The Identification of B-Lymphocyte Epitopes of Herpes Simplex Virus Type 1 Glycoprotein H

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for the Degree of Doctor of Philosophy

University of Leicester

October 1991

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Abstract

Herpes simplex virus type 1 glycoprotein H is a minor viral constituent which has been implicated in viral mechanisms of cell entry and exit. Study of this molecule has been hindered by a relative lack of specific immunological reagents. This study reports the generation and characterisation of specific polyclonal antibodies. The purification of gH and the generation of polyclonal serum is described. Antigenic determinants reactive with polyclonal antibodies were determined using synthetic peptides derived from the primary amino acid sequence of the molecule and a similar approach was applied using available monoclonal antibodies. A series of 833 hexapeptides, with five residue overlap, were synthesised representing all potential continuous epitopes comprised of six residues or less. Peptides, displaying structural homology with epitopes of the native molecule were identified by screening for antibody binding. To further validate the identity of epitopes, corresponding homologous peptides were conjugated to carrier molecules and used to generate anti-peptide antibodies. The biological and immunological properties of the resulting anti-peptide antibodies were determined. Three antipeptide antibodies appeared reactive with the glycoprotein either following western transfer or in immunoprecipitation confirming the identity of gH-1 epitopes.

Acknowledgements

This work was carried out under the auspices of the Vaccine Research and Production Laboratory, Public Health Laboratory Service, Centre for Applied Microbiology and Research, Porton Down, Salisbury, UK and the Department of Medical Microbiology, University of Leicester, UK.

My grateful thanks to Professor Jack Melling (Divisional Director, VRPL) for his support during this work.

Special thanks to Dr. Peter Andrew (University of Leicester), Dr John North (Porton International) and Professor Tony Minson (Department of Virology, University of Cambridge) for their encouragement and constructive criticism. I am particularly endebted to Tony Minson for making available scarce reagents and Dr. Wayne Conlan (University of Southampton) for guidance during initial peptide synthesis, and not least Dr. Karen Blake for her criticism of the text. Thanks also to Dr. R Philpotts.

My gratitude is extended to Mr Steve Elvin for his technical expertise during serum generation, and finally Jane Gosney and Kirsty Waterman for excellent typing.

The provision of post-graduate registration funding from Porton International is gratefully acknowledged.

Abbreviations

3A9EC	3-amino-9-ethylcarbazole
AA	amino acid
ABTS	2,2'-Azino bis 3-ethylbenzthiazoline sulphonic acid
Alpha-TIF	Alpha-trans inducing factor
BSA	bovine serum albumin
CDR	complimentavity determining regions
CNS	central nervous system
CPE	cytopathic effect
CTL	cytotoxic T lymphocyte
DMF	dimethylformamide
DMSO	dimethylsulphoxide
DNA	deoxyribonucleic acid
E	early
EBV	Epstein-Barr Virus
EDTA	ethylenedinitrilotetra acetic acid
ER	endoplasmic reticulum
FCA	freund's complete adjuvant
FICA	freund's incomplete adjuvant
FMOC	fluoremylmethyloxcarboxyl
g	glycoprotein
HCMV	human cytomegalovirus
HOBT	hydroxybenzotrizole
HPLC	high performance liquid chromotography
HRP	horseradish peroxidase
HSV	Herpes Simplex Virus
ICP	infected cell protein
IE	immediate early
KLH	keyhole limpet haemocyamin
L	late
LAT	latency associated transcript
LSAP	large scale antigen preparation
MBS	m-maleimido-benzoyl-N-hydroxysuccinimide ester
MEM	minimum essential medium

MOI	multiplicity of infection
Mr	molecular ratio
npt	non permissive temperature
NRS	normal rabbit serum
OD	optical density
OPD	o-phenylene diamine
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
Pfp	penta fluorophenyl
pfu	plaque forming units
PMSF	phenylmethylsulfonyl fluoride
ppm	parts per million
RNA	ribonucleic acid
r.t.	room temperature
SDS	sodium dodecyl sulphate
TFA	trifluoroacetic acid
TMB	3, 3', 5, 5' tetramethylbenzidine
Tris	tris (hydroxymethyl) amino methane
t.s.	temperature sensitive
UL	unique long
US	unique short
UV	ultra violet
v/v	volume/volume
VZV	Varicella Zoster Virus
w/v	weight/volume

CONTENTS

Amino Acid Codes

Amino Acid	Abbreviation	Single Letter Code
Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartic acid	Asp	D
Cysteine	Cys	C
Glutamic acid	Glu	E
Glutamine	Gln	Q
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V

Preface

Several Herpes Simplex Virus type-1 glycoproteins have been identified both on the envelope of the virus and at the extra cellular surface of infected cell membranes. The precise roles of Herpes virus glycoproteins are uncertain although they have been implicated in attachment, penetration, envelopment, virus egress and fusion of infected membranes. Three HSV-1 glycoproteins, designated gB, gD and gH, have been shown to be essential for virus infectivity *in vitro*. Although a quantitatively minor glycoprotein, several lines of evidence indicate a particularly important role for gH in the mechanisms of cell penetration and the egress of viral progeny.

The following study focuses primarily on glycoprotein H-1, and by identifying epitopes of the molecule attempts to increase understanding of the structure of this glycoprotein and how this may relate to function. Where relevent observations concerning gH-2 will also be outlined.

Chapter 1

INTRODUCTION TO HERPES SIMPLEX

	Primary Disease	Consequences of latent Virus Reactivation.	Other diseases in which herpes virus infection is/may be a factor
Herpes simplex type 1	Orolabial Infection (Blistering Vesicles)	Cold Sores	-
Herpes simplex type 2	Genital Infection (Painful Pustular Vesicles)	Recurrent genital lesions	Possible co-factor in cervical neoplasia
Varicella Zoster virus	Varicella (Chicken Pox) (Mild itching rash of childhood)	Zoster (shingles, intensely painful skin eruption of the abdo-thorax)	-
Human cytomegalovirus	Often asymptomatic in normal individuals	Reactivation (or primary infection) following immunosuppression can lead to severe disseminated infection.	Possible co-factor in AIDS. (HCMV transactivation of HIV.)
Epstein-Barr virus	Infectious Mononucleosis (glandular fever of adolescence.)	EBV Lymphocyte Immortalisation	Co-factor in Burkitt's Lymphoma(Equatorial Africa) Nasopharyngeal Carcinoma (Southern Indo-China)
Human Herpesvirus 6.	**exanthem subitum**	**Unknown**	**Isolated from lymphoid cells of patients with lymphoreticular disorders**

Table 1. Diseases associated with Human Herpesviruses.

1.1 Background

Humans are the natural hosts of at least six herpes viruses and the clinical diseases most commonly associated with these infections are described in Table 1. Herpes viruses have been classified into three subfamilies largely on the basis of biological characteristics (Table 2) and this taxonomy has been supported by subsequent data on the structure of the viral genomes. (Roziman, 1982).

The two serotypes of Herpes Simplex Virus, type-1 (HSV-1) and type-2 (HSV-2) are included in the alpha herpes sub-family. Their genomes show 50% homology (Roizman, 1979), and are colinear (Davison and Wilkie, 1983). Transcriptional ordering is similar (Easton and Barklie-Clements, 1980) and many of their proteins are functionally interchangeable. (Esparze *et al.*, 1976).

1.2 HSV Infection

Primary HSV infection in humans usually results in lesions localised in the epithelial tissue at moist mucosal surfaces of the mouth, eye, or genital areas. Occasionally serious complications, such as viral encephalitis can occur and in the immuno-compromised severe disseminated infection can arise. HSV-1 and HSV-2 are transmitted by different means involving different body sites, although overlap can occur. HSV-1 is usually associated with infection of oro-facial areas whereas HSV-2 is most often associated with genital infection. Sexually transmitted herpetic disease has reached epidemic proportions world wide and is a major public health concern (Corey and Spear, 1986).

HSV disease of the new born, contracted from secretions of an infected birth canal, involves a broad range of tissues and the prognosis of infants with disseminated disease is poor with approximately 50% mortality (Nahmias, 1981).

Following infection, local lesions begin as blistering vacuoles which progress to an eruption of painful ulcerative sores leading to crusting and eventual healing. Asymptomatic individuals infected with HSV are capable of shedding infectious virus (Corey and Spear, 1986) offering a mobile reservoir of infectivity.

A latent reservoir of virus becomes established in ganglia which innervate the site of primary lesions. Once established, latent virus persists for the life of the host and an individual may suffer recurrent lesions when latent virus in sensory ganglia is reactivated. About half of those harbouring latent virus

	Example	Host Range	Life Cycle Duration	Site of Latent Infection	Characteristic Cytopathology
Alpha herpes Subfamily	HSV	Wide in vitro and in vivo	Relatively short	Sensory Ganglia	Syncitial strains cause cell fusion producing large multinucleated cells
Beta herpes Subfamily	HCMV	Narrow in vivo Frequently Natural Host restricted	Relatively long	Secretory Glands Kidneys Lymphoreticular cells	Cell enlargement and intranuclear inclusion bodies
Gamma herpes Subfamily	EBV	Natural Host restricted	Variable		EBV Lymphocyte Immortilisation

Table 2. Biological Properties of Herpesvirus Sub-families.

will suffer recurrence with varying degrees of frequency and severity (Wildy and Fell, 1985). HSV-1 infection of the eye is a major cause of corneal scarring and blindness, and in the USA approximately 300,000 annual cases of herpes keratoconjunctivitis have been reported (Whiteley, 1986). Therapy for HSV infection is limited to the application of nucleoside analogues (acyclovir) which selectively inhibit viral replication however recurrent infection is common even after treatment (Darby, 1988).

1.3 Morphology and Physical Properties

Electron microscopic studies of HSV-1 have revealed four distinct structural components. The genomic core is comprised of double stranded linear DNA arranged around an electron dense fibrillar structure. This genomic core is encapsulated by a protein capsid constructed of 150 hexameric and 12 pentameric capsomeres arranged so as to form an icosahedral structure. Surrounding the capsid is a globular, electron dense region termed the tegument which is approximately 100nm in diameter and often of irregular appearance. Enclosing these inner structures is a bilayered membranous lipid envelope studded with distinct surface projections which electron microscopy allied to protein gold labelling have indicated to be viral glycoproteins (Stannard *et al.*, 1985).
The complete infectious virion is estimated to contain approximately thirty distinct protein species (Heine, *et al.*, 1974).

Seven HSV-1 glycoproteins have been identified, which are named according to international convention in alphabetical order as discovered, gB, gC, gD, gE, gG, gH and gI. Missing letters in the alphabetical sequence are due to erroneous identification of precursors as distinct protein species. Homologues found in HSV-2 are termed gB-2, gC-2 etc, and two, gB and gH, have homology with glycoprotein species of Varicella Zoster virus (VZV), Human Cytomegalovirus (HCMV) and Epstein-Barr virus (EBV) (Cranage *et al*, 1986, 1988, McGeoch and Davison 1986, Davison and Taylor 1987, Pellet *et al.* 1985).

The exposed position of HSV-1 glycoproteins on the surface of infected cells and the viral envelope suggests a role in viral infectivity as well as offering potential targets for host immune responses. These factors together with glycoprotein involvement in viral attachment and penetration are discussed in detail later.

1.4 Replicative Life Cycle

Infecting virus attaches to receptors present on the outer surface of susceptible cells and there is evidence that one type of receptor able to fulfil this role may resemble heparin sulphate (WuDunn and Spear, 1989). More recent reports have also implicated the fibroblastic growth factor receptor as an attachment site for HSV-1 (Kaner, *et al.*, 1990). Once attached the process of penetration is initiated and although the role of viral and cellular proteins is uncertain available evidence indicates virus entry into the cytoplasm is mediated by fusion of the envelope and plasma membrane rather than phagocytosis (Spear, *et al* 1989). The de-enveloped capsid having reached the cytoplasm is translocated to the nucleus where released viral DNA enters via nuclear pores. The initial fusion of viral envelope and plasma membrane releases at least two proteins from the infecting particle. Viral host shutoff (VHS) protein closes down cell protein synthesis, and whilst the Alpha gene trans-inducing factor (Alpha-TIF) moves to the nucleus in readiness for the first step in viral protein synthesis (Roizman, 1990).

HSV gene expression takes place in regulated temporal sequence and genes are consequently grouped together according to the timing of their activation (Honess and Roizman, 1975). The five Alpha genes (immediate early) induced by Alpha-TIF are the first to be transcribed using cellular enzymes. Following translation of the Alpha proteins a second round of transcription takes place involving Beta genes (early) many of which are needed for viral DNA synthesis, followed in turn by gamma genes (late) which encode primarily viral structural proteins including glycoproteins. Capsid assembly proceeds and newly synthesised viral DNA is inserted. Glycoproteins and tegument proteins are aquired with envelope as progeny capsid bud through the inner nuclear membrane, (Minson, 1990). In addition glycoproteins are also transported to and inserted within the plasma membrane.

Mature virus can be released by budding through plasma membrane, however viral progeny are also capable of passage directly from cell to cell thus avoiding a potentially hostile extracellular environment (Wildy and Gell, 1985). The processes which control and facilitate virion egress are at present obscure, however, what is certain is that the events described have fatal consequences for an infected cell.

1.5 Latency

Several puzzling observations confronted early workers studying Herpes simplex infections. Herpetic lesions which had completely healed could recur at the same site, although during normal periods virus could not be isolated from epithelial tissue. The appearance of lesions appeared co-incident with trauma, (especially sectioning of the trigeminal tract) and yet independent of serum antibody levels which remained constant.

This led to the hypothesis (Goodpasture E W, 1929) that recurrent lesions may be due to a protected reservoir of virus which could reactivate to produce recurrent disease. Thus the concept of latency, a feature now known to be characteristic of all known human herpes viruses, was developed. Although latency plays a major role in the successful perpetuation of the viral parasite, it has proved difficult to study *in vivo*, and *in vitro*. However progress has been made in understanding which tissues harbour virus, and in what state latent virus is maintained, although the underlying molecular mechanisms which initiate reactivation remain obscure.

Early suspicions that HSV-1 was neurotropic appear correct, with nerve tissue the preferred site of latency. Little evidence has been found for the establishment of latency at extra- neural sites such as previously infected skin tissue (Hill *et al.*, 1985).

To establish latent infections in sensory ganglia, primary infection must first be established in peripheral tissue. Virus then enters a nerve ending and moves (translocates) via nerve axons to the ganglion (Wildy and Gell, 1985). The mechanism of viral movement within nerve tissue is unknown. In mice, cutting peripheral nerves supplying a site of primary infection prevents viral spread to the central nervous system (CNS) (Wildy, P., 1967).
Indications that neurons are preferred amongst the collection of cells which comprise the ganglia, were confirmed by elegant experiments using a temperature sensitive (ts) mutant of HSV. When reactivated at non-permissive temperature (npt.), this mutant expressed viral antigens but was unable to infect other cells. Following reactivation, viral antigens were only detected on neurons (McLennan and Derby, 1980).

Two possibilities for the nature of latent virus have been postulated. The dynamic state in which a low level of productive infection is chronically maintained, or the static state in which the viral genome is totally or partially repressed and integrated into the host cell genome (Roizman, 1974). Late (gamma) proteins have been undetectable in latently infected rabbit ganglia, and viral thymidine kinase, a presumed requirement for the dynamic state, has not been detected in latently infected cells

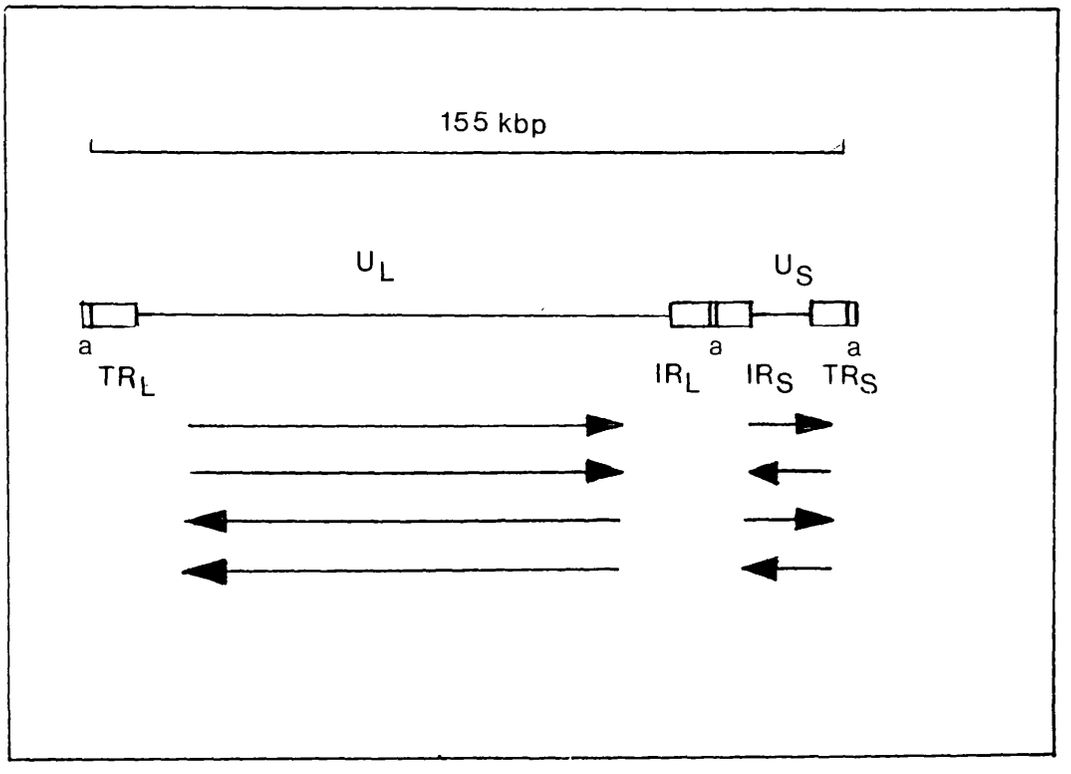


Fig 1. HSV-1 genome arrangement - see text 1.6.

(Fong and Scriba, 1980). The present weight of evidence therefore favours latent virus in the static state, and this raises the question how is productive replication prevented?

The means by which this is achieved are uncertain however latency associated transcripts (LAT's), distinct from any previously detected during *normal* productive infection, have been identified although their significance is not known. (Stevens, *et al.*, 1987, Spivak and Fraser, 1987, Spivak and Fraser, 1988, Steiner, *et al.*, 1988).

During normal HSV-1 replication *in vitro*, alpha gene products are required for the expression of beta and gamma genes. LAT's by contrast appear to be expressed in the absence of alpha gene transcripts which suggests that regulation of LAT transcripts occurs by some other mechanism. (Spivak and Fraser 1988).

1.6 The HSV-1 Genome

The complete sequence of the HSV-1 genome has been determined and shown to consist of 152,260 residues with a G plus C content of 68.3% (McGeoch, *et al.*, 1985 and 1988). Within the double stranded DNA genome 72 genes have been recognised which encode 70 protein species.

The genome is comprised of two unique sections, one long (UL) the other short (US) (Fig. 1). Each section can invert relative to the other giving four possible genomic isomers and a population of HSV-1 virions contains equimolar concentrations of each isomer. One isomer has been chosen to represent the protype used in all genomic map presentations. Each unique sequence is flanked by a pair of repeat sequences and each copy of the repeat sequence is inverted relative to its twin. The paired repeat sequences which sandwich the UL region are distinct from those which sandwich the US region. At each end of the molecule is an apparently redundant sequence of approx 400 base pairs termed the 'a' sequence. Several copies of the 'a' sequence may be present at the junction of UL and US regions, slotted between the flanking inverted repeats. In the publication of the UL sequence, completing the elucidation of the entire genome, the presence of a single copy of the 'a' sequence at each site was assumed (McGeoch, *et al.*, 1988).

HSV-1 gene expression takes place in sequential groups forming a co-ordinated and regulated cascade of protein synthesis (Honess and Roizman, 1974 and 1975).

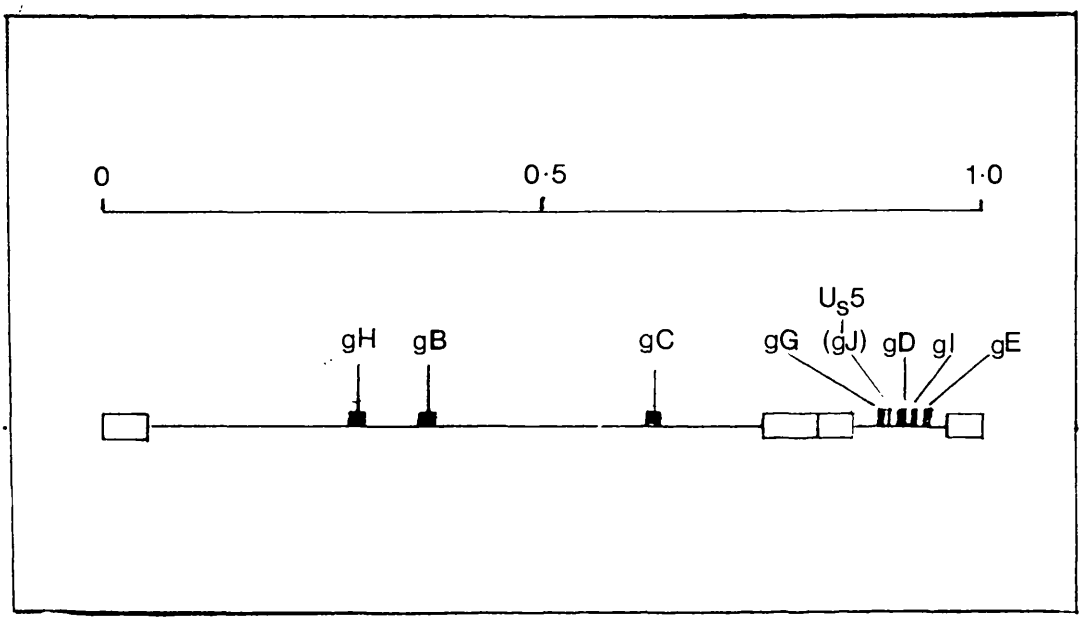


Fig 2. Relative positions of glycoprotein genes within the HSV-1 genome.

By definition, alpha or immediate early peptides are the first to be produced following infection and require no prior *de novo* viral protein synthesis, although their expression is stimulated by an infecting virus particle component, the alpha trans inducing factor (Batterson and Roizman 1983). Five immediate early proteins are known, ICP 0, 4, 22, 27, 47 and there is evidence implicating three of these in transcriptional activation (Everett, 1986).

The beta or early protein group are next in the order of synthesis and reach a peak rate 5 to 7 hours post infection. For synthesis of early proteins to proceed functional immediate early proteins are required, but *de novo* viral DNA replication is not. Included in the group of beta proteins are enzymes required for DNA replication, and regulation of gene expression.

The gamma or late group of proteins are predominantly virion structural components. They can be sub-divided into gamma-1 proteins which require only prior protein synthesis and gamma-2 proteins which additionally require viral DNA synthesis. Peak rate of synthesis is reached approximately 14-16 hours post infection.

HSV-1 glycoproteins belong to the beta or gamma proteins and analysis of the viral genome has identified eight potential glycoprotein genes. Seven glycoproteins have now been isolated from infected cells, and their relative position within the viral genome is shown in Fig 2. The protein product of the US5 gene has yet to be identified (Minson, 1990).

The genome is thought to replicate by a rolling circle mechanism, producing long concatameric DNA which is then processed and packaged into nascent nucleocapsids. Circularisation of the genome is facilitated by exposure of complementary 'a' sequences by exonuclease activity. An attractive feature of the model is its ability to account for the existence of the 4 genomic isomers seen in equimolar quantities in progeny virus (Jacob, *et al.* 1979).

Two possible origins of DNA replication were indicated by early electron microscopic studies (Friedman, *et al.*, 1977 and Hirsch, *et al.*, 1977).

Subsequently it has been shown that HSV-1 DNA contains two copies of one origin termed *ori* S located in the 'a' and 'a' inverted repeat sequences, and a copy of a second more complex origin termed *ori* L located near the centre of UL (McGeoch, 1987). The existence of two types of defective virus containing either *Ori* S or *Ori* L which are both able to replicate *in vitro* indicates a capability for independent operation (Polvino-Bodner, 1987).

