides to inhibit cell adhesion, in particular cell spreading, in order to determine whether or not this activity is specific for fibronectin.

2. Attempting to demonstrate a direct interaction between fibronectin and gangliosides adsorbed to a plastic support. This involved the use of either ^{125}I -labelled fibronectin or a sensitive radioimmune detection system.

3. Investigating whether a cell surface ganglioside is capable of supporting stable cell adhesion by analysing cell spreading on substrata coated with cholera toxin, a ganglioside-binding ligand. Cholera toxin-induced cell spreading is compared with that on fibronectin and the glycoprotein reactive lectin concanavalin A in an attempt to assess the relative importance of glycoproteins and gangliosides in mediating fibronectin-induced cell spreading.

4. Finally I have investigated the relationship between the levels of cellular gangliosides and the ability to interact with fibronectin. This involved:-

(i), a comparison of the ability of Balb/c 3T3 cells and four ganglioside-deficient variant cell lines to interact with FN,

(ii), investigating the effect of adding back exogenous gangliosides to a cell line lacking gangliosides on its ability to retain fibronectin at the cell surface, and

(iii), conversion of cellular di- and tri-sialogangliosides into monosialogangliosides by neuraminidase treatment and examining the cell's ability to spread on fibronectin-coated substrata.

In general this data is not consistent with the proposal that gangliosides act as the sole receptor for fibronectin.