1.7 Adaptive Immune Responses

Host reaction to viral infection involves activation of a wide range of humoral and cellular responses. Lymphocytes proliferate following the stimulation of antigen specific receptors at their surface (immunoglobulins for B lymphocytes, specialised T cell receptors for T lymphocytes).
Antibodies secreted by activated B lymphocytes may neutralise free virus whereas activated cytotoxic T lymphocytes (CTL) identify and kill infected cells with the activity of both B lymphocytes and CTL's controlled by T helper lymphocytes via the excretion of cytokines.

Immunoglobulins on the surface of B lymphocytes are capable of binding directly with virus antigens whereas T cell receptors only recognise viral antigens as processed peptides, presented within a groove on the extracellular domain of major histocompatability complex (MHC) molecules expressed on the surface of antigen presenting cells. For virus infections, it is suspected that infected cells may also be capable of presenting antigen in addition to more specialised cells (e.g. macrophages) (Randle and Souberbielle, 1990).

The interaction between adaptive host immune responses and HSV infection is incompletely understood, although much work has been carried out using animal models, particularly mice. Both B and T lymphocyte activation are induced in the control of infection.
Wildy (1985) has pointed out that there are probably only two occasions when virus is exposed to antibody. These occur following primary infection as virus moves from infected epithelial tissue to invade sensory nerve endings, and when virus reappears from nerve endings during spread of primary infection or following reactivation (Wildy and Gell, 1985). In animal experiments administration of neutralizing antibodies directed against viral glycoprotein can prevent invasion of sensory nerves, but in normal infectious circumstances natural neutralising antibodies appear too late to be effective. Although antibodies are probably important in preventing recurrent infection and recrudescence, evidence has accumulated indicating that cellular immunity is required for viral clearance. The properties of antibodies elicited by individual glycoproteins are discussed in section 1.8.

Uncertainty surrounds which viral antigens are most important in triggering T lymphocyte responses. Many studies have assumed that glycoproteins exposed on the plasma membrane of infected cells would be likely candidates, and several studies have reported glycoproteins acting as CTL targets (Maingay, 1988). In contrast other herpes virus studies have described CTL responses directed toward non-structural proteins. The immediate early gene products of human (Borysiewicz, 1987) and murine (Reddehase and Koszinowski, 1984) cytomegalovirus have been reported capable of inducing CTL responses. In addition HSV-1 specific CTLs have been shown to recognise target cells

Glycoprotein	Gene designation	Residues predicted from gene	Mr of unprocessed translation product	Apparent Mr of Mature glycoprotein in P.A.G.E.
gH	UL22	838	90,361	110K
gB	UL27	904	100,287	120K
gC	UL44	511	54,995	130K
gG	US4	238	25,236	59K
(gJ)	US5	92	9,555	-
gD	US6	394	43,344	60K
gI	US7	390	41,366	65K
gE	US8	550	59,090	80K

Table 3. Biophysical Properties of HSV-1 glycoproteins

expressing only HSV-1 immediate early gene products (Martin, *et al.*, 1988). The identity of the epitopes of HCMV major immediate early protein required to elicit the *in vitro* proliferation of sensitized lymphocytes is currently being investigated using a series of over lapping synthetic peptides (Sissons, 1990 pers. comm).

Experiments using vaccinia virus recombinants containing the gH-1 gene have indicated that in this system gH is not a CTL target (Minson, 1989 pers. comm, Blacklaws *et al.* 1990).

1.8 HSV Glycoproteins

HSV-1 glycoproteins (Table 3) display the characteristics of integral membrane proteins. Their gene sequences predict amino terminal signal sequences which are cleaved during processing, a large extracytoplasmic domain often heavily modified, a membrane spanning hydrophobic domain which serves to anchor the molecule within lipid bilayers, and a short hydrophilic region at the carboxy termimus.

The processing and synthesis of herpesvirus glycoproteins proceeds using host cell enzymes with nascent polypeptides synthesised at ribosomes attached to the endoplasmic reticulum (E.R). Glycosylation takes place both coincident with translation and subsequently as glycoproteins are transported via the Golgi apparatus to the plasma and nuclear membranes (Minson 1990).

Both N-linked and O-linked glycosylation occurs. N-linked glycosylation is facilitated by transfer of preformed glycans from dolichol phosphate lipid carriers to asparagine residues present in the amino acid sequence Asn-Residue-Thr/Ser. of the polypeptide. (Kornberg and Kornberg 1988). O-linked glycosylation begins with the binding of N-acetyl-galactosamine to the hydroxyl group of serine or threonine, followed by the addition of galactose and sialic acid at the Golgi apparatus (Roizman, 1990). The function of the carbohydrate component of glycoproteins is unknown, although it appears essential since blocking of the initial step in N-linked glycosylation using tunicamycin prevents the formation of infectious particles (Peake *et al.* 1982, Pizer *et al.* 1980).

Four HSV-1 glycoproteins, gC-1, gE-1, gI-1 and gG-1 appear unnecessary for virus infectivity in vitro. (Richman et al. 1986, Neidhardt et al. 1987, Longnecker *et al.* 1987).
With the exception of gC, the genes for these non-essential glycoproteins are clustered in the short unique region of the genome along with the open reading frame US5 which predicts a small and as yet undetected glycoprotein (gJ).

The functions of these glycoproteins are unclear however the properties of some suggest they may be influential in promoting HSV pathogenesis.

Glycoproteins E and I have been demonstrated to form a complex able to bind the Fc region of immunoglobulin G (IgG) (Bauke and Spear 1979, Johnson and Feenstra 1987). The significance of glycoprotein Fc receptors is unknown, but their presence at the surface of infected cells may adversly affect Fc mediated host immune responses. Recently it has been shown that gE alone is capable of IgG Fc binding albeit at lower levels than the gE/gI complex (Bell *et al.* 1990).

Glycoprotein C-1 is one of the more abundant glycoprotein species and can be readily detected in immunoblots and precipitation assays. The gC-1 gene maps to the long unique region of the viral genome and sequence data predicts a nascent polypeptide of Mr. 55,000 (Frink *et al*, 1983).
Newly synthesised gC polypeptide undergoes substantial glycosylation with the attachment of both N and O linked oligosaccharides, the latter being most prevalent (Dallio *et al.* 1985) giving the mature glycoprotein an Mr of approximately 130 000 in PAGE. Although gC appears to be non-essential for virus infectivity in vitro , the pathogenicity of mutants which fail to produce gC is reduced and viral attachment appears superior in virus which contains the molecule (Holland *et al.* 1984, Herold *et al.* 1991). Glycoprotein C-1 has also been reported as a target for host immune responses, eliciting complement dependent neutralising antibodies (Eberle and Mou 1983, Norrild 1985) and cytotoxic T lymphocyte responses (Glorioso *et al.* 1985, Rosanthal *et al.* 1987). In addition gC-1 presented at the surface of infected cells will bind the C3b component of the complement system (Friedman *et al.* 1984).

This has led to speculation that binding of this component may assist the pathogenesis of the virus by interfering with complement dependent neutralisation (Fries *et al.* 1986).
Some studies have implicated gC-1 in decreased complement mediated lysis of infected cells and a fall in complement dependent antibody neutralisation (McNearney *et al.* 1987, Muller-Eberhand 1986).

The antigenic structure of gC has begun to be defined with both glycosylation dependent and independent epitopes confirmed (Sjoblom *et al.* 1987). Early work using synthetic peptides identified a type common continuous epitope between residues 128-134 of the gC-1 sequence which was available only on denatured glycoprotein (Zweig *et al.* 1984). Marlin and colleagues (1985) used a panel of gC specific neutralising monoclonal antibodies together with a series of monoclonal antibody resistant virus mutants to categorise binding sites. On the basis of neutralisation patterns nine

epitopes were operationally detected and futher divided into two antigenic sites on the basis of competitive binding studies. Using truncated polypeptides antigenic site II was located within the N-terminal half of the molecule whereas site I lay toward the C- terminus. Subsequent sequencing of the mutant genes has identified predicted AA changes between residues 307-373 in group I mutants and changes between 129-247 for group II mutants (Wuu *et al.* 1990).

Regions of the gC-2 molecule involved in C3b binding have been investigated using insertion mutagenesis within the gene. Transfection of the altered gene and expression of the mutant proteins at the cell surface allow the effects on C3b binding to be observed. Three regions which influence binding were located, one of which lay between residues 307-379.
(Seidel-Dugan *et al.* 1990).

Indirect evidence from studies with viral mutants defective in particular glycoprotein genes and which are not independently viable has indicated three HSV-1 glycoproteins as indispensable for virus growth in tissue culture (Desai *et al.* 1988, Ligas and Johnson 1988, Cai *et al.* 1988). Roizman (1990) has postulated that they represent the minimum glycoprotein components necessary to carry out the essential viral functions of attachment, penetration and egress.

Glycoprotein D-1 is a quantitatively abundant glycoprotein which maps to the unique short region of the genome, in contrast to the other essential glycoproteins, gB and gH, which map to the long unique region.

The gD-1 gene codes for a polypeptide of 369 amino acids (Watson *et al.* 1982) and sequencing of the gD-1 and gD-2 genes has revealed sequence homology of 85% (Lasky and Dowbenko 1984, Watson *et al.* 1983). The primary amino acid sequence encompasses three N-linked glycoprotein sites, all of which are utilized, and at least two O-linked sites (Campadelli-Fiumie *et al.* 1982, Johnson and Spear 1983).

It has been known for several years that glycoprotein D was particularly important for inducing neutralising antibodies (Cohen *et al.* 1972) and gD-1 has also been identified as a target for cellular immune responses (Martin *et al.* 1987, Torseth *et al.* 1987, Zarling *et al.* 1986). Animals inoculated with various gD derivatives have proved resistant to viral challenge with either serotype (Marsden 1987).

The function of gD-1 is not clear although the molecule has been implicated in the processes of adsorbtion (Fuller and Spear 1985, Johnston *et al.* 1990) penetration, (Fuller and Spear 1987, Highlander *et al.* 1987) and the fusion of infected cells (Noble *et al.* 1983).

Several groups have taken the view that the identification of gD-1 epitopes would be a useful early step in the investigation of structure function relationships within the molecule and extensive data on the antigenic determinants of gD has accumulated. Eisenberg, Cohen and colleagues (1985) used a large panel of monoclonal antibodies to map the antigenic sites of gD and attempted to indirectly relate the biological properties of antibodies with domains of the glycoprotein.

Monoclonal antibodies have been arranged in groups according to their type specificity, neutralisation, reactivity with native or denatured gD and the ability to interact with truncated gD molecules or peptidase fragments.

Antibody groups I, III, IV and VI neutralize virus but fail to recognise denatured gD and are therefore deemed to recognise discontinuous epitopes. For reasons outlined later discontinuous epitopes are particularly difficult to define (Cohen *et al.* 1986) Antibodies included in groups II, V and VII interact with native and denatured gD and thus recognise continuous epitopes.

Group V antibodies recognise a type specific continuous antigenic site between residues 340 and 356 located between the transmembrane domain and the carboxy terminus (Eisenberg *et al.* 1982 and 1985), are non-neutralising and will not protect animals against viral challenge (Rector *et al.* 1984). Group II antibodies react with a continuous antigenic determinant between residues 268 and 287 at an extra cellular location close to the transmembrane region and are weakly neutralising. Group VII antibodies have proved particularly interesting in so far that they recognise a type common continuous epitope within residues 11-19 and are strongly neutralising (Bosch *et al.* 1987). Synthetic peptides homologous with this region elicit type common neutralising antibodies (Cohen *et al.* 1984, Eisenberg *et al.* 1988).

Transfection of viral genes into cell lines and the expression of authentic gene products has permitted the generation of HSV null mutants which although they lack essential genes are able to grow in the transfected cell lines.

The viral progeny produced are infectious in normal permissive cell lines but the absence of the full complement of essential genes results in subsequent viral progeny being non-infectious. The system

provides a means by which the effects of gene mutations on function and structure of essential proteins can be studied (Cohen et al. 1985). Complementation assays have been developed in which a mutated essential gene is transfected into cells and the ability of the resulting protein to complement an infecting null virus adjudged by infectivity of the resulting viral progeny.

Using similar approaches regions of the gB and gD gene influential in function have begun to be identified (Muggeridge et al. 1990). As outlined later a similar approach for gH-1 resulted in transfected cell lines producing a non-authentic glycoprotein.

The continuous epitopes of gD antigenic site VII and their importance in protein function were of particular interest, since this information could indicate the means by which monoclonal antibodies recognising this site bring about neutralisation. Using complementation assays it has been established that a region of the gD molecule encompassing site VII is not essential for gD function (Feenstra *et al.* 1990) and suggests that neutralisation brought about by antibodies within this group is not due to direct binding to a functional site.

Glycoprotein B-1 is a major glycoprotein component of virus envelope which maps to the unique long region of the genome (Pellet *et al.* 1985, Bzik *et al.* 1984).
The gene is conserved throughout human herpes viruses and gB-1 homologues have been identified in HSV-2 (Bzik *et al.* 1986, Stuve *et al.* 1987), VZV (Davidson and Scott 1986), HCMV (Cranage *et al* 1986) and EBV (Pellet *et al.* 1985).

The characteristics of gB t.s. mutants have implied a role in viral entry, with mutant viruses formed at the non-permissive temperature able to bind but not penetrate cells (Little *et al.* 1981). These observations have been supported by complementation studies in which gB null virus could adsorb to cells but not penetrate (Cai *et al.* 1988).

Glycoprotein B has also been implicated in fusion of infected cell membranes to form large multi-nucleated syncitia, a process which may be related to the mechanisms of viral penetration. Genetic studies have identified several genes and loci which are involved in the syncitial phenotype (Ruyechan *et al.* 1979, Little and Schaffer 1981, Pogue-Geile *et al.* 1984, DebRoy *et al.* 1985, Bond and Person 1984) however two of these reside in the cytoplasmic domain of the gB gene (Cai *et al.* 1988).

HSV-1 gB has been shown to exist as a dimer within infected cells, (Claesson-Welsh and Spear 1986) and analysis of the epitopes of gB using monoclonal antibodies has indicated that dimerisation gives

Mab	Antigen Source	Complement Independent Neutralisation	gH recognition in Immunopreciption Western	
LP-11	Infectious Virus	HSV-1	HSV-1	None
52S	Infectious Virus	HSV-1	HSV-1	None
53S	Infectious Virus	HSV-1 only	HSV1 and HSV-2	None

Table 4. Properties of gH specific monoclonal antibodies

rise to epitopes which are unavailable on the monomer (Chapsal and Pereira 1988).

Using neutralising monoclonal antibodies and virus mutants resistant to neutralisation a map of the antigenic structure of gB has begun to be constructed. Comparison of the neutralisation pattern of mutants with a panel of monoclonal antibodies resulted in antibodies being assigned to one of five groups each corresponding to an antigenic site. Only antibodies recognising site III were able to neutralise virus in the absence of complement, however they did not affect virus attachment (Marlin *et al.* 1988).
Complement dependent neutralising antibodies recognising sites I and IV were able to slow the rate of viral penetration in the absence of complement, implicating the antibody binding sites with protein function.

Subsequent experiments involving radio immunoprecipitation of truncated forms of gB localised binding sites I, III and IV to within residues 241-441. Antibodies recognising site II which have no apparent affect on viral penetration map to a region close to the transmembrane region situated between residues 596 to 737 (Highlander *et al.* 1988).

1.9 Glycoprotein H

Glycoprotein H a minor component of HSV-1 has been identified on the virus envelope and at the surface of infected cell membranes. It has an apparent Mr in SDS PAGE of 110,000 and has been mapped to the UL region of the viral genome (Buckmaster 1984). The open reading frame predicts a translation product of 838 AA residues containing an N-terminal signal sequence, several possible N-glycosylation sites, a transmembrane region, and a hydrophilic C-terminus (Gompels and Minson, 1986 and McGeoch and Davison, 1986). Both HSV serotypes encode distinct but antigenically cross reactive forms of the glycoprotein (Showalter, *et al.*, 1981).

Glycoprotein H-1 appears poorly antigenic and despite many attempts, only a few gH specific monoclonal antibodies (2 in USA - 52S and 53S and 1 in the UK - LP-11) are available, the properties of which are summarised in Table 4. These gH specific murine monoclonals are able to neutralise virus in the absence of complement. LP-11 has also been shown to inhibit plaque enlargement *in vitro*, and prevent the formation of multinucleated syncitia in viral strains noted for this effect (Buckmaster, *et al.*, 1984).
Similar observations have been made with antibodies against the varicella zoster virus gH homologue (Keller, *et al.*, 1987) and a monoclonal antibody raised against the HCMV gH homologue by Booth and colleagues (Baboonian *et al.* 1989) also exhibited properties similar to those of LP-11.

Vaccinia virus recombinants containing the gH-1 gene have been constructed, although attempts to raise specific gH antibodies using the recombinant have been disappointing. Monoclonal antibodies generated using the recombinant were able to recognise the vaccinia gene product but were unable to recognise native gH-1 by immunoprecipitation (Minson, 1989 Pers. Comm, Blacklaws *et al.* 1990)

Using a fusion protein, derived from part of the Trp E gene product of *Escherichia coli* and amino acids 270 to 690 of gH-1, a type specific rabbit polyclonal serum has been raised which will recognise denatured gH-1 in Western blot analysis (Minson, 1989 pers. comm).

Gompels and Minson (1989) also constructed a transient cell expression system which overproduced a form of gH recognised in immunoprecipitation by the type 1 specific monoclonal antibodies 52S but not the type common 53S or the type 1 specific LP-11. This was unusual since other transfected HSV viral protein genes for example gD, are known to be antigenically correct when expressed in similar systems (Cohen, *et al.* 1988, Isola, *et al.* 1989 and Muggeridge, *et al.* 1988). However following superinfection with either HSV-1 or HSV-2, 53S and LP-11 would immunoprecipitate the gH gene product and these results suggest that correct processing of gH is dependent on other viral products. This view was supported by other studies in which the gH gene constitutatively expressed in celllines produced an immature form of the molecule which was not fully glycosylated. Superinfection with HSV-2 allowed the gene product to be processed into its mature form.
The identity of the co-operative proteins required for gH-1 processing is uncertain although superinfection with viral mutants containing defects in gB, gD, gC, gE and gI genes did not prevent gH-1 maturation (Foa-Tomasi, *et al.* 1991).

Although a quantitatively minor glycoprotein, evidence points to an important role for gH in the viral life cycle. HSV-1 gH gene homologues have been identified in other human herpes viruses, including varicella zoster virus, Epstein-Barr virus (McGeoch and Davison, 1986), Human cytomegalovirus (Cranage, *et al.*, 1988), as well as herpes virus saimiri (Gompels, V.A., 1988), and the conserved nature of gH is paralleled only by gB.

Little is known concerning the function of gH-1 or how monoclonal antibodies bring about virus neutralisation, although it has been established that the neutralising mabs LP-11, 52S, 53S do not prevent viral attachment to cell surfaces (Fuller, 1989). There is little information on gH-2. The gene has not been sequenced and the function of the protein is unknown.

Studies of the phenotypic characteristics (outlined in Fig.3) of Q26 (Desai, *et al.*, 1988), a temperature sensitive HSV mutant with a single amino acid substitution within the polypeptide

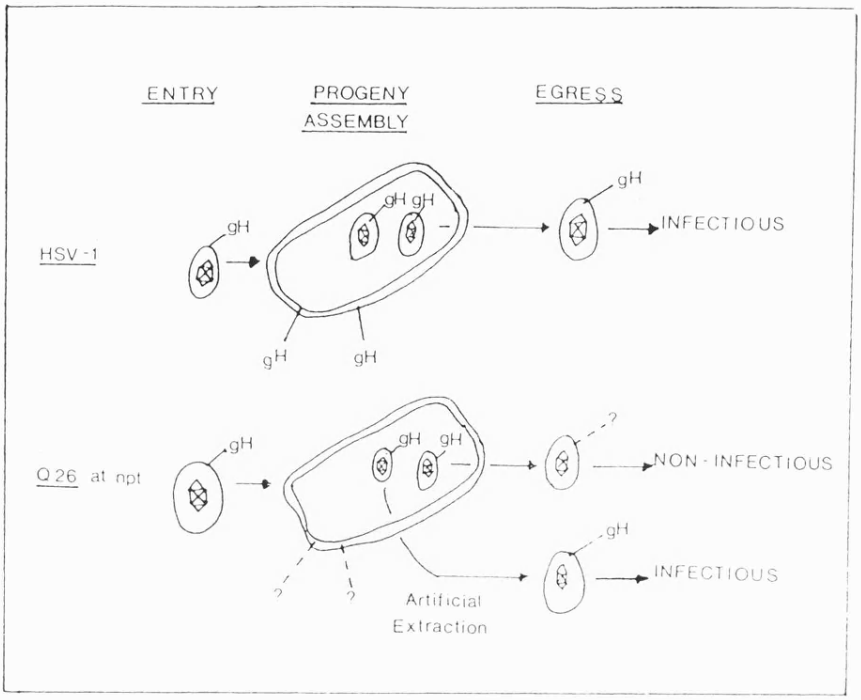


Fig 3. Phenotypic characteristics of Q26.

(Weller *et al.* 1983), may offer insight into the role of gH. At npt Q26 was found to infect (enter) cells normally and infection proceeded to yield normal quantities of intracellular virus particles. Normal lytic plaques were not evident although artificially extracted intra-cellular progeny proved infectious. Glycoprotein H of slightly lower M.Wt. was detected on these intracellular progeny and pulse labelling experiments have suggested this to be a precursor form (Gompels and Minson, 1986). By contrast excreted Q26 viral progeny were non-infectious and gH could not be detected on these particles using available immunological reagents. Further, unlike wild type infection, gH was not detected on cell plasma membrane following Q26 infection at npt.

The implications are that gH plays a role in the entry and exit of virus from cells. A simple explanation suggested by Desai (1988) for the apparent absence of gH-1 in both plasma membrane and extracellular Q26 progeny is that gH is degraded as it moves toward the cell surface via the endoplasmic reticulum.
This argument assumes that progeny virions arriving at the intra cellular surface of cells are capable of exit in the absence of membrane associated or viral envelope gH. If viral exit is a gH dependant process however, the degradation interpretation is not appropriate.

An alternative untested hypothesis suggested by this author which fits the reported phenotypic characteristics of Q26 speculates an essential role for gH in the mechanisms which facilitate viral passage across plasma membrane irrespective of direction. In other words the same mechanism may operate in the exit of viral progeny and the entry of infecting virus. Passage across cell plasma membrane results in loss of virus associated gH unless, as in the case for normal exiting viral progeny, gH is already present at the plasma membrane. This could explain the apparent loss of gH from emerging Q26 progeny. For an infecting virus loss of gH is unimportant, but Q26 progeny exiting from infected cells at npt are rendered non infectious by the loss of gH.

1.10 Epitope Mapping, Objectives and Rationale

As discussed, monoclonal antibodies which bind gH-1 are able to neutralise virus in the absence of complement although viral attachment to cells is unaffected (Fuller *et al.* 1989). This implies a role for gH-1 in post attachment events, perhaps in conjunction with other membrane proteins. Possible viral candidates include gD and gB which like gH have been demonstrated to be essential for infectivity. How gH might facilitate virus entry is unknown.
It may form an intrinsic part of a mechanism or, in view of its quantitatively minor representation, operate in a controlling function. Whatever the role of gH in entry, any co-operative interaction

between molecules raises the question of protein interactive sites.

Although it is not known how gH specific monoclonal antibodies neutralise virus in the absence of complement, antibody binding at these sites may result in disruption which could lead to neutralisation. It is possible to envisage at least four ways in which this may be brought about. At the virus particle level, multivalent antibodies may simply cause aggregation of virus. At the molecular level disruption of gH interactions could be instigated in at least three ways. Firstly by steric hinderance due to antibody binding at or near an interactive site. Alternatively antibody binding might prevent necessary functional changes in molecular structure, fixing the molecule in an unfavourable conformation. Conversely antibody binding may cause adverse conformational changes with similar undesirable effects.

A role for gH in viral entry and exit also raises questions concerning the nature of the involvement and how these processes can be investigated. One way in which this can be approached is by identifying the epitopes of a protein. Identifying and defining the epitopes of a molecule may provide useful clues to the whereabouts of functional protein domains. In addition this information may assist in addressing related questions concerning the mechanisms of neutralisation, protein-protein interactions and the indirect relationship between epitopes and the biological properties of their corresponding antibodies.

The aim of this study was therefore to extend the limited knowledge of gH-1 epitopes and so improve understanding of the molecule and how it may function.

Epitopes are presently described as continuous (sequential) or discontinuous (non-sequential). Continuous epitopes have been operationally defined as linear series of amino acids in the primary sequence and are thought to range between 5 and 8 amino acids in length. Discontinuous epitopes are comprised of small groups or individual amino acids remote from one another in the primary sequence which are brought into juxtaposition by protein folding. Such epitopes are often lost when disruption of protein structure occurs (Van. Regenmortel, 1989).

The epitopes recognised by LP-11, 52S and 53S are probably discontinuous since these antibodies will immunoprecipitate gH-1 from infected cell material but will not recognise gH-1 in Western blots, presumably because the electrophoresis procedure causes epitope denaturation. Since 52S is capable of precipitating only gH-1 whereas 53S will immunoprecipitate both gH-1 and gH-2 (Showalter, *et al.*, 1981) it is probable that different epitopes are involved. This view is reinforced by the finding that LP-11, 52S and 53S do not exhibit competitive binding (Minson, 1990-pers comm).

In an attempt to map the epitopes of LP-11 and 52S Gompels (1990) selected eight HSV-1 variants resistant to neutralisation for comparison of gH gene sequences. In these studies LP-11 resistant virus were reported as susceptible to 52S neutralisation and immunoprecipitation further emphasising the likelihood that epitopes recognised by these antibodies are distinct. Sequencing the gH gene of these variants showed that in each case a single point mutation giving rise to a single amino acid (AA) substitution confered resistance to neutralisation. Substitutions at either AA 86, 168 or 329 gave rise to LP-11 resistance whereas substitutions at AA 536 or 537 confered resistance to 52S. A problem with this type of study is the uncertainty which exists concerning the effect of the AA substitutions and whether their influence occurs directly at the binding site or indirectly by influencing secondary or tertiary structure.

As sequence data in general has accumulated so attempts have been made at forecasting potential antigenic sites from the nature of primary amino acid sequences. Various computer algorithms are available for predicting the antigenicity of regions of primary AA sequence based on hydrophilicity (Hopp and Woods, 1981, Hopp, 1986), and likelihood of structural features such as ß turn potential (Chou and Fasman, 1979, Krchnak *et al*, 1987). However the ability to predict antigenic domains from any primary AA sequence is not yet sufficiently refined for this to be achieved with certainty. Thus choosing potential antigenic sites from algorithm data alone, whilst often useful, can be unreliable.

An alternative empirical approach to epitope identification involves the use of synthetic peptide homologues derived from primary AA sequence. Antibodies recognise and bind to synthetic peptides with conformations which resemble epitopes of the nascent molecule.

The idea that a structural antigenic determinant of a protein might be accurately represented in a relatively short peptide was given credence by Anderer (1963) with the demonstration that a small peptide representing an antigenic determinant of Tobacco Mosaic virus could elicit antibodies capable of recognising the native molecule. These experiments laid the foundation for more recent classical studies of viral antigenicity. Most notably synthetic peptides have allowed knowledge of the epitopic repertoire of Foot and Mouth Disease virus (Brown, 1988) and Hepatitis B virus to be advanced (Howard, 1988).

A recent development in synthetic peptide technology (Geysen *et al.* 1987) has allowed this approach to be widened. This technique makes possible the synthesis of numerous peptides attached at their carboxy terminus to a solid supporting medium.

By screening a sequential array of peptides with appropriate antibodies, synthetic peptides homologous with epitopes of the native protein can be identified. This method has the advantage that once a screening procedure is complete antibody-peptide interactions can be disrupted and peptides used in further screening.

1.11 Experimental Strategy

At the outset a major obstacle was the shortage of gH specific immunological reagents. The first step was therefore to purify gH-1 suitable for the generation of specific antibodies. Having generated specific antibodies it was planned to characterise their immunological and biological properties and along with other available antibodies identify their corresponding epitopes. To achieve this it was proposed that the method described by Geysen (1987) be used to synthesise a series of overlapping peptides encompassing the entire primary AA sequence of gH. Overlap of all but one AA of each successive peptide would enable all potential continuous epitopes of a given length to be represented. Peptides would be probed for reactivity with available antibodies and the significance of potential epitopes further validated by characterising the biological properties of antibodies raised against homologous free peptides (Geysen, 1985).

Chapter 2

MATERIALS AND METHODS

2.1 Reagents and Materials.

Reagents and materials were obtained from the following UK sources unless otherwise stated.

Aldridge Chemical Co. Ltd., Gillingham, Dorset

Laboratory chemicals

Amersham International, Aylesbury, Buckinghamshire

Radiochemicals, Immunological reagents,

ß Max Photographic film

BDH Chemicals Ltd., Eastleigh, Hampshire

Laboratory chemicals

Biorad Laboratories, Watford, Hertfordshire

Western Blotting materials

Cambridge Research Biochemicals Ltd., (ICI) Harston, Cambridge

All chemicals and reagents required for peptide synthesis

Corning, New York, USA

Tissue culture flasks

Dupont (UK) Ltd., Stevenage, Hertfordshire

Scintillation cocktail and tubes

Dynatech Laboratories Inc., Chantilly, Virginia, USA

Microtitre plates

European Collection of Animal Cell Cultures, CAMR, Porton Down

All cell types

Flow Laboratories, Irvine, Scotland

Tissue culture media

Gibco - BRL, Uxbridge, Middlesex

Tissue culture media

Imperial Laboratories, Andover, Hampshire

Tissue culture media

Kodak Limited, Hemel Hempstead, Hertfordshire

X-Ray film, developer and fixer

Nunc, Kamstrup, Denmark

Tissue culture flasks

Pharmacia, Uppsala, Sweden

Protein A - sepharose beads. Sephadex G-25

Romil Chemicals, Shepshed, Loughborough, Leicestershire

Solvents required for peptide synthesis

Sigma Chemical Co. Ltd., Poole, Dorset

Laboratory chemicals

Whatman Labsales Ltd., Maidstone, Kent

Filter paper

2.2 Cell Culture

All cell lines (BHK 21, Vero, MRC5) were cultured in Dulbeccos minimal essential medium, supplemented with 2mM glutamine, 0.1mg/ml kanamycin and 10% (v/v) foetal calf serum. Culture medium was maintained in equilibrium with an atmosphere of 5% CO_2 in 95% air to give a media pH of 7.2. Cell monolayers were grown in 175cm^2 flasks or 850cm^2 roller bottles (unless otherwise stated). When confluent cells were passaged at a ratio of 1:4 following treatment with Trypsin/EDTA solution following the removal of medium and washing with warm phosphate buffered saline (PBS).

Cells were periodically checked for mycoplasma contamination in the normal manner. Following growth of cell monolayers on coverslips, cells were fixed using Carnoy's solution. Fixed cells were steeped in Hoechst stain (r.t. for 10 min) and examined under incident U.V. illumination (Leitz Dialux

20 E.B. microscope) for the presence of mycoplasma DNA.

Buffers and Reagents

Trypsin/EDTA	-	0.25% Trypsin 0.05% EDTA (w/v) in PBS.
Carnoy's solution	-	methanol: acetic acid 3:1 (v/v).
Hoechst stain	-	diluted 1:2000 in Hanks buffered saline.

2.3 Virus Stocks and Titration

Virus stocks were grown following infection of confluent monolayers at a multiplicity of infection (moi) of 5. Virus was harvested from infected cells after 48 hr following disruption with glass beads. Disrupted cell material was removed from the medium by low speed centrifugation (10,000g at $4^{o}C$ for 20 min). Routinely HSV-1 titres in excess of 1×10^{8} plaque forming units (pfu) per ml were obtained.

The virus strains used in this study were HSV-1 Troisbel strain or HSV-2 25766 strain kindly supplied by Dr. A.C. Minson, University of Cambridge.

Virus titration was carried out essentially according to the methods of Wentworth and French (1970). Vero cell monolayers were grown in 24 well plates until confluent and infected with tenfold dilutions of virus sample at $37^{o}C$ for 1 hr. Medium containing virus was replaced with overlay medium and infection allowed to proceed. Following plaque development, cells were fixed for 20 min at room temperature (r.t.), washed, stained for 15 min at r.t., washed, dried and plaques counted. All assays were performed in triplicate.

Buffers and Reagents

Overlay Medium	-	Dulbeccos MEM: Carboxymethylcellulose (4%), 1:1 by volume.
Fixing Agent	-	10% Formalin in PBS.
Stain	-	0.1% Crystal Violet in 20% ethanol.

2.4 Radiolabelling of HSV Glycoproteins.

Cell monolayers were grown to confluence as previously described. Medium was removed and cells were washed in PBS at 37°C. Fresh culture medium was added containing virus seed at a moi of 5pfu per cell. Infecting media was removed after 1 hr, cells washed in PBS at 37°C and MEM containing [14C] glucosamine hydrochloride radiolabel (to give an activity of 2.5 uCi/ml) was added. Following CPE infected cells and virus were harvested using glass beads. Disrupted cells were pelleted from media by centrifugation at 10,000g, 4°C for 20 min. Virus in the supernatant was pelleted by centrifugation at 80,000g, 4°C for 40 min. Lipid membrane associated glycoproteins were solubilized by resuspending pellets in extraction buffer for 1 hr at 4°C with gentle mixing.

Buffers and Reagents

Extraction Buffer - 20mM Tris HCl, pH 7.0
0.3M NaCl
10% glycerol v/v
1mM $CaCl_2$
0.5mM $MgCl_2$
2mM EDTA
0.5% NP-40
300ug PMSF

2.5 Scintillation Counting

Activity of radiolabelled samples were determined using a Packard Miniaxi Tricab scintillation counter (model 400) following addition to 2ml of aqueous scintillation cocktail (Scintillator 299™ - Packard).

2.6 Large Scale Antigen Preparations

Three large scale antigen preparations (LSAP) were carried out using confluent Vero cells infected with HSV-1 at an MOI of 0.1. Each preparation consisted of 24 roller bottles, one of which was uninfected and acted as a negative control and another was infected in the presence of medium containing 125uCi of [14C] glucosamine hydrochloride. Following complete CPE, medium was removed and cells were harvested in a total of 50ml of extraction buffer, in which 0.1%

octylglucoside replaced NP.40 as the detergent component. Virus was pelleted from the medium by centrifugation at 80,000g, 4^{o}C for 40 minutes and resuspended in extraction buffer. Insoluble debris was pelleted from extraction buffer by low speed centrifugation, 10,000g at 4^{o}C for 30 minutes.

2.7 Polyacrylamide Gel Electrophoresis.

SDS polyacrylamide gels were prepared essentially according to the method of Laemmli (1970). Running gels comprised 7.5% polyacrylamide whilst stacking gels were 4% polyacrylamide, unless otherwise stated. Protein samples were prepared by incubation with an equal volume of sample buffer at 100^{o}C for 2 min loaded onto gels with electrophoresis carried out using LKB Electrophoresis apparatus at 200 volts for 4 hr. Gels were fixed at r.t. for 30 minutes, stained and destained as required. Storage was facilitated by drying gels onto Whatman No. 3 filter paper under heat and vacuum using a slab gel dryer (Biorad).

Apparent molecular weights of protein samples run on SDS-PAGE were determined relative to the migration of marker proteins of known molecular ratio (Mr).

Buffers and Reagents

Tank Buffer	- 0.05M Tris : 0.53M Glycine : 0.0035M.SDS
Stacking Gel Buffer	- 0.5M Tris : 0.015M SDS pH6.7
Running Gel Buffer	- 1.5M Tris : 0.015M SDS pH8.9
Reducing Sample Buffer	- 0.0625M Tris : 2% SDS (w/v) 15% glycerol (v/v) 5% mercaptoethanol (v/v)
Non Reducing Sample Buffer	- 0.625M Tris : 2% SDS 15% glycerol
Fixer/Stain	- Methanol:acetic acid:water. 3:1:6 v/v Coomassie Blue R.250 0.25% w/v in above
Destain	- Methanol:acetic acid:water. 3:1:10 v/v

2.8 Autoradiography

Autoradiography of dried polyacrylamide gels or nitrocellulose membrane utilized Dupont Cronex L X-Ray Film or Amersham Hyperfilm-ß-max. Following exposure, film was developed using Kodak Universal Developer and Kodak FX-40 fixer according to the manufacturers instructions.

2.9 Fluorography

Following destaining, gels were washed twice in neat dimethyl sulphoxide (DMSO) at r.t for 30 min, followed by a single wash in scintillation solution at r.t. for 20 min. Gels were then washed three times in distilled water and dried onto Whatman No. 3 filter paper.

Buffers and Reagents

Scintillation Solution - 30% 2.5 diphenyloxazole w/v in DMSO

2.10 Immunoprecipitation

Non specific immune interactions were minimized by preincubation of sample with an appropriate volume of normal rabbit serum (NRS) at r.t. for 1 hr, followed by incubation with Protein A Sepharose beads at r.t. for 15 min. Beads were pelleted at 10,000g, r.t. for 5 min.

Pre-treated supernatant was then incubated with the antibody of choice at r.t. for 2 hr. Immune complexes were adsorbed by incubation with Sepharose-Protein A beads at r.t. for 30 min. Sepharose-Protein A antibody-antigen complexes were pelleted and washed 3 times in washing buffer. Immune complexes were eluted from Sepharose-Protein A by incubation with gel sample buffer (100ul) at 100^{0}C for 2 min.

Buffers and Reagents

Washing buffers:

a. 0.1M Tris HCl pH 9.0
   0.5M lithium chloride
   1% v/v mercaptoethanol

b. 50mM Tris HCl pH 8.3
0.6M NaCl
0.5% Triton X-100

2.11 Western Transfer (Blot)

Electrophoretic transfer of proteins separated by SDS PAGE onto nitrocellulose was carried out according to the method of Burnette (1981). Nitrocellulose membrane was immersed in transfer buffer at r.t. for 2 min and then positioned adjacent to the gel. Whatman No 3 filter paper was then placed on either side followed by a scotchbrite pad.

The complete sandwich was clamped and placed in a Biorad Transblot Western Transfer tank filled with transfer buffer ensuring that the nitrocellulose membrane was situated between gel and anode. Transfer was carried out at r.t. overnight at 30v, 0.1A.

Buffers and Reagents

Transfer Buffer - 20mM Tris
150mM glycine
20% methanol (v/v)

2.12 Probing of Western Transferred Proteins

After removal from the blotting tank, membrane was washed with blocking buffer at r.t. for 1 hr and sliced into strips longitudinally.
A strip was incubated with blocking buffer for 1 hr at r.t. followed by probing antibody diluted as appropriate in washing buffer at r.t. for 2 hr or $4^{o}C$ overnight. Strips were removed, washed three times and probed with anti-species-IgG-horseradish peroxidase (HRP) conjugate at r.t. for 1 hr. Finally, strips were washed three times in washing buffer and antibody binding revealed using O-phenylene diamine (OPD) substrate or 3-amino-9-ethylcarbozole (3A9EC) which gave brown or red colouration respectively in the presence of HRP.

Buffers and Reagents

Washing Buffer - 150mM NaCl
10mM Tris pH 8.0
0.1% Tween 20

Blocking Buffer - as above with addition of 1% BSA and 1% Milk Powder.

2.13 Virus Neutralisation Assays

Neutralisation assays were carried out according to the method described by Minson *et al.* (1986) unless otherwise stated. Assays utilized confluent Vero cells both in the absence of complement (serum heated to 56^{o}C for 30 min) or in the presence of active complement. Serial dilutions of serum in 2ml of tissue culture media were incubated with 2 x 10^{3} pfu of virus at r.t. for 1 hour. Samples of 500ul and 50ul of virus suspension were then incubated with Vero cells, grown to confluence in 24 well plates, in a total volume of 1ml at 37^{o}C for 2 hr. Infecting medium was removed and replaced with overlay medium containing carboxy methylcellulose (see section 2.3). Cells were incubated at 37^{o}C for 3 days after which medium was removed and cells fixed using 10% formalin solution for 20 minutes. Formalin solution was discarded and cells washed with water and stained using 0.1% crystal violet for 15 min. Crystal violet was washed off with water and viral plaques counted.

Buffers and Reagents

Formalin Solution - 10% in PBS
Crystal Violet - 0.1% in 20% ethanol

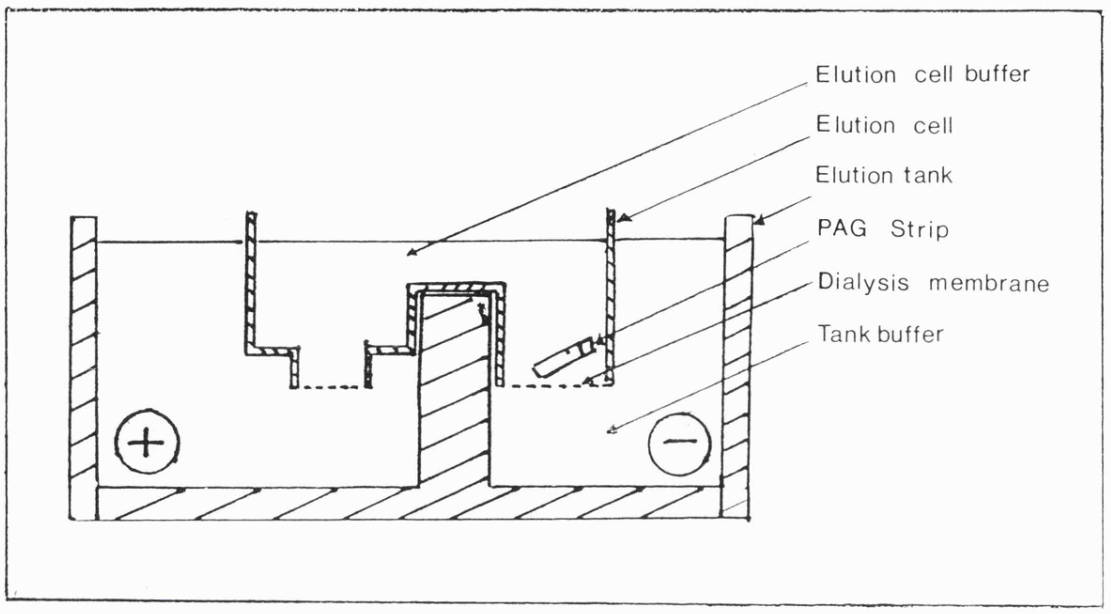


Fig 4. Diagramatic cross section of Electrophonetic concentrator.

2.14 Electroelution of Proteins

Proteins were eluted from preparative polyacrylamide gels using an ISCO-1750-Nebraska electrophoretic concentrater (illustrated in Fig. 4) with elution buffers adapted from Hunkapiler (1983) and North (1983). Polyacrylamide gel containing protein of interest was immersed in soaking buffer at r.t. for 30 min and then placed in compartment 4 of an eluting cell adjacent to the cathode. Each eluting cell utilized Spectrapor dialysis membrane with a Mr limitation of 50,000. Eluting cells were placed across the eluting tank bridge and filled with soaking buffer. Eluting tank compartments were filled with eluting buffer A and electroelution allowed to proceed at 250 volts, 4^{o}C, overnight. Following elution overnight, tank buffer A was replaced twice by eluting buffer B at 4^{o}C, for 2 hr and then twice by buffer C at 4^{o}C for 1 hr. Eluted fractions were then collected from cell compartments.

Buffers and Reagents

Elution Buffers

A. 0.05M Tris acetate pH 7.5
0.1% octylglucoside
4M urea
0.1% SDS

B. As in A except SDS and pH adjusted to 8.5

C. As in B except Urea

Soaking Buffer

0.2M Tris acetate pH 7.5
0.2% SDS
0.1% octylglucoside
4M urea

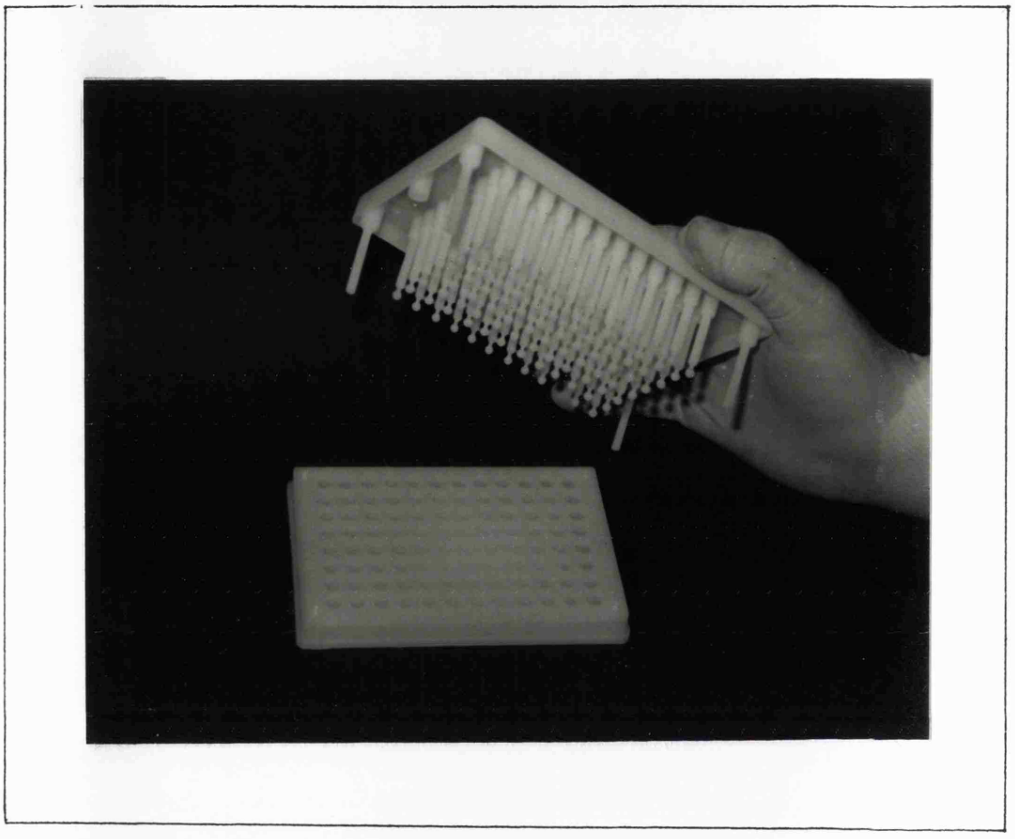


Fig 5. High density polythene pin system used as solid phase in multiple peptide synthesis.

2.15 Immunization Schedule and Serum Collection

New Zealand White rabbits were immunized according to the protocol described by Goding (1983). All injections were given subcutaneously usually in no more than two sites with a maximum inoculum of 0.5ml per site. Priming doses used antigen emulsified in an equal volume of Complete Freund's Adjuvant followed by boosting doses in Incomplete Freund's Adjuvant. Boosting injections were delivered 3 weeks after the primary dose with animals bled 6-10 days later. Any additional boosting injection was given at 2 week intervals with serum similarly collected 6-10 days post injection. All animals were bled prior to inoculation. All rabbits were bled via a marginal ear vein which in skilled hands allowed rapid collection of 20mls of blood without distress. Final exsanguination was carried out by cardiac puncture under anaesthetic.

2.16 Peptide Synthesis

Peptides were synthesised according to the methods described by Geysen *et al.* (1984, 1987). Each cycle of synthesis attached a single AA residue with the resulting peptide covalently linked at its carboxy terminus to the solid phase.

2.16.i The Solid Phase

The solid phase is supplied as a moulded high density polythene pin by Cambridge Research Biochemicals (CRB). Each pin carries protected beta alanine residues which allow covalent attachment of the initial peptide residue. The beta-alanine residues are covalently linked to the solid phase pins by hexamethylene diamine and acrylic acid linkers. Pins are arranged in blocks of 96 (Fig. 5) so that a 96 well microtitre tray will accommodate a 96 pin block. This allowed synthesis reactions and screening of peptides attached to pins to be carried out in microtitre trays.

2.16.ii Residues and Deprotection

Synthesis utilized pentafluorophenyl (Pfp) active esters of all L-amino acid residues except serine and threonine for which oxo-benzotriazole replaced Pfp. The N-terminus of each residue was protected by fluorenylmethyloxcarboxyl (FMOC) groups. Side chain protection was afforded by t-butyl

derivatives except for arginine in which methoxy-trimethylphenylsulphonyl performed this function.

All synthesis operations used dimethylformamide (DMF) cleared of contaminating amines by storage in the presence of molecular sieve type 4A (a crystalline sodium alumin-silicite) Amine contamination was assessed by method of Steward and Young (1984).

To 1ml of a 1mg/ml solution of fluorodinitrobenzine in 1,4 dioxam was added 1 ml of DMF. After 30 min U.V. absorbance was measured at 381 nm. and multiplied by a conversion factor 5.38 to give amino levels as ppm. Typically levels of 4-5 ppm. were deemed satisfactory for experimental purposes.

Terminal FMOC protecting groups were removed by immersing pins in 20% (v/v) piperidine in DMF at r.t. for 30 mins.
Deprotected pins were then washed in DMF (100 mls per block) at r.t. for 5 mins followed by four washes in methanol at r.t. for two mins and allowed to air dry. Prior to immersion in reaction mixture, pins were washed in DMF at r.t. for 5 mins.

2.16.iii **Amino Acid Coupling**

The practical details concerning the quantity of each AA required for each cycle of synthesis were detailed in a schedule generated by microcomputer programme (CRB). The synthesis schedule also directed the order in which AA residues were dispensed (coupled).

Reaction mixtures containing AA residues at a concentration of 30mM were made up immediately prior to dispensing into wells of a microtitre reaction tray. Quantities of each residue were dissolved in a solution of 1 hydroxybenzotrizole (HoBt) in DMF, and aliquots of 100 ul dispensed into appropriate wells. Blocks of pins, with FMOC deprotected residues attached, were carefully lowered into the wells, and the reaction allowed to proceed in sealed bags at r.t. for 18 hr. On removal from reaction wells, pins were washed once in DMF at r.t. for 2 mins followed by four two minute washes in Methanol (100mls) at rt and finally another DMF wash for two minutes at rt.

Newly attached FMOC protected residues were then subjected to the deprotection procedure followed by another cycle of AA coupling. These events were repeated until peptides of the desired length were obtained. A final FMOC deprotection cycle was carried out prior to acetylation.

2.16.iv **Acetylation**

N-terminal AA residues were acetylated by immersing peptides in acetylation solution at r.t. for 90 mins. Upon removal, pins were washed in DMF at r.t. for 2 mins followed by four washes in methanol at r.t. for 2 mins and allowed to air dry.

Acetylation solution:

DMF, acetic anhydride, triethylamine 5:2:1 (v/v/v)

2.16.v **Side Chain Deprotection**

Side chain protecting groups were removed by treatment with cleavage solution at r.t. for 4 hr. Pins were then washed twice in dichloromethane at r.t. for 5 mins, twice in 5% (v/v) diisopropylethylamine in dichloromethane at r.t. for 5 mins, and again in dichloromethane at r.t. for 5 mins and allowed to air dry.

Finally peptides were washed in sterile distilled water at r.t. for 2 mins followed by a methanol bath at r.t. for 18 hr. Blocks of pins were then dried *in vacuo* over silica gel for 18 hr and stored in sealed polypropylene boxes over silica gel at 4^{o}C until required for screening.

Buffers and Reagents

Cleavage Solution - Trifluoroacetic-acid:phenol:ethanedithiol 95:2.5:2.5 (v/w/v)

2.17 Epitope Mapping

All newly synthesised peptides were subjected to disruption procedures (detailed below) prior to screening.

2.17.i **Peptide Screening**

As previously described, blocks of pins with peptides attached were accommodated in 96 well microtitre trays. This allowed convenient methods of peptide screening in which pin tips were immersed in appropriate dilutions of antibody or antibody conjugate dispensed into microtitre trays. Essentially, pins were incubated in microtitre trays containing blocking buffer 200 ul per well at r.t. for 1 hr, followed by incubation with screening antibody diluted in blocking buffer at 4^{o}C overnight. On removal, pins were washed four times at r.t. followed by incubation with anti-species antibody enzyme linked (HRP) conjugate with agitation at r.t. for 1 hr. After four further washes in PBS/Tween, the presence of HRP conjugate was revealed by pin immersion in ABTS substrate solution. Colour reaction was stopped by pin removal. Microtitre plate reactions were assessed by absorbance at 405nm (Titertek plate reader). Pins were subjected to the disruption procedure prior to further screening.

Buffers and Reagents

Washing buffer - Phosphate buffer saline.
0.1% Tween 20.

Blocking buffer made up with PBS 1% Ovalbumin
1% Bovine Serum albumin
0.1% Tween 20

2.17.ii **Disruption**

After each screen peptide-antibody interactions were disrupted by sonication of pins in disruption buffer at 60^{o}C for 30 mins, followed by a wash in sterile distilled water at 60^{o}C for 1 min and in boiling methanol for 2 mins. Blocks of pins were allowed to air dry and stored.

Buffers and Reagents

Disruption buffer - 0.1M sodium dihydrogen orthophosphate - pH 7.2
1% SDS
0.1% 2-mercaptoethanol

2.18 Synthesised Antigenic Peptides.

2.18.i Specification

Potential antigenic peptides from the primary AA sequence of interest were synthesised as free peptides on request by C.R.B. (ICI) Ltd. To facilitate conjugation to Keyhole limpet haemocyanin (KLH) all peptides incorporated a C-terminus cysteine residue. During synthesis thiol (sulphydryl) groups were protected by S-trityl groups which were simultaneously removed from the peptide as it was cleaved from the resin support. All peptide N-termini were acetylated.

2.18.ii HPLC Analysis

HPLC of peptides was carried out using a Beckman HPLC system utilizing twin 114M Pumps, a 421A control unit, and a model 165 dual channel detector, under the following conditions:

Column:
Brownlee ODS Spleri-5 C18 (Reverse phase) 10cm x 2.1 mm.

Gradient:
0 to 100%

Solvents:
A. 0 to 0.1% TFA
B. 0.09% TFA/80% Acetonitrile

Wavelength:
220 nm

Flow:
0.2 ml min^{-1}

Chart Speed:
0.5 cms min^{-1}

HPLC Peptide Solvent:

Solvent was adjusted according to peptide solubility using one of the following:

Water
Water: Acetonitrile 1:1
Water: Acetonitrile:TFA 1:1:1

2.19 Peptide Conjugation to Carrier Protein

Peptides were coupled via their cysteine residues to KLH according to the method described by Green, *et al.* (1982).

2.19.i Activation of KLH

Four mg of KLH were solubilized in 250 ul 10mM sodium phosphate buffer (pH 7.2) and activated by adding dropwise 0.7ug of m-maleimido-benzoyl-N-hydroxy succinimide ester (MBS) dissolved in 100ul DMF. The reaction mixture was stirred at r.t. for 30 mins.

2.19.ii Purification of activated KLH

The reaction product KLH-MBS was separated from free MBS using a 25ml Sephadex G.25 gel filtration column. The column was equilibrated and eluted with 50mM sodium phosphate buffer pH 6.0. Reaction mixture was added to the column using a peristaltic pump (Pharmacia) at a flow rate of 1ml min^{-1} with eluent collected in 1ml fractions. Fractions containing KLH-MBS were determined by absorbance peak at $^{OD}280nm$ (Pye-Unicam).

2.19.iii Conjugation

Conjugation was facilitated by dissolving 5mg of each peptide in 1ml of PBS (pH 7), 0.1M sodium borate buffer (pH 9.0) or 1M sodium acetate buffer (pH 4.0) as appropriate. Dissolved peptide was added to KLH-MBS solution and the mixture titrated to between pH 7 and 7.5. The mixture was stirred for 30 mins and divided into aliquots for inoculation.

2.20 Preparation of Peptide-Adsorbed Microtitre Plates

Peptides were bound to microtitre plates essentially according to the methods described by Stott, G.J. and Tyrrell, D.A.J., (1986) using preactivated plates (Immunlon-2). Peptides were weighed out in 1mg quantities and dissolved in 50% acetic acid - Water (v/v). Peptide solutions were diluted to 10ug per ml in 50mM sodium bicarbonate buffer pH 9.5 with 100 ul added to each microtitre plate well (to give 1ug per well) and incubated at 4^{o}C overnight. Solutions were removed from wells, which were allowed to dry before storage at 4^{o}C prior to use.

2.21 Anti-peptide Serum ELISA

Peptide coated microtitre plates were washed three times in PBS/0.1% Tween 20 prior to incubation with 150ul blocking buffer at r.t. for 1 hr. Following removal of blocking buffer, wells were incubated with anti-peptide serum diluted in PBS/0.1% Tween at r.t. for 2 hours. After a further three washes wells were incubated with anti-species Fab-fraction antibody conjugate (HRP) at r.t. for 1 hr. Conjugate was removed, plates washed three times in PBS/0.1% Tween 20 before addition of conjugate reaction substrate (TMB). Absorbance values at 450nm were recorded using a microtitre plate reader.

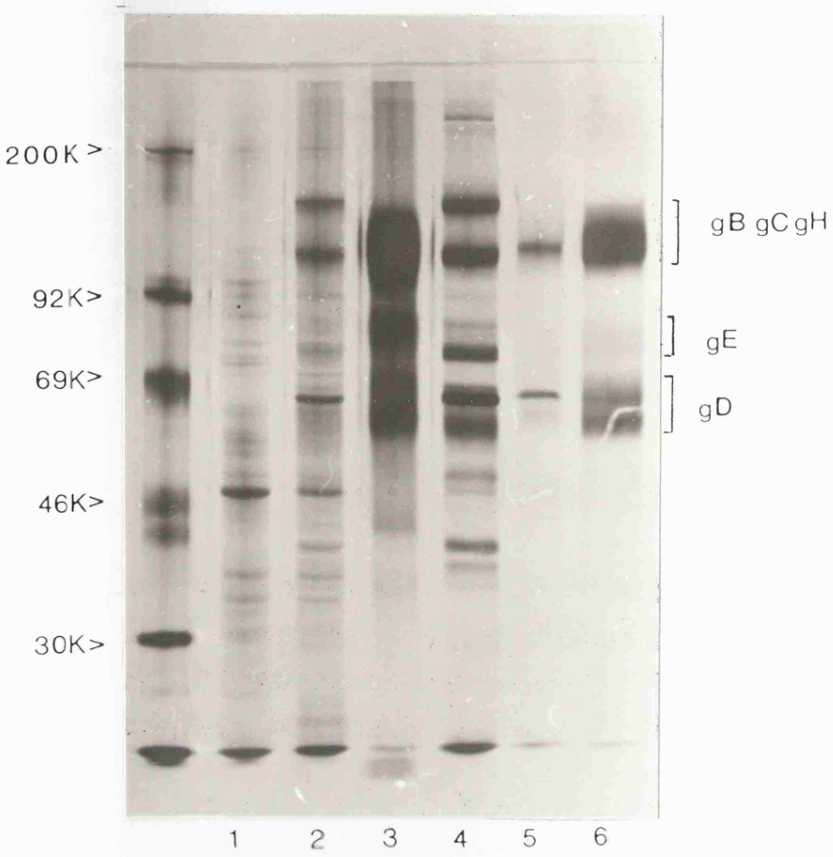
Buffers and Reagents

Blocking buffer - PBS
0.1% Tween 20 (v/v)
5% Skimmed milk powder (w/v)

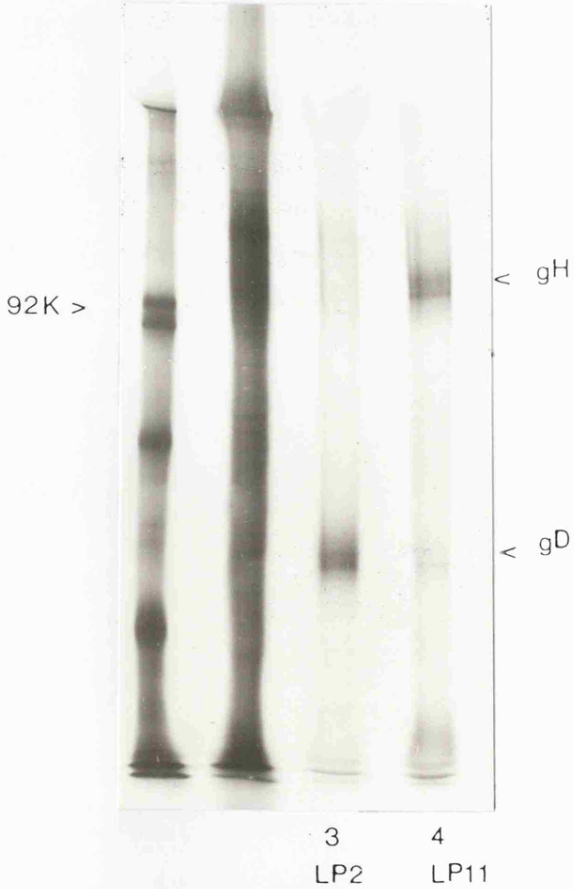
Chapter 3

PURIFICATION OF gH-1 AND THE CHARACTERISATION OF MONOSPECIFIC POLYCLONAL SERUM

PANEL 1



PANEL 2



3.1 Background and Objectives

The initial aim was the generation of monospecific antibodies. As previously outlined, attempts at producing gH specific antibodies using recombinant techniques had met with limited success. The purification of gH-1 provided an alternative source of antigen for the generation of anti gH-1 sera.

The immunoprecipitation properties of monoclonal antibody LP-11 made feasible the purification of gH-1 by immunoaffinity methods, and to this end small quantities of LP-11 the only gH-1 specific monoclonal antibody then available in Europe was kindly made available by Professor A.C. Minson, (University of Cambridge).

3.2 Immunoprecipitation of Radiolabelled gH using LP-11

Glycoprotein H-1 is a minor glycoprotein which requires radiolabelling and autoradiography for routine detection in PAGE. HSV glycoproteins were radiolabelled following infection of confluent cell monolayers in the presence of [14C] glucosamine hydrochloride. This is demonstrated in Fig. 6 Panel 1 in which the characteristic pattern of [14C] labelled HSV glycoproteins is seen in autoradiograph following PAGE.

Of interest are track 3 which represents a [14C] labelled infected cell sample and track 6 a preparation of purified virus particles. Viral glycoproteins can been seen to have migrated to three regions, the 110 - 130K Mr region incorporating gB, gC and gH, the 85K Mr region of glycoprotein E and the 50-60KMr region of gD. Additional panel 1 tracks represent [35S] labelled preparations of uninfected BHK cell (track 1), HSV-1 infected cells (track 2), purified HSV-1 virions (track 4) and HSV-1 envelope proteins (track 5).

The immunoprecipitation of [14C] gH-1 using LP-11 and [14C] gD-1 using monoclonal antibody LP-2 (also from Professor A C Minson) from a heterogeneous mixture of solubilized protein derived from, purified virus, followed by PAGE and autoradiogrpahy is illustrated in Fig. 6 panel 2 tracks 3 and 4 respectively. Glycoprotein H appears as a closely associated doublet band with an apparent molecular ratio (Mr) of approx 110 - 115K.

Fig 6. Autoradiography of [14C] radiolabelled HSV-1 virus proteins, infected cell proteins, and immunoprecipitated HSV-1 glycoproteins D and H following PAGE - see text 3.2.

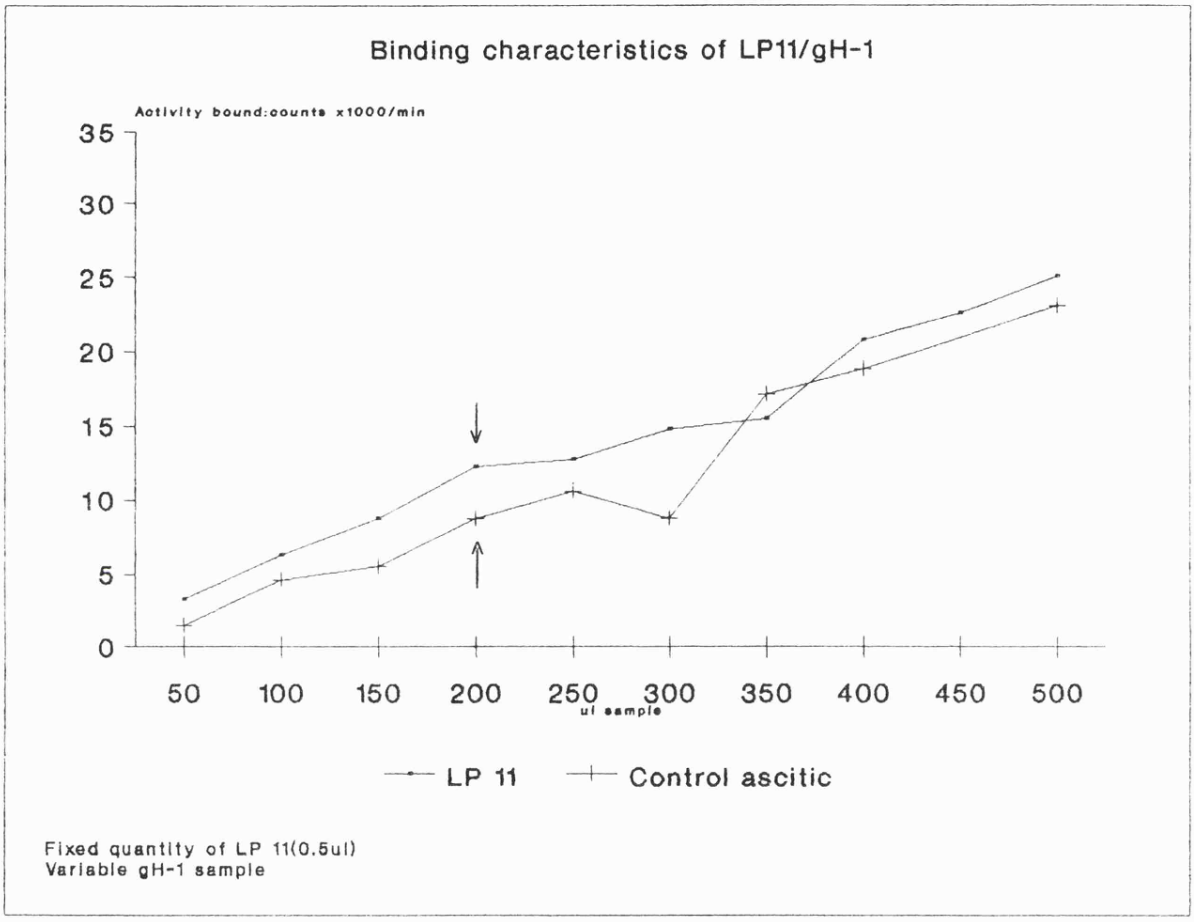


Fig 7. Binding characteristics of LP-11 and gH-1 - see text 3.3.

3.3 Quantative Estimates

To estimate the optimum binding characteristics of LP-11 and gH-1 a series of experiments were undertaken in which the quantity of gH-1 able to be immunoprecipitated from an extract derived from a known number of infected cells was determined.

Cell monolayers were infected with HSV-1 in the presence of [14C] glucosamine hydrochloride. After complete CPE, cells and excreted pelleted virus were solubilized in extraction buffer to give a dilution of 2×10^7 infected cell equivalents per ml. Using a fixed amount of LP-11 the radioactivity of immunoprecipitated gH-1 was determined by scintillation counting, and compared with a control ascitic fluid, over a range of sample cell equivalents in a fixed volume of 0.5 ml.

Results shown in Fig. 7 indicate most favourable binding of gH was achieved from a 200ul sample representing 4×10^6 cell equivalents. The ratio for optimum immunoprecipitation therefore occurred when an 8×10^6 cell equivalent sample interacted with 1 ul of LP-11.

A quantitative estimation of the gH-1 immunoprecipitated by LP-11 was established in comparative Coomassie blue staining experiments relative to protein standards (not shown). Staining of gH-1 immuno precipitated from a sample of 8×10^6 cell equivalents provided a signal which corresponded with protein standards containing 0.1 ug of protein, the limit of detection in this system (Read and Northcote, 1981). Thus from a sample representing 8×10^6 cell equivalents it was estimated approximately 0.1ug of gH could be precipitated by 1ul of LP-11.

3.4 Purification Strategies

Two similar strategies were adopted for the purification of antigen. Both techniques had previously been used successfully by others (Knudson et al., 1985, North et al., 1983). Quantities of HSV-1 gD purified by similar methods had also been used successfully to generate monospecific rabbit polyclonal antibodies (Chadwick and Philpotts, Unpublished data 1988)

Common to both approaches was the extraction of gH-1 from infected cell preparations using immuno-affinity methods based on the optimum binding ratio previously determined. Protein complexes were then dissociated in non-reducing PAGE sample buffer and separated by preparative PAGE. After separation, proteins contained within gels were either Western transferred to nitrocellulose membrane which was utilized directly as an antigen source, or were electroeluted from gel and then used as the basis for inoculum.

Difficulties common to both methods included location of antigen on membrane or in gel and the removal of SDS. Removal of antibody at this stage was attempted to circumvent the generation of anti murine IgG antibodies which could be troublesome in future immunological assays. Subjecting immunoprecipitated gH-1 to PAGE followed by Western transfer or elution from gel theoretically allowed SDS to be stripped from proteins, and because antigen and antibody migrate at different rates, antigen isolated from LP-11 could be obtained as a basis for inoculum.

3.4i Large Scale Antigen Preparations (LSAP)

Three large scale antigen preparations were used in the production of antigen each consisting of 24 roller bottles of confluent Vero cell monolayers infected with HSV-1 at an MOI of 0.1. One roller bottle from each LSAP was infected in the presence of 125 uCi [14C] glucosamine hydrochloride, and another roller bottle containing uninfected Veros acted as a negative control. Infected cells and virus were harvested when the monolayers exhibited complete CPE. Each roller was estimated to contain 1 x 10^8 cells, which from previous estimates would generate approximately 10 ug of gH-1 by immunoprecipitation. Assuming optimum binding criteria a 22 roller LSAP would yield 220 ug of immunoprecipitated antigen and it follows from binding ratios previously determined that 2200 ul of LP-11 would be required for each LSAP.

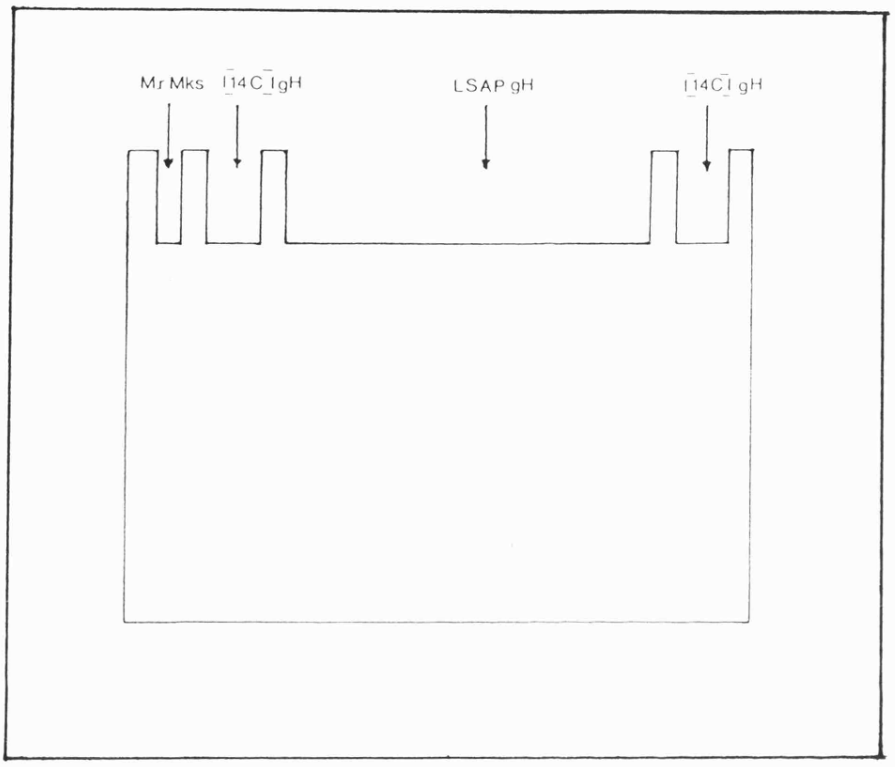


Fig 8. Design of Preparative Polyacrylamide gels - see also Figs 9, 10 and 11.

Satisfactory antigen generation was checked by immunoprecipitation of [14C] gH-1 from a positive control radiolabelled infected roller bottle and confirmation of virus titre at normal levels in cell supernatant confirmed by plaque assay. After clearance of insoluble debris each LSAP was added to 2200 ul of LP-11 and incubated with very gentle mixing at 4^{o}C overnight.

Antigen-antibody complexes (assuming an ascitic IgG content of 15mg/ml - Goding 1983) were batch bound to 1800ul of Protein A sepharose (binding capacity 2mg IgG per 100ul - Pharmacia). Protein A sepharose complexes for use in preparative western blot or electroelution were allowed to sediment out and washed twice.

3.4ii Design of Preparative Gels

Preparative gels for both Western transfer and electroelution experiments were of similar construction to that illustrated in Fig. 8. Preparative sample wells accommodated Protein A sepharose antigen-antibody complexes treated with sample buffer.

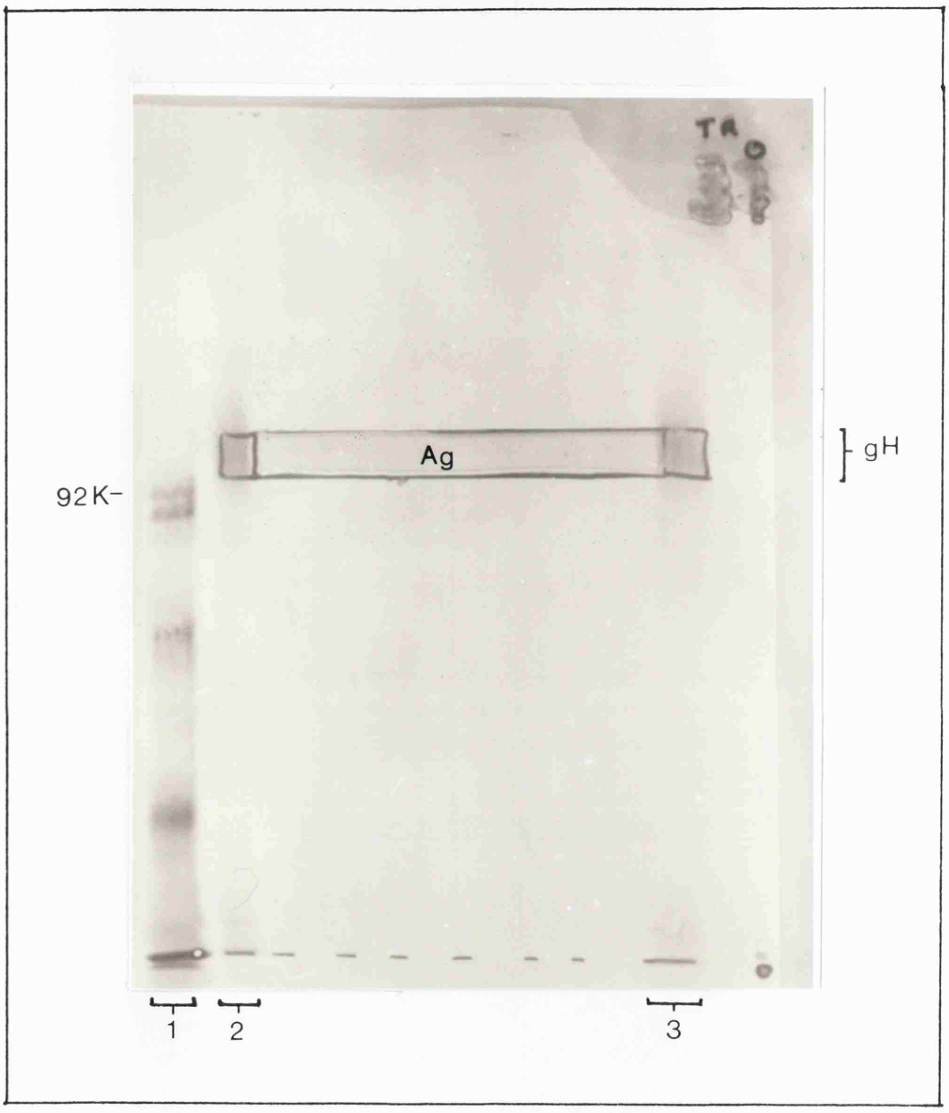


Fig 9. Autoradiograph location of Western transferred non-radioactive antigen using [14C] labelled gH-1 - see also Figs 10 and 11.

3.4iii Western Transferred Antigen

The results of a preparative Western transfer are illustrated in Fig. 9 which shows nitrocellulose membrane (binding capacity 80-100 ug per cm^2) underlying a corresponding autoradiograph. Track 1 of the autoradiograph represents molecular wt. markers with tracks 2 and 3 representing LP-11 immunoprecipitated [14C] gH-1 derived from the infected cell control performed in parallel to each LSAP. The presence of radiolabelled gH-1 marker confirmed satisfactory protein transfer and allowed corresponding preparative gH to be located. The panel of nitrocellulose (Ag) adjudged to contain gH was excised and used as a basis for inoculum. Reagent which would have allowed direct detection of Western transferred gH was not available at this juncture.

The nitrocellulose panel was solubilized in 1ml of DMSO and divided into aliquots. A priming dose containing half the available material was inoculated subcutaneously using a glass syringe as previously outlined and the remaining material administered as 3 further boosting doses.

Subsequent rabbit serum antibodies proved unreactive with gH-1 in immunoprecipitation experiments and following Western transfer.

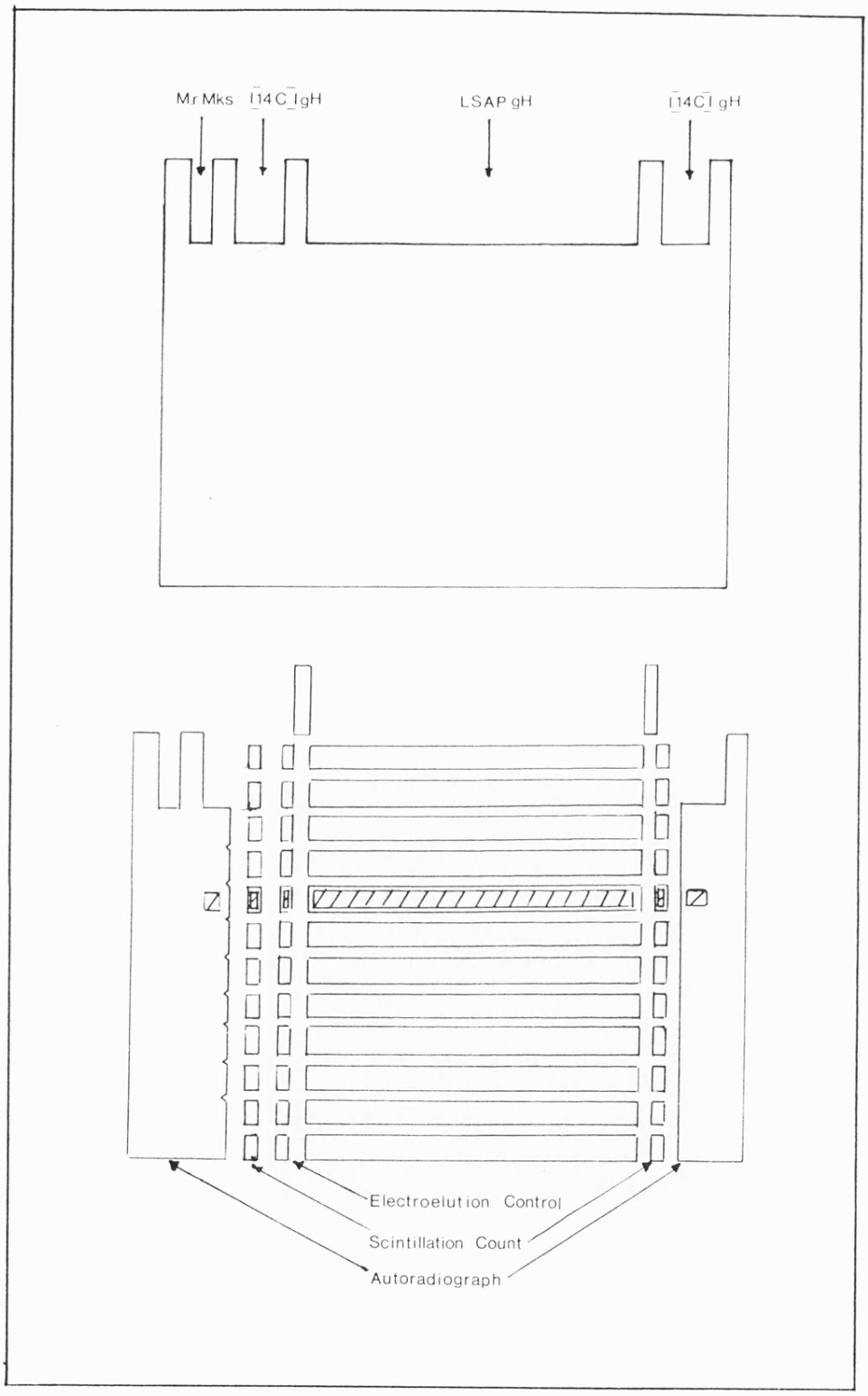


Fig 10. Sectioning of Preparative gels - see also Fig 11.

3.4iv. Electroelution

Prior to electroelution, preparative gels were processed in accordance with the diagram shown in Fig. 10. The end sections of the gel were sliced away longitudinally, bisecting gel tracks containing radiolabelled [14C] gH-1. The central preparative section was cut horizontally into 1cm wide strips which were stored dry at 4^{o}C. The limits of each horizontal strip were marked on the end sections by a small groove. Location of radiolabelled gH-1 was determined by autoradiography..

Scintillation activity derived from small gel samples containing radiolabelled glycoprotein proved inconclusive (Fig 12)

Fig. 11 shows an autoradiograph of the longitudinal section from the left of a preparative PAGE with radiolabelled gH positioned adjacent to horizontal strip 5.

The scintillation data presented in Fig. 12 confirmed the presence of [14C] gH-1 in strip 5.

The progress of electroelution was monitored using [14C] radiolabelled glycoprotein contained in gel section 5.

Electroelution was carried out as previously described. In outline, gel was placed in eluting cells containing eluting buffer (as illustrated in Fig. 4) which were in turn positioned across the bridge of an eluting tank. Tank compartments were filled with appropriate buffer and electroelution commenced. Only four cells from a possible six were required for each preparative elution.
An additional cell was used for elution of radiolabelled [14C] gH from control gel which was monitored by scintillation count, with samples of 10 ul from each compartment of the eluting cell assayed for activity after compensation for volume. The assay results presented in Fig. 13 suggested that although the greatest concentration of antigen was present in compartment 1 of the eluting cell, activity was also evident in other compartments. In view of this, and following successive changes of tank buffer to dialyse remaining SDS detergent and urea to promote correct secondary and tertiary folding, the contents of the preparative cells were pooled and concentrated by freeze drying. Samples were reconstituted to a final volume of 3 mls in PBS and this material formed the basis for inoculation.

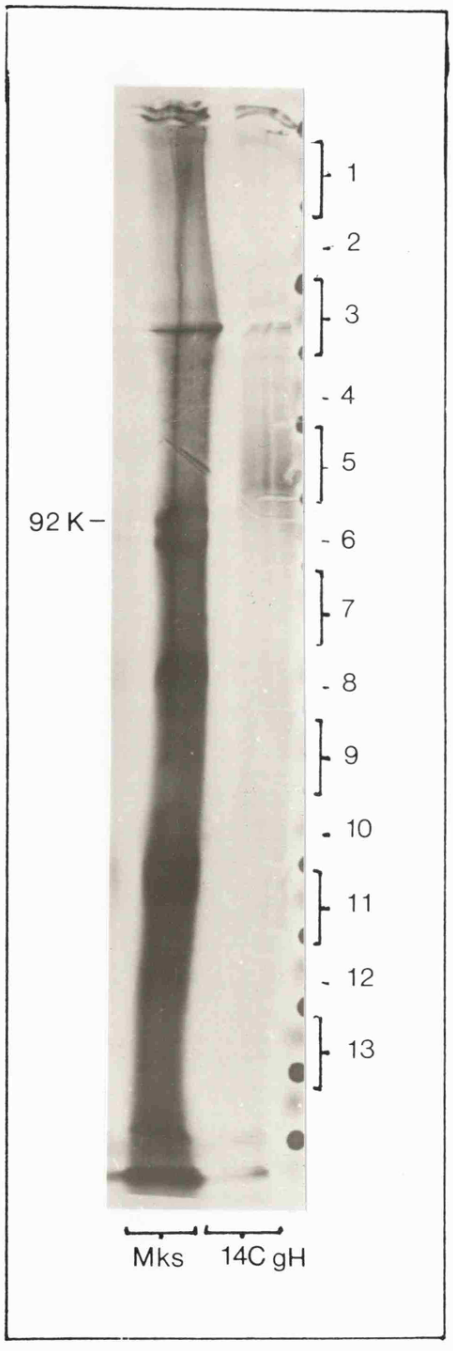


Fig 11. Location of non-radioactive gH-1 in preparative polyacrylamide gel using [14C] radio labelled gH-1 as an indicator - see also Fig 10.

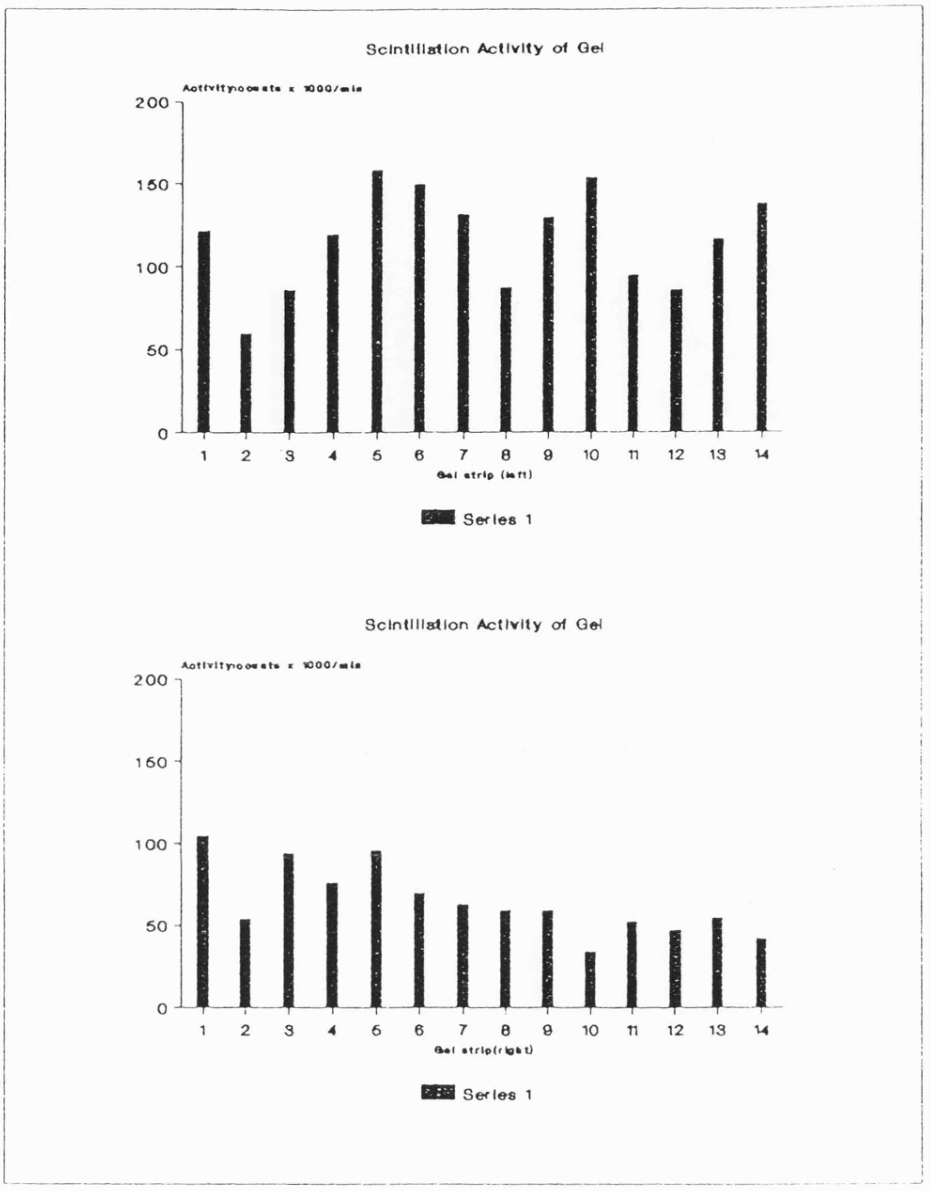


Fig 12. Location of preparative gH-1 using scintillation activity of marker [14C] radiolabelled gH-1 - see text 3.4iv, and also Fig.10

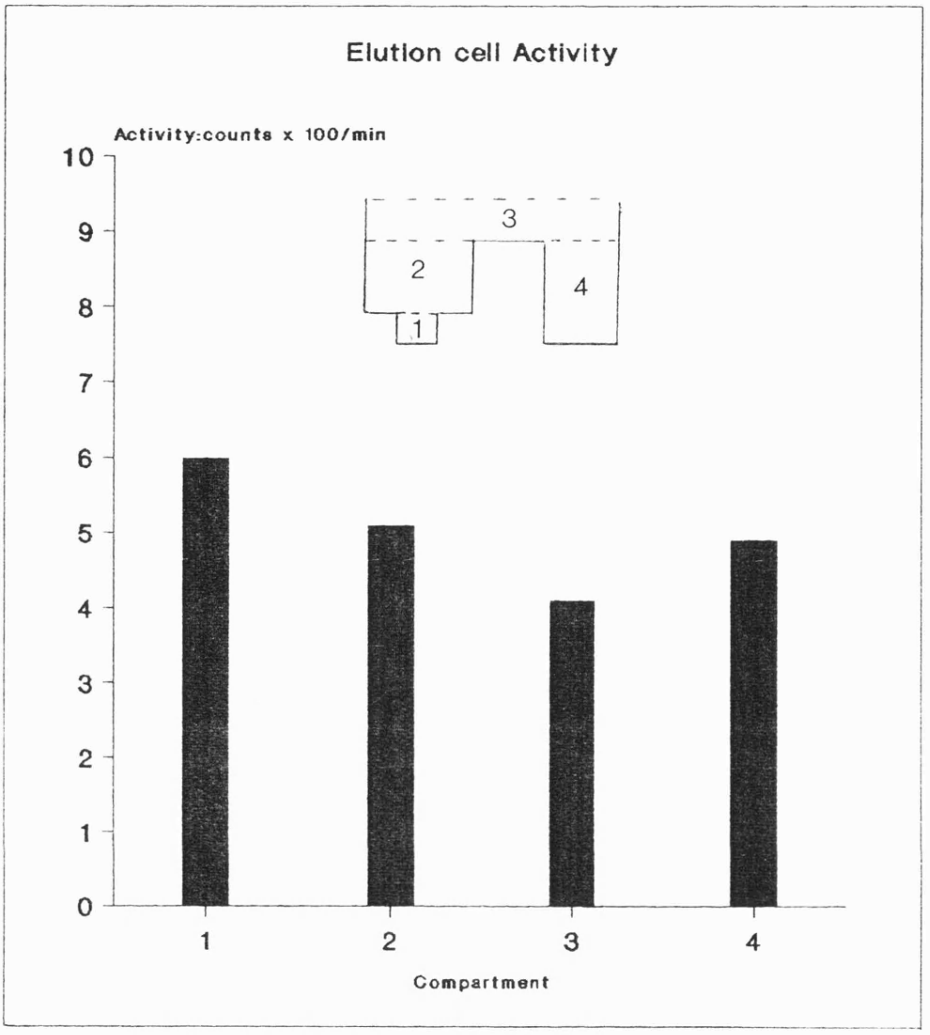


Fig 13. Scintillation activity of samples from control eluting cell compartments.

Rabbit R3a inoculated with electroeluted material derived from the third LSAP, elicited antisera which gave positive results when screened against gH in immunoprecipitation and Western transfer.

3.4v Properties of Electroeluted Antigen

Pilot experiments using radiolabelled gH-1 had shown that following electroelution the apparent molecular wt of the protein remained unchanged. Several attempts to re-immunoprecipitate eluted gH-1 using LP-11 were unsuccessful.

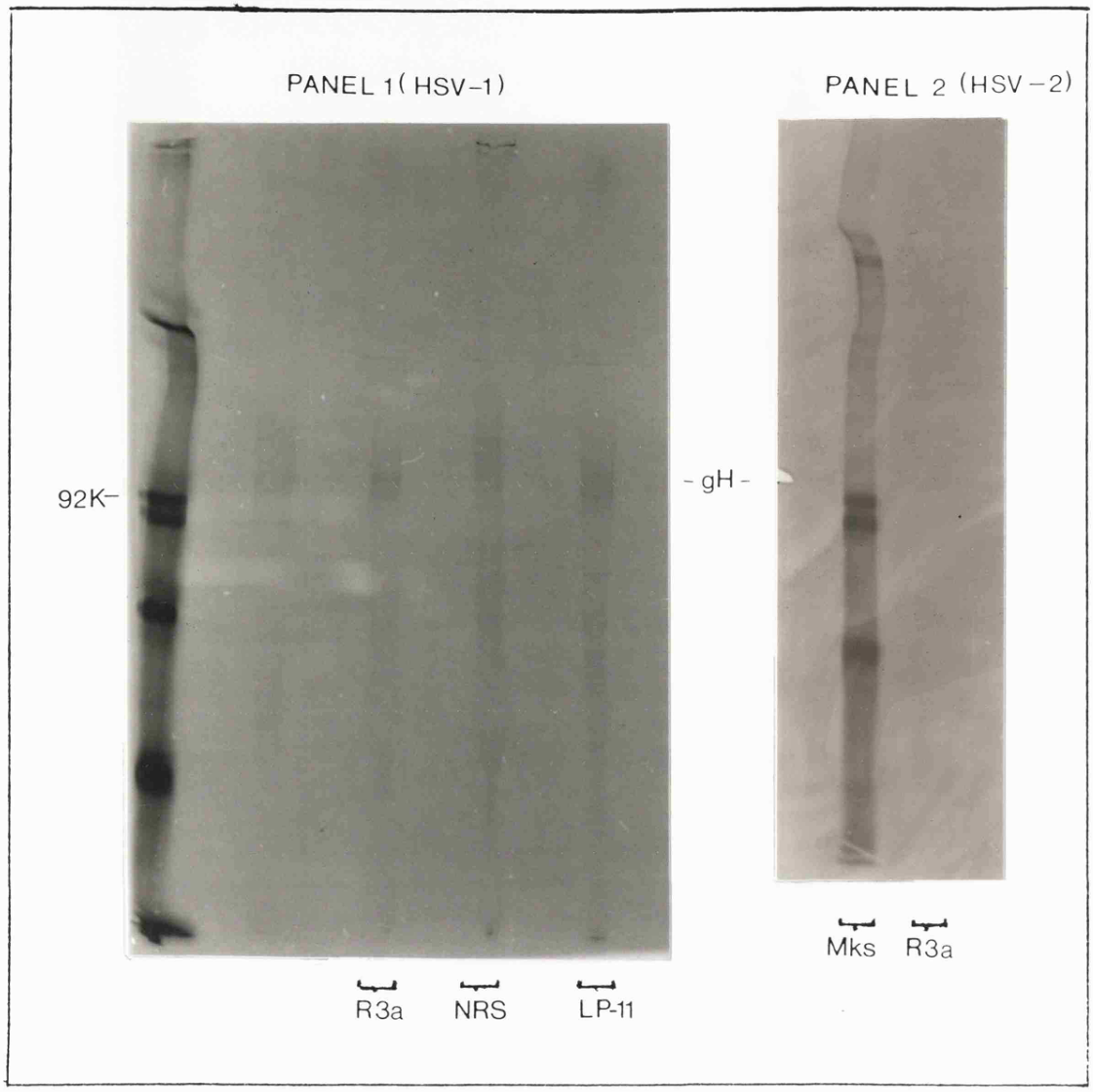


Fig 14. Immunoprecipitation properties of R3a Antibodies - see text 3.5:

3.5 Properties of Polyclonal Serum R3a

3.5i Immunoprecipitation

Screening of R3a polyclonal serum antibodies for immunoprecipitation of gH was performed using [14C] labelled HSV-1 virus, pelleted and solubilized with extraction buffer. In addition type common cross reactivity was screened in a similar manner using HSV-2 virus.

The immunoprecipitation properties of R3a are illustrated in Fig. 14 panel 1 an autoradiograph of PAGE in which the protein species recognised by R3a can be seen to have migrated at an identical rate to gH-1 immunoprecipitated by LP-11.

Some non-specific binding with N.R.S. can also be seen although the gH doublet band is not present. Similar experiments with [14C] radiolabelled HSV-2 derived extracts show the immunoprecipitation of a minor glycosylated protein species with an apparent Mr in PAGE of 110K (illustrated in Panel 2 Fig. 14). This protein species may represent the HSV-2 gH homologue.

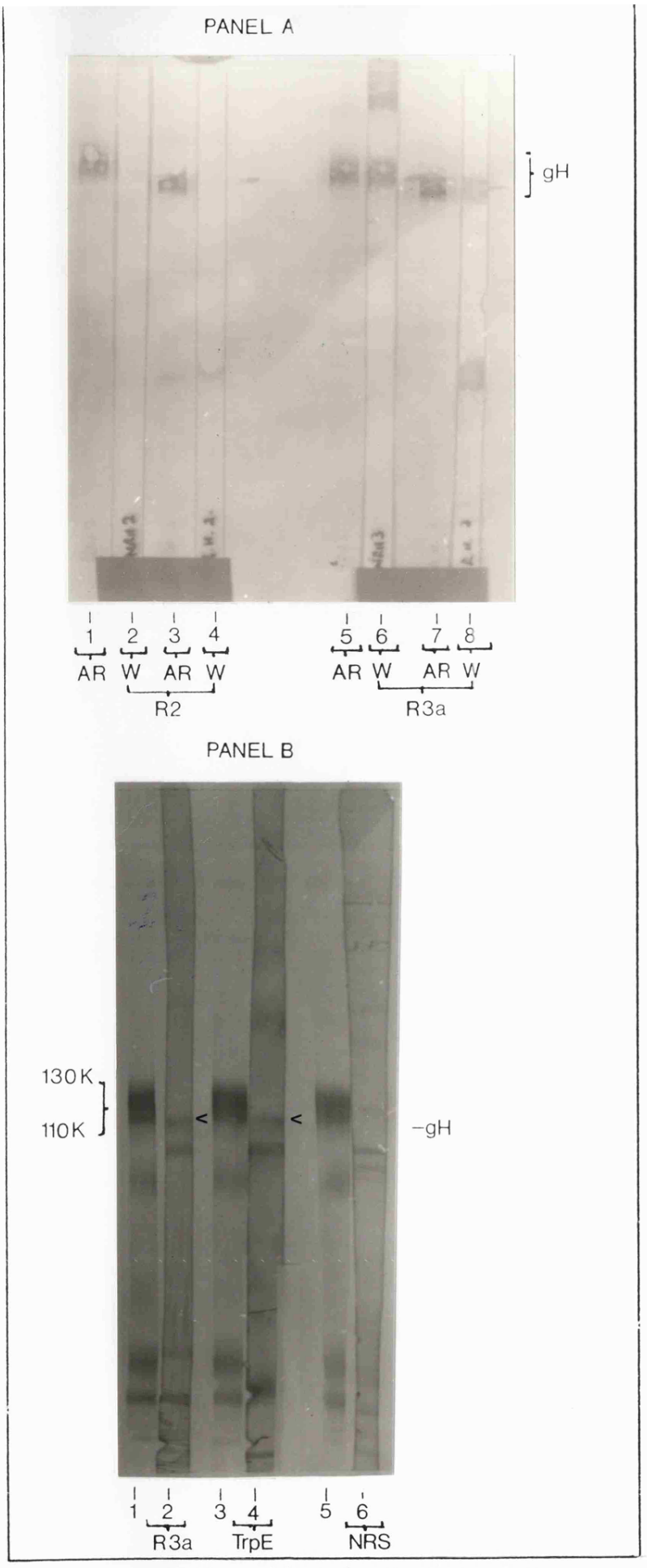


Fig 15. Antibody reactivity against Western transferred immunoprecipitated radiolabelled gH-1 (Panel A) and radiolabelled infected cell proteins (Panel B). Autoradiographs (AR) with corresponding Western blots (W) are shown for each antibody probe - see text 3.5ii.

3.5ii Western Transfer

The results of experiments designed to investigate the reactivity of R3a against gH-1 are illustrated in Fig. 15. LP-11 immunoprecipitated [14C] radiolabelled gH-1 was subjected to PAGE under reducing (Panel A, tracks 4 and 8) and non-reducing (Panel A, tracks 2 and 6) conditions and Western transferred to nitrocellulose. R3a probing of strips from westerns identified protein bands (Panel A, tracks 6 and 8) which aligned precisely with the position of radiolabelled gH-1 as revealed by autoradiograph (Panel A, tracks 1, 3, 5 and 7). By contrast R2 rabbit serum antibodies derived from an animal inoculated with gH-1/nitrocellulose material (see section 3.4iii) were unreactive (Panel A, tracks 2 and 4).

This result was subsequently confirmed using gH-1 specific rabbit polyclonal antibodies (kindly made available by Professor Minson) raised against a TrpE/gH-1 fusion protein (see section 1.9) and able to bind gH-1 following western transfer. The results of probing western transferred [14C] labelled HSV-1 infected cell proteins are illustrated in Fig. 15 Panel B. The protein bands recognised by R3a and TrpE/gH-1 antibodies proved identical (Panel B, tracks 2 and 4) and aligned with the region of western blot revealed by autoradiograph to contain glycoprotein from the 110-130K Mr region (Panel B, tracks 1, 3 and 5).

Closely related doublet bands of Mr 110K were apparant. In addition to a lower band with an apparant Mr of approximately 95K.
This lower band is not apparent in protein material derived from purified virus, (rather than infected cells) and is presumed to represent a partially degraded form of the molecule.

Several attempts were made to identify western transferred HSV-2 protein species using R3a antibodies but without success.

3.5.iii Neutralisation

No evidence for *in vitro* neutralisation of infectious virus by R3a over a range of dilutions, in the presence and absence of complement could be detected.

Chapter 4

PEPTIDE SYNTHESIS AND EPITOPE MAPPING

4.1 Backgound and Objectives

It is widely accepted that peptides are useful tools in the structural and functional analysis of proteins. Antibodies raised against native antigen are often reactive with synthetic peptides and the corollary, that antibodies raised against synthetic peptides can recognise and bind native protein is also true (Audibert *et al.* 1982, Jacob *et al.* 1983, Lerner *et al.* 1981, Bittle *et al.* 1982). A problem with the conventional peptide approach to the location and analysis of epitopes has been the limited number of peptides which can be produced. Even using semi-automated synthesis the methods are highly labour intensive and costly in terms of time, facilities and equipment. Furthermore, choice of peptide is often based on predictive algorithms of protein structure and although the quantity and quality of synthesised peptides can be excellent, they frequently represent only a small part of any antigen.

A recent development by Geysen and colleagues (1987) has dramatically changed this situation. Geysen has shown that when screening antibodies for peptide recognition, the specificity of the antibody is the essential parameter, rather than the purity of the peptide. Since only p.mole quantities of a correctly represented epitope are required as a target for antibody reactivity in an ELISA based system, synthesis conditions which yield u.molar quantities of peptide would suffice if 1% of peptides are correctly synthesised. Thus when producing peptides for epitope mapping purposes the stringent synthesis conditions used in conventional methods can be relaxed. This has allowed the development of a synthesis method able to produce large numbers of peptides relatively quickly and made possible a systematic empirical approach to epitope identification and analysis. Several studies have used these methods to identify epitopes, screening a series of overlapping peptides for antibody reactivity. (Geysen *et al*, 1984, Conlan *et al.* 1988, Lew *et al.* 1989, Jacobs *et al.* 1990, Langedijk *et al.* 1990, Wong *et al.* 1990, Scopes *et al.* 1990).

4.2 Peptides and controls

The generation of new gH specific antibodies (R3a) increased the likelihood that a peptide screening approach for identifying and defining antigenic sites of gH-1 would be successful. In addition gH specific monoclonal antibodies 52S and 53S became generally available from the American Type Culture Collection. These reagents together with the TrpE/gH-1 fusion protein polyclonal serum and LP-11 widened the spectrum of antibodies available for screening.

Epitope mapping experience (Conlan, *et al.*, 1989) had indicated that superior resolution and definition of epitopes resulted from screening smaller peptides (down to 5 residues) with the maximum overlap. Based on the gH-1 AA sequence of HSV-1 strain 17 determined by McGeoch and

Davison (1986) a series of 833 six residue peptides were synthesised representing the entire molecule. Successive hexapeptides overlapped by 5 residues and thus the series represented all potential continuous epitopes of six AA residues or less. (Listed in Appendix 1).

Control peptides PLAQ and GLAQ were synthesised in parallel with experimental peptides. Peptide PLAQ was recognised by control monoclonal antibody (CRB supplied) whereas GLAQ was not. In addition ready-synthesised control peptides (CRB supplied) allowed assay conditions to be checked.

4.3 Mapping with R3a polyclonal antibodies

The results of screening with rabbit serum R3a are presented in Figs 16 to 24. Serum was diluted 1:500 and all assays were performed in duplicate. In all figures peptides are numbered along the x axis according to the residue of the primary sequence which occurs at the peptide carboxy terminus. Thus the first peptide in the series is numbered 6. Absorbance values are represented along the y axis with background levels deducted (lowest 25% of readings - Geysen 1987). Each figure represents a block of 94 peptides unless otherwise stated.

Strong and consistent antibody binding occurred with the following peptides:

QTDPWF 31
TDPWFL 32
DPWFLD 33

GFLAPP 69
FLAPPD 70

FSAVPD 168

HPSGPR 190

GPRDTP 193
PRDTPP 194
RDTPPP 195
DTPPPR 196

RPPVGA 201
PPVGAR 202

ALYAEF 504

LDESVF 602
DESVFI 603

HLLAFD 794

FFWRRE 838

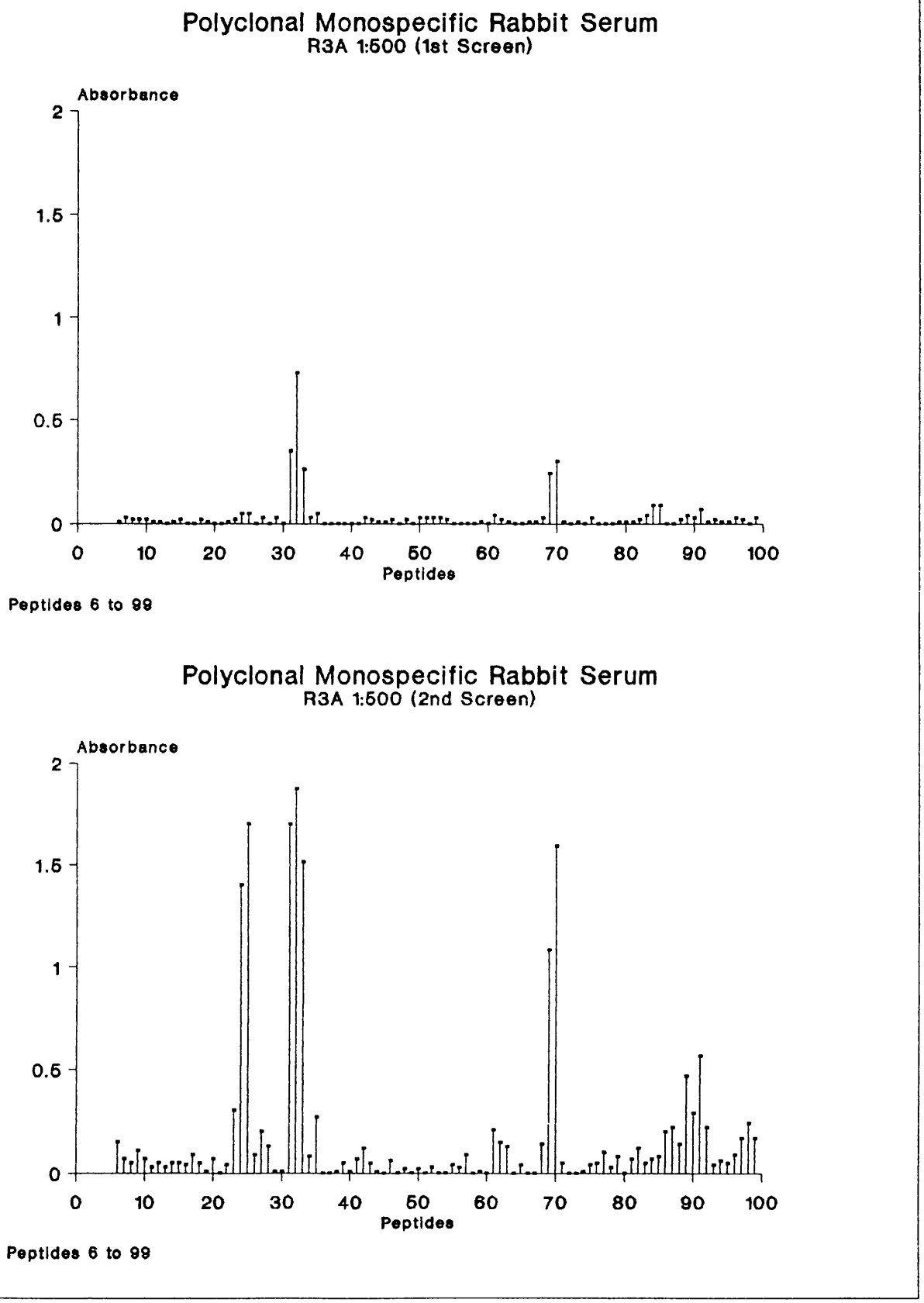


Fig 16. Reactivity of R3a antibodies with synthetic hexapeptides. 6-99

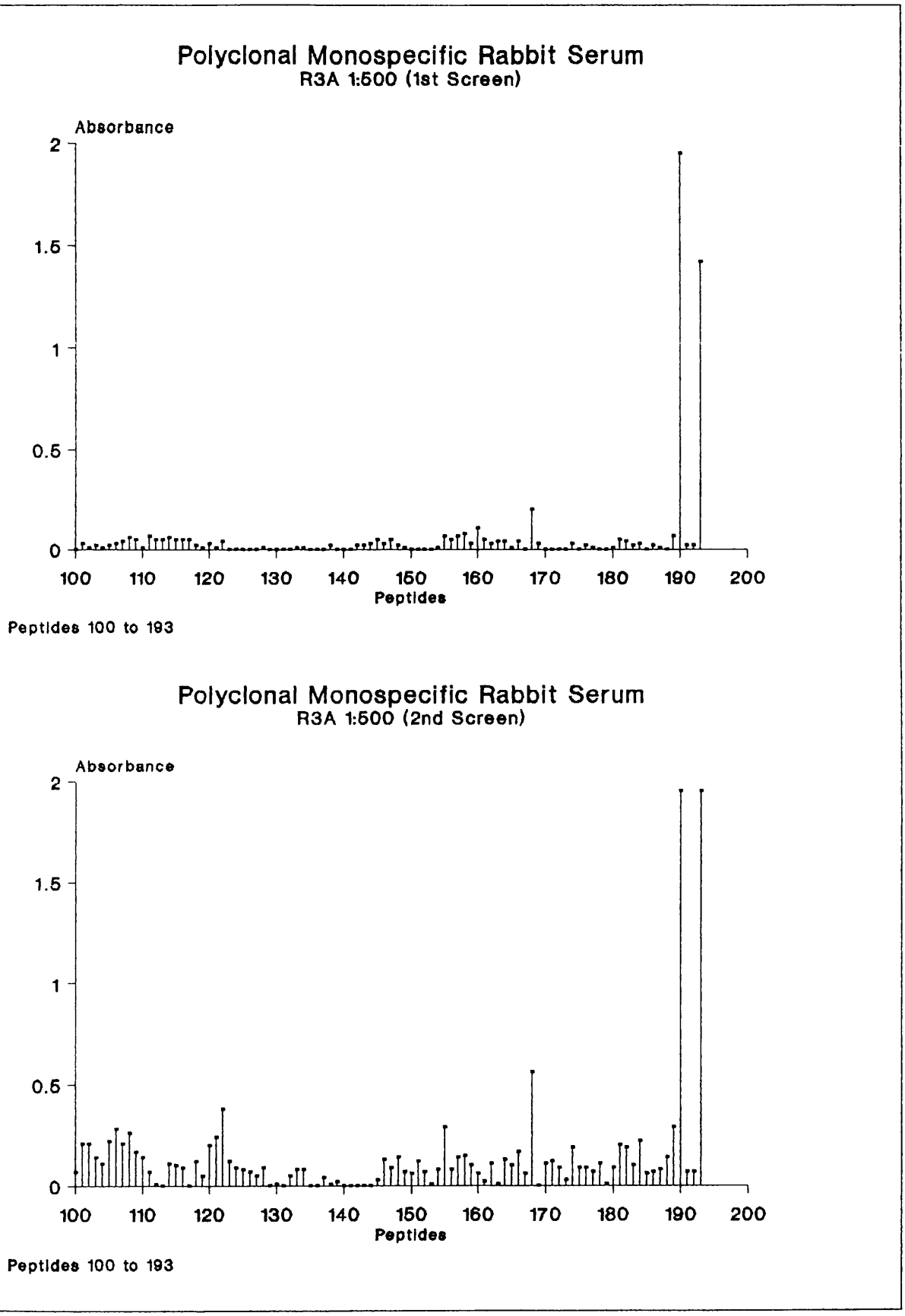


Fig 17. Reactivity of R3a antibodies with synthetic hexapeptides. 100-193

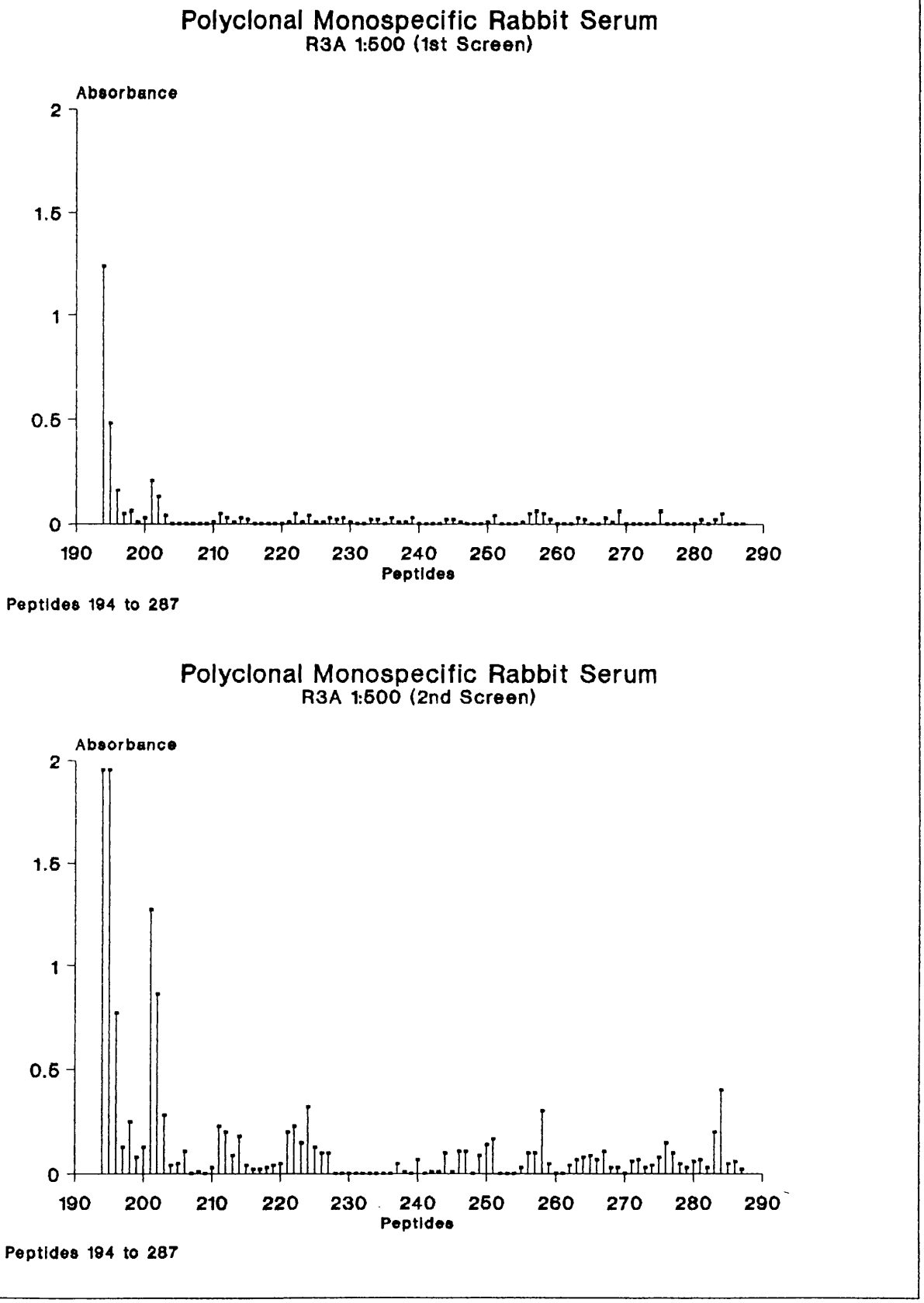


Fig 18. Reactivity of R3a antibodies with synthetic hexapeptides. 194-287

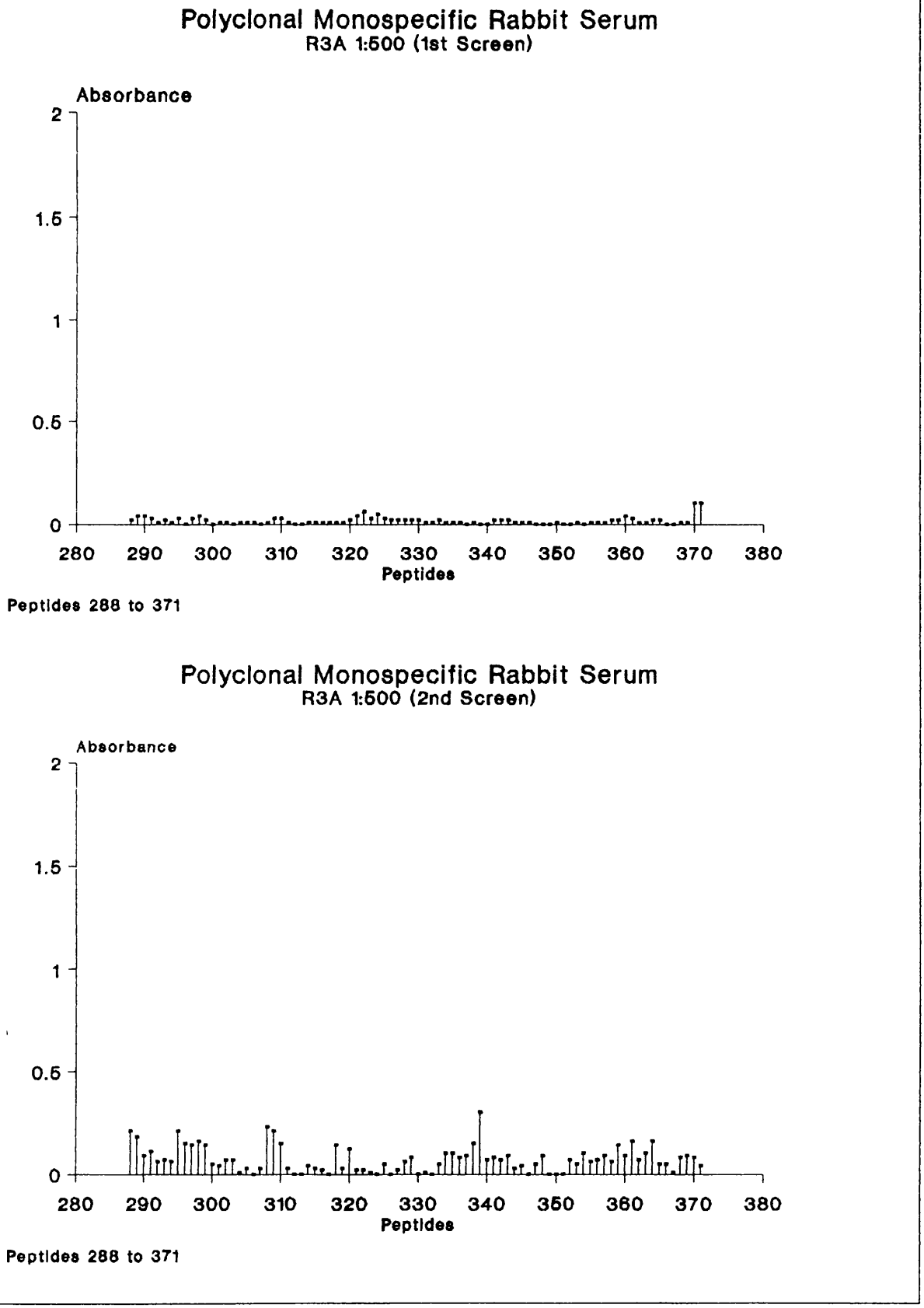


Fig 19. Reactivity of R3a antibodies with synthetic hexapeptides. 288-371

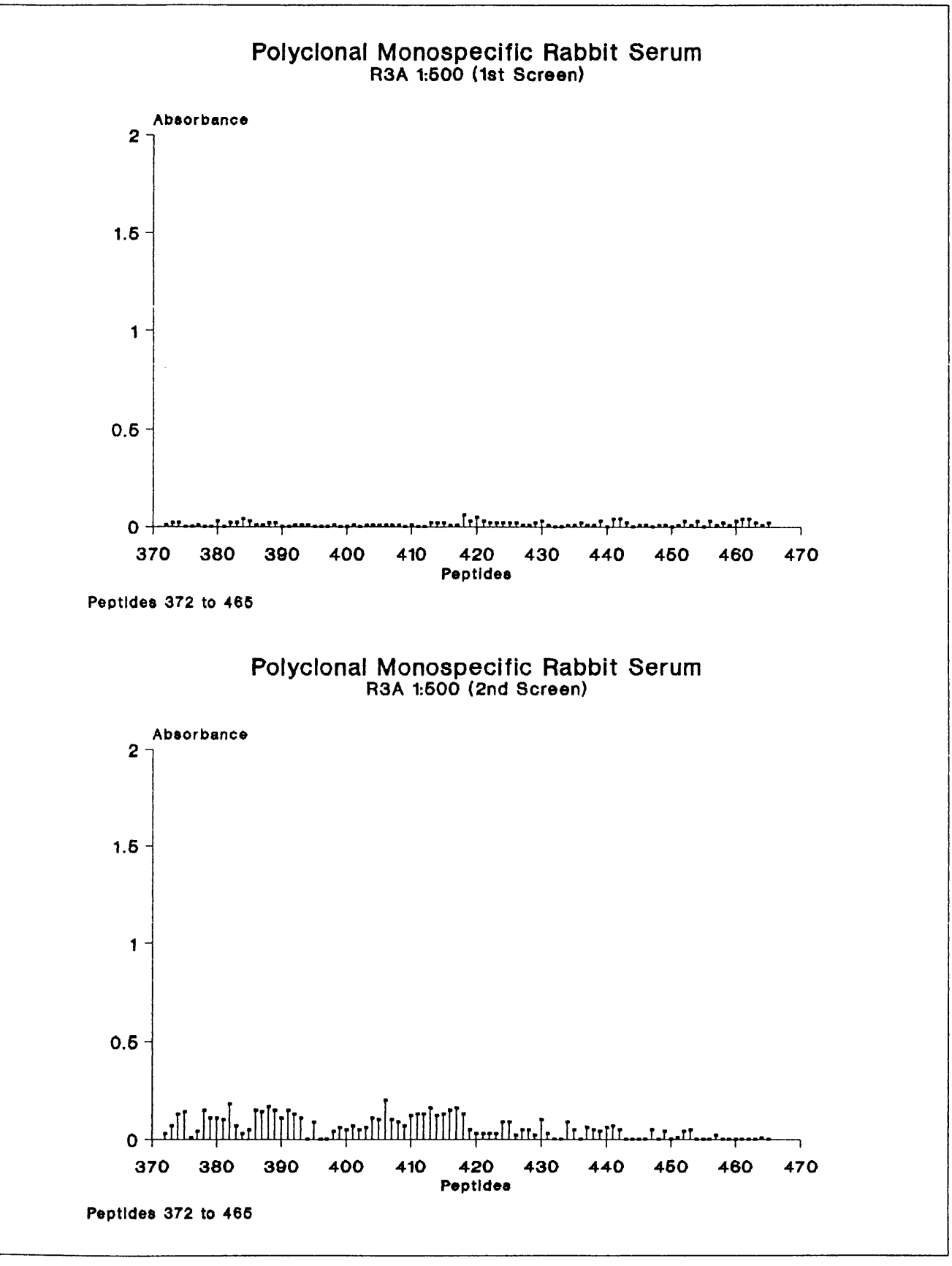
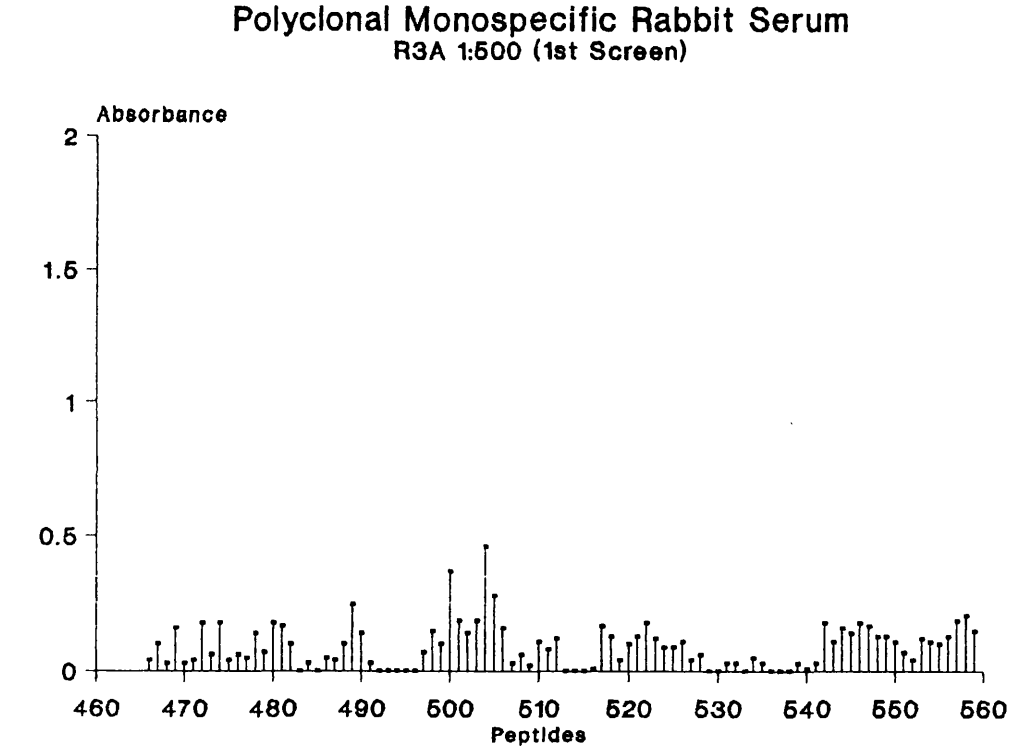


Fig 20. Reactivity of R3a antibodies with synthetic hexapeptides. 372-465



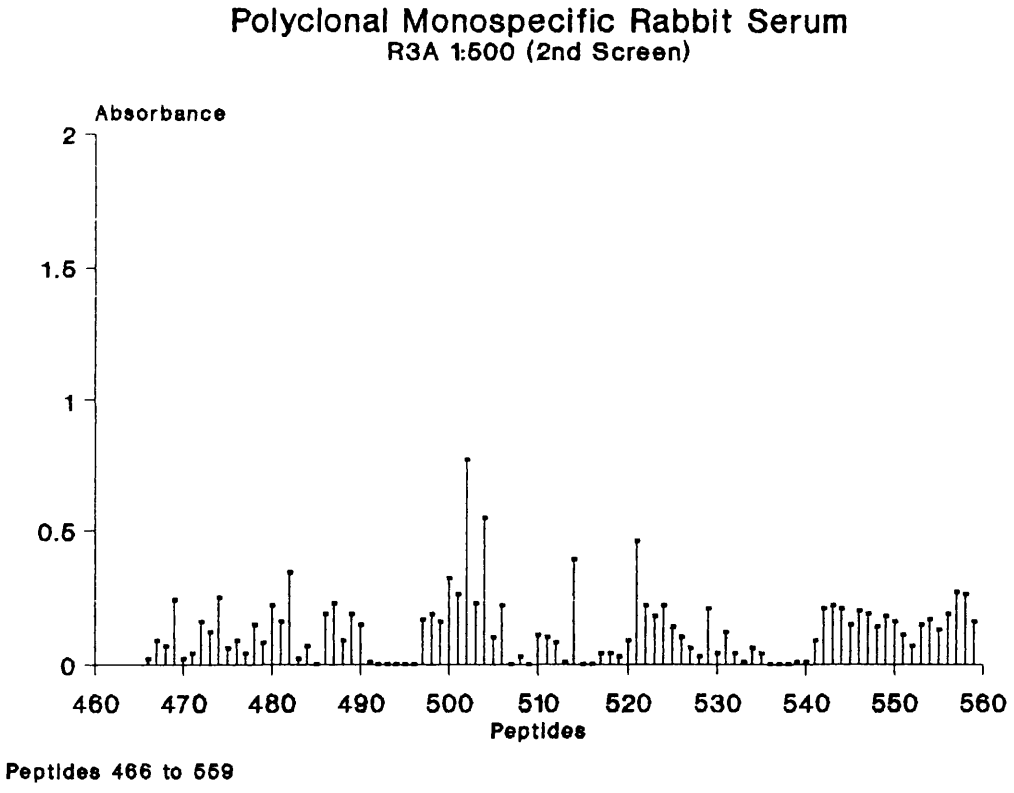
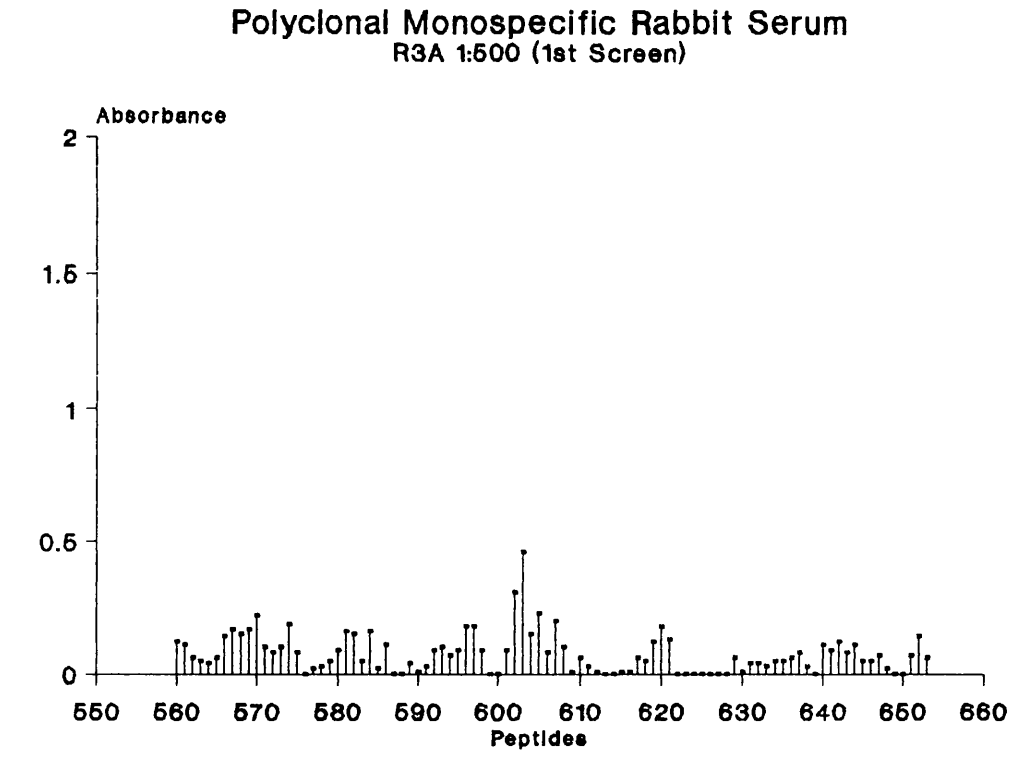


Fig 21. Reactivity of R3a antibodies with synthetic hexapeptides. 466-559



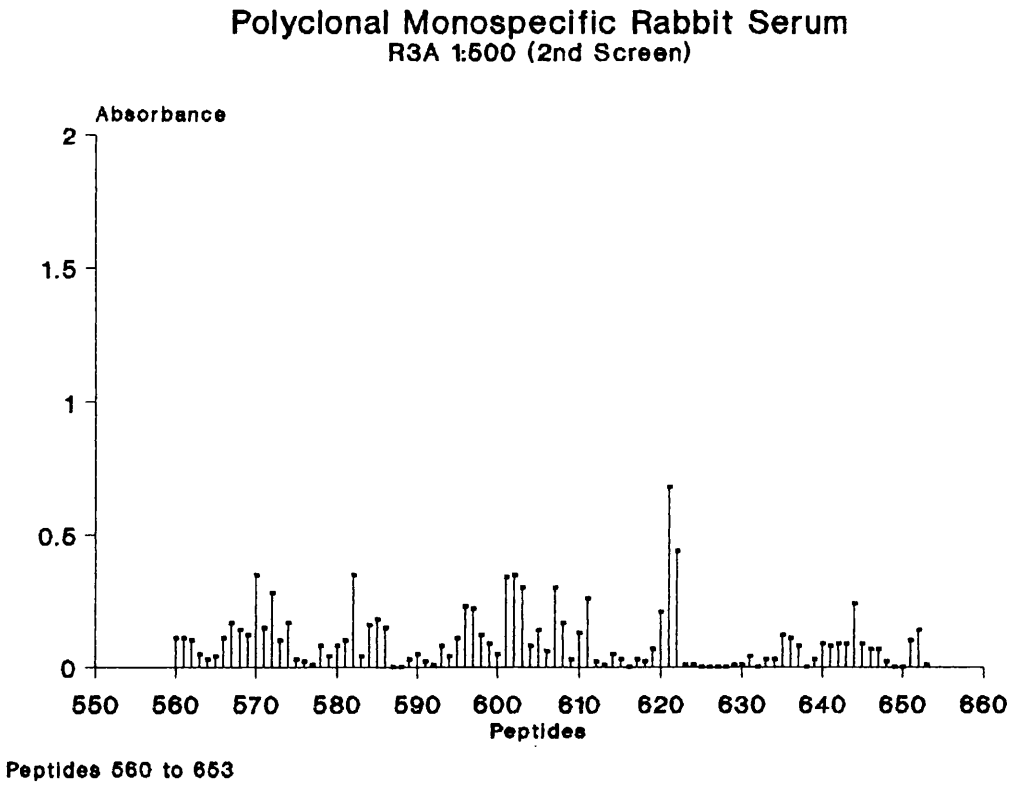


Fig 22. Reactivity of R3a antibodies with synthetic hexapeptides. 560-653

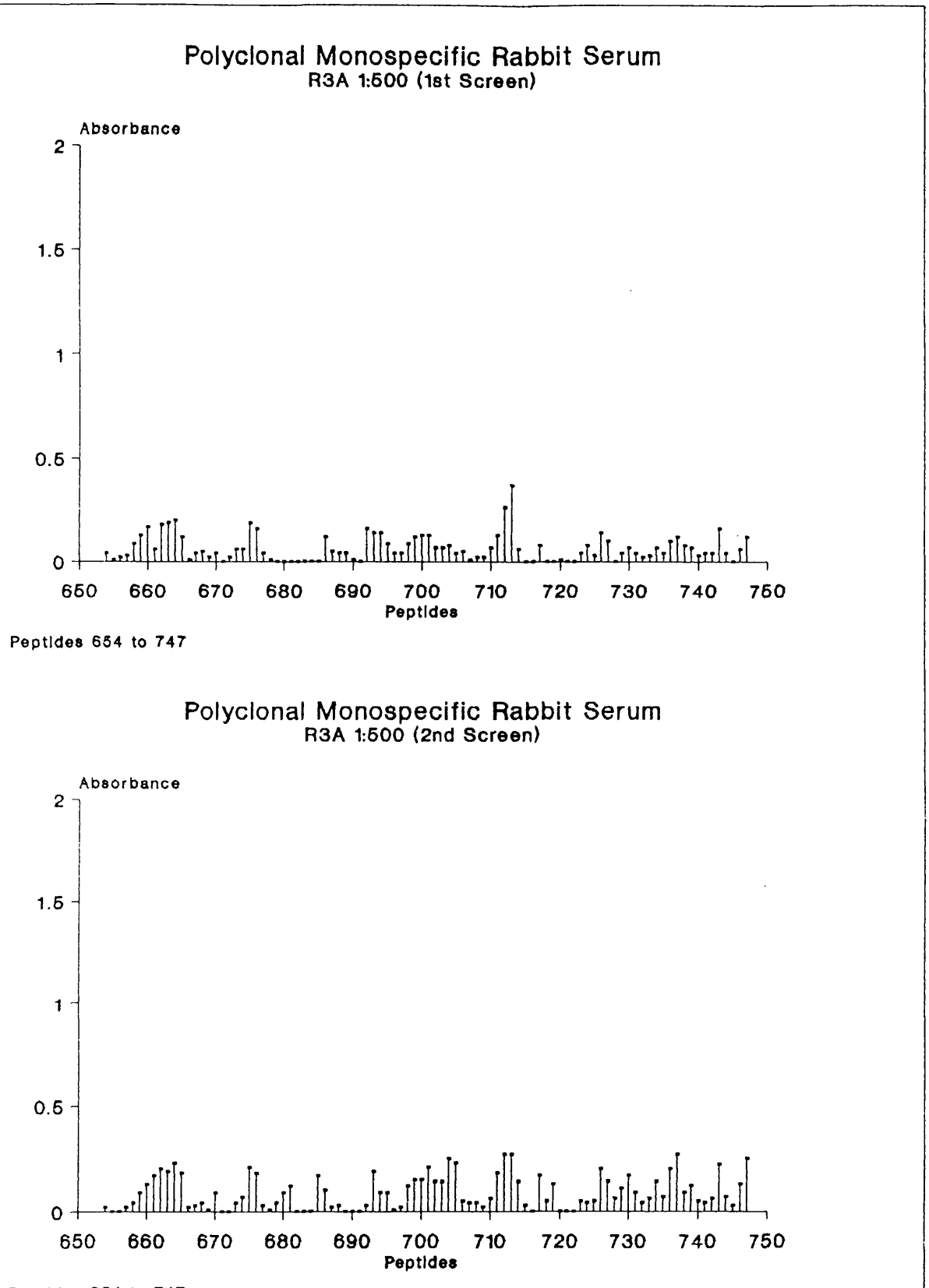


Fig 23. Reactivity of R3a antibodies with synthetic hexapeptides. 645-747

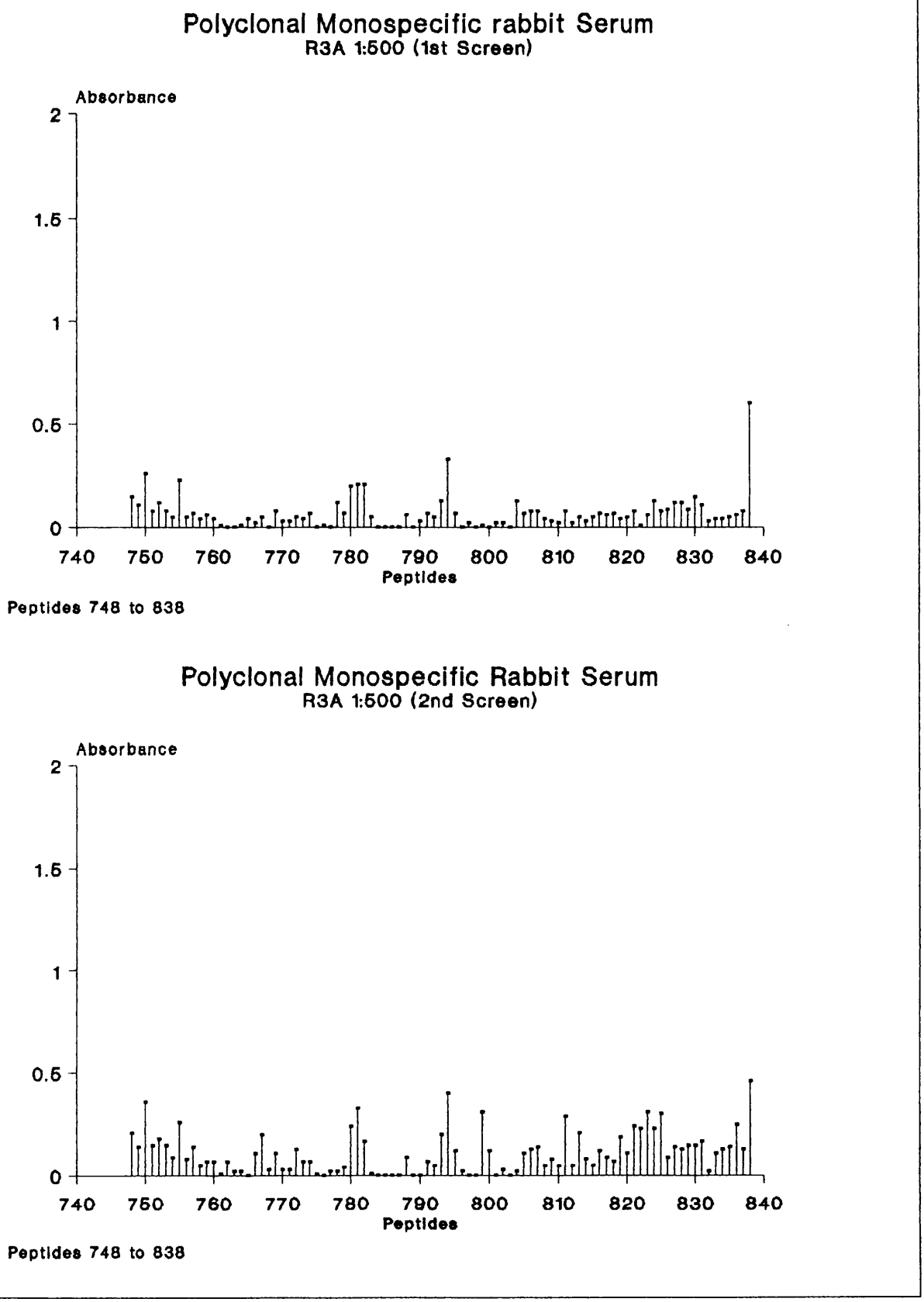


Fig 24. Reactivity of R3a antibodies with synthetic hexapeptides. 748-838.

4.4 Mapping with LP-11

Screening of the gH peptide series was carried out using monoclonal antibody LP-11 ascitic fluid at a dilution of 1:250. Assays were performed in triplicate although the data presented in Figs. 25 to 33 show duplicate assays only. The peptide recognition pattern of the third assay proved consistent with the first two assays. The parameters for each figure are similar to those previously described in section 4.3.

Outstanding antibody reactivity occurred with the following peptides:

GQDLDR 297
RVGDPA 302

DENPPG 308
ENPPGA 309

TRADNG 333

EGTNYA 353
GTNYAQ 354

FSGDAG 369

It should be noted that background binding levels were also elevated in the vicinity of the most reactive peptides. (Fig 28)

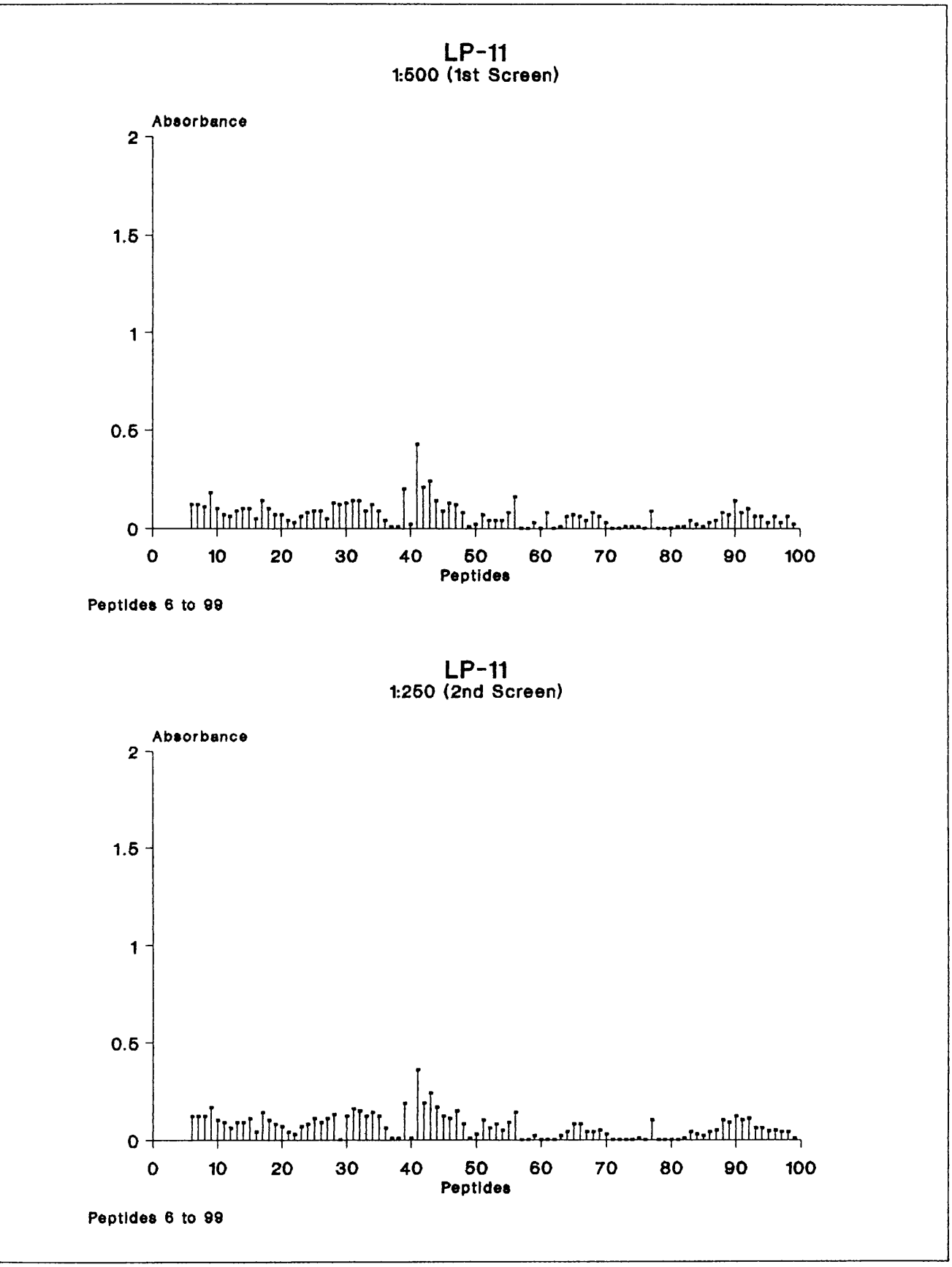


Fig 25. Reactivity of LP-11 antibody with synthetic hexapeptides. 6-99

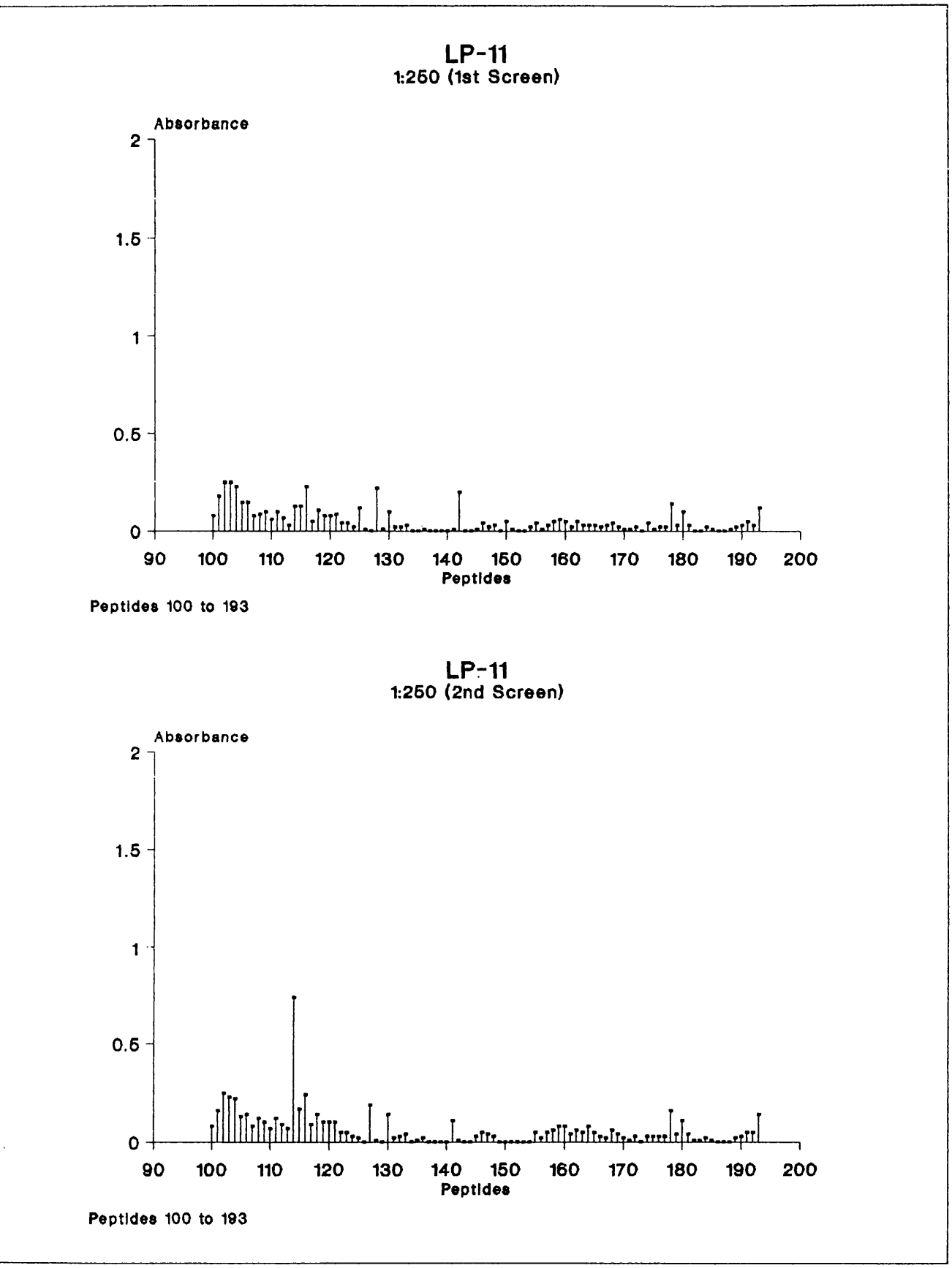


Fig 26. Reacitivity of LP-11 antibody with synthetic hexapeptides. 100-193

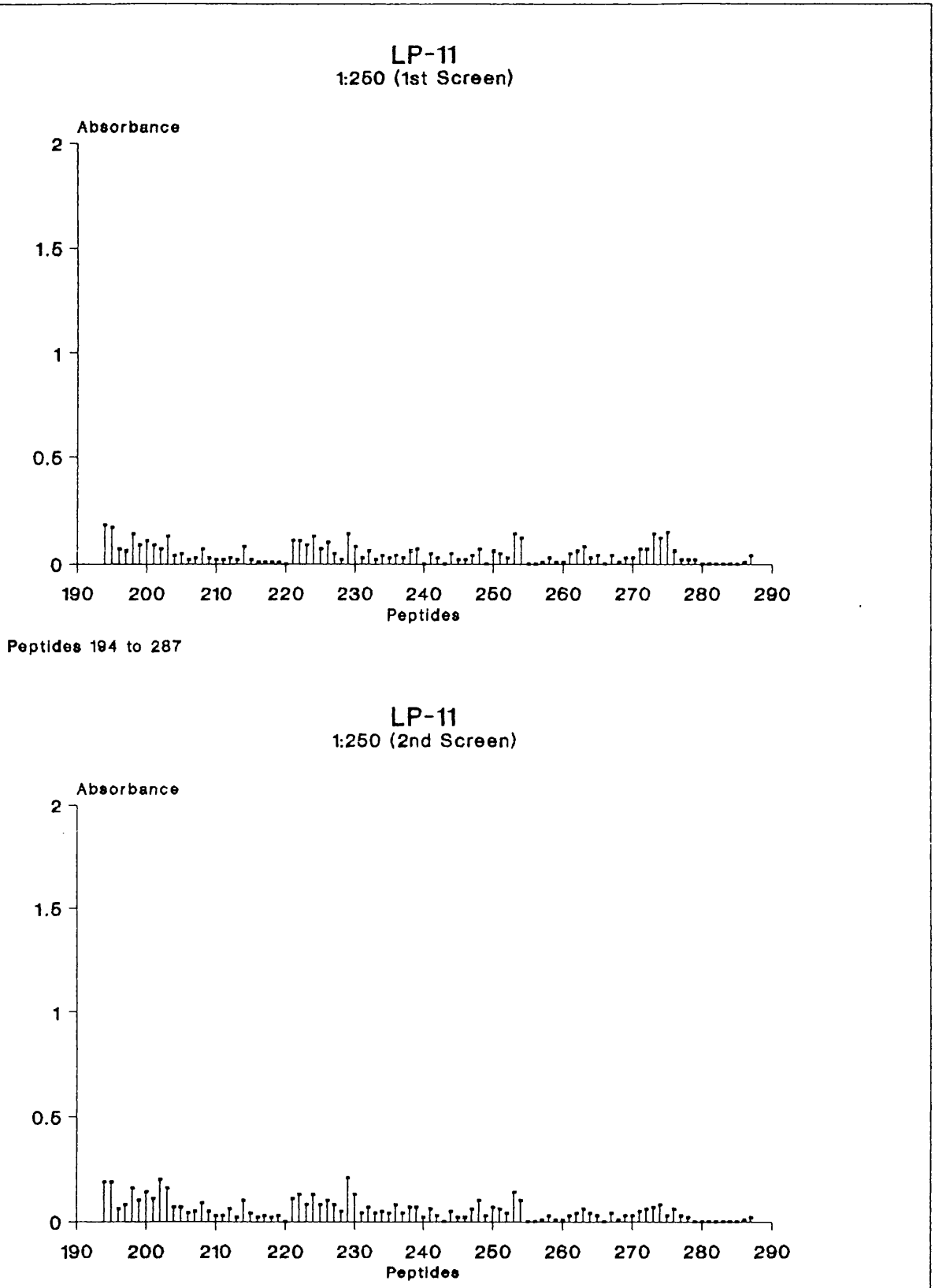
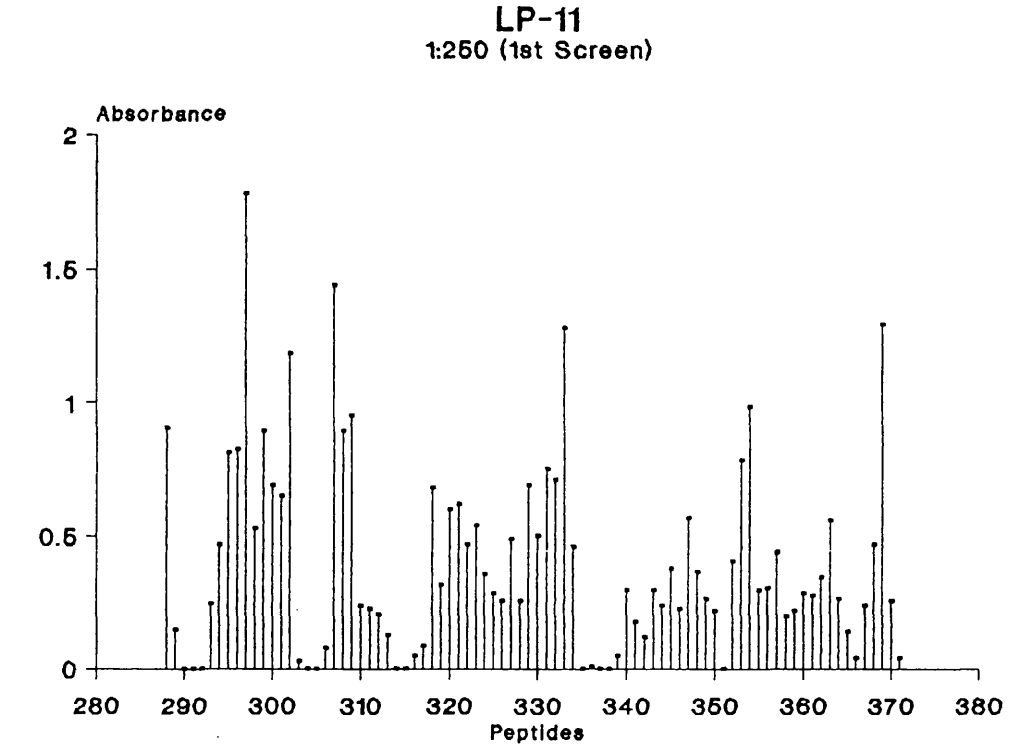


Fig 27. Reactivity of LP-11 antibody with synthetic hexapeptides. 194-287



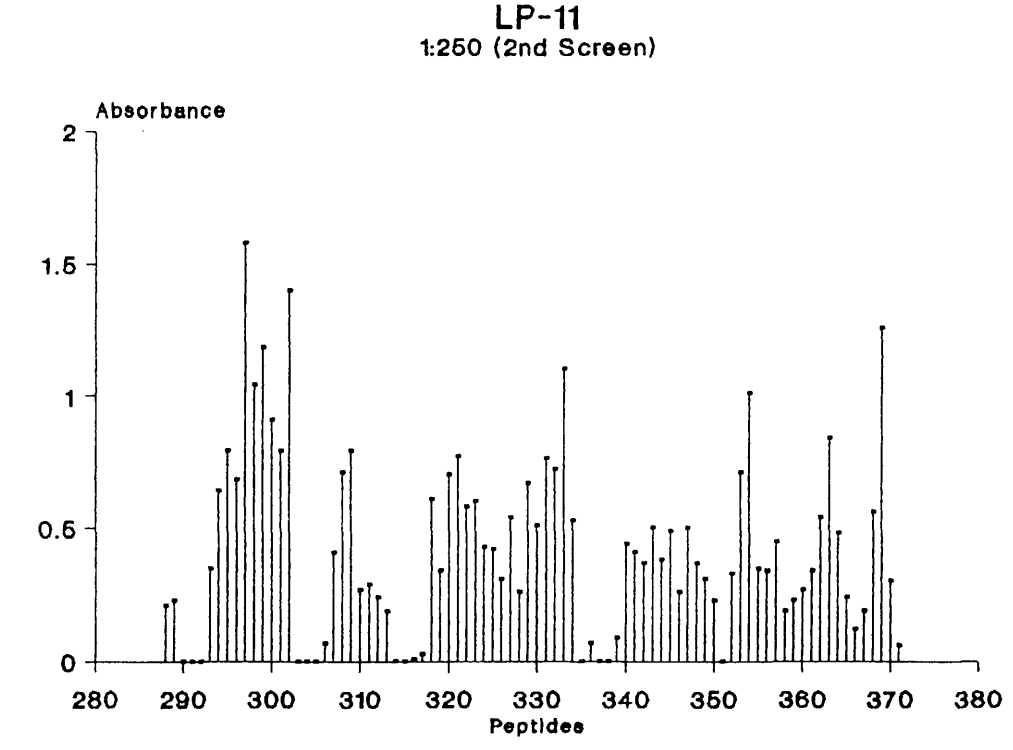


Fig 28. Reactivity of LP-11 antibody with synthetic hexapeptides. 288-371

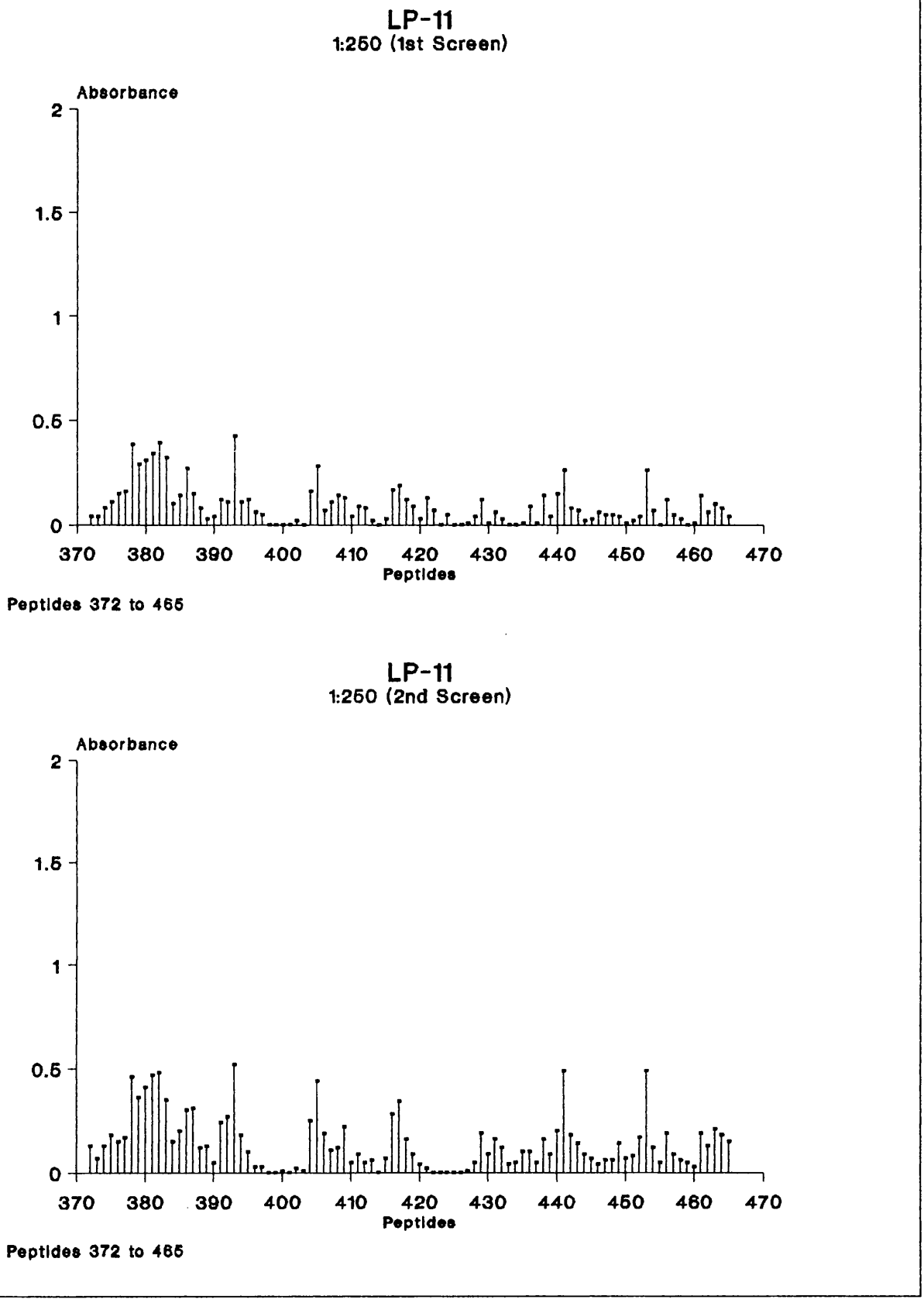
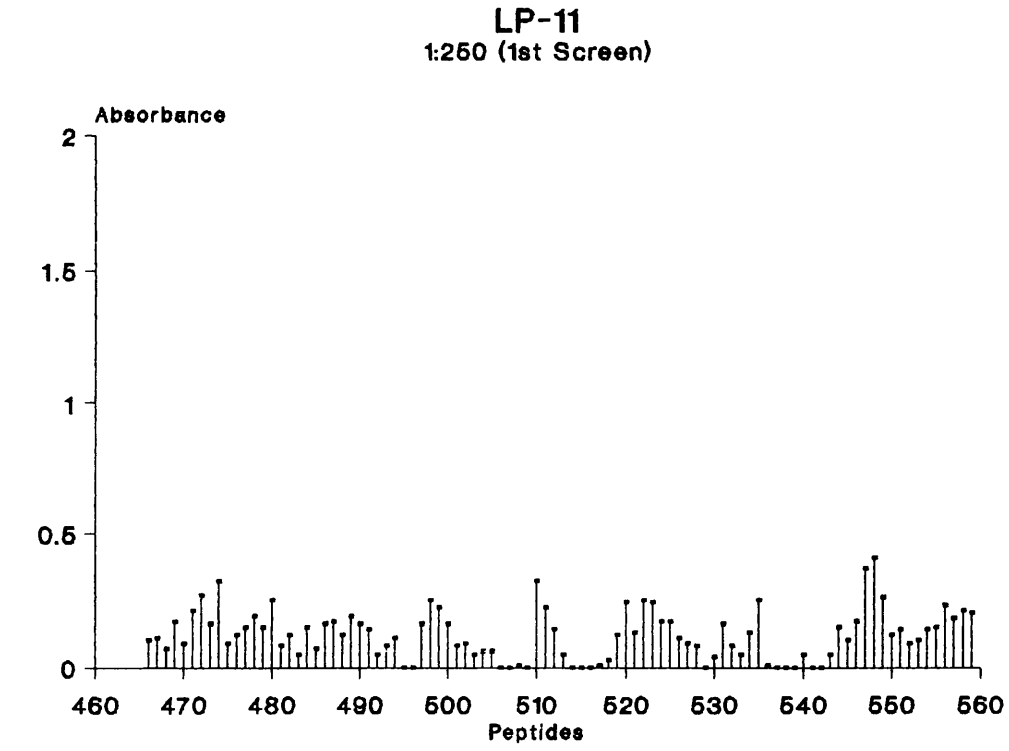


Fig 29. Reactivity of LP-11 antibody with synthetic hexapeptides. 371-465



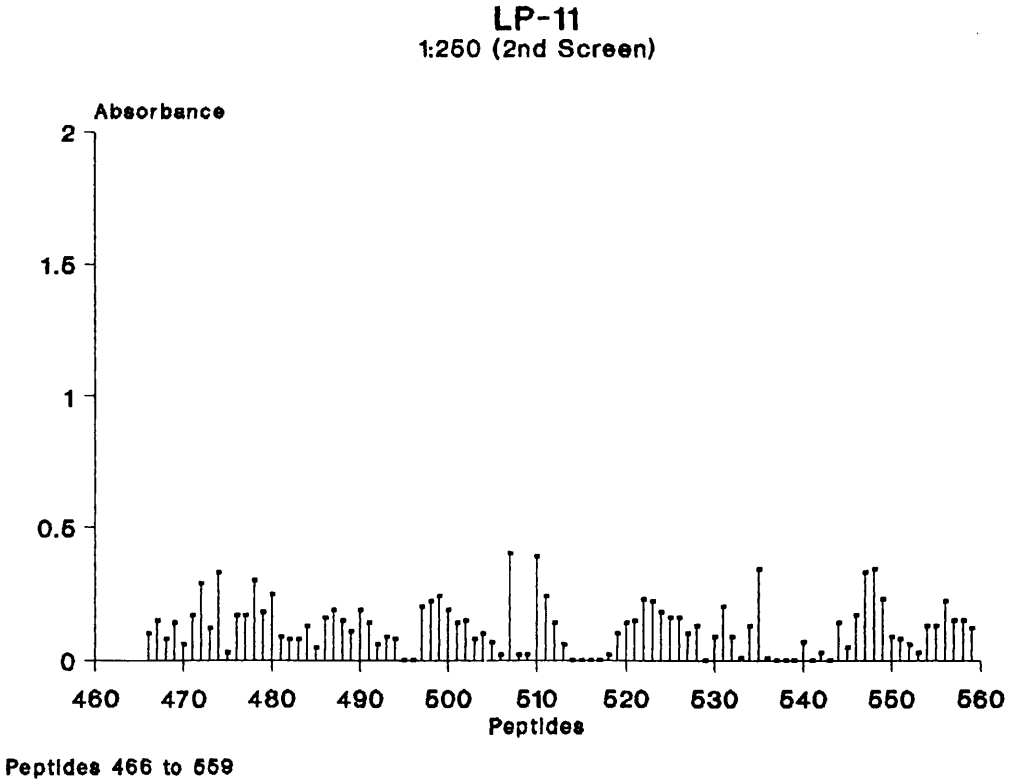


Fig 30. Reactivity of LP-11 antibody with synthetic hexapeptides. 466-559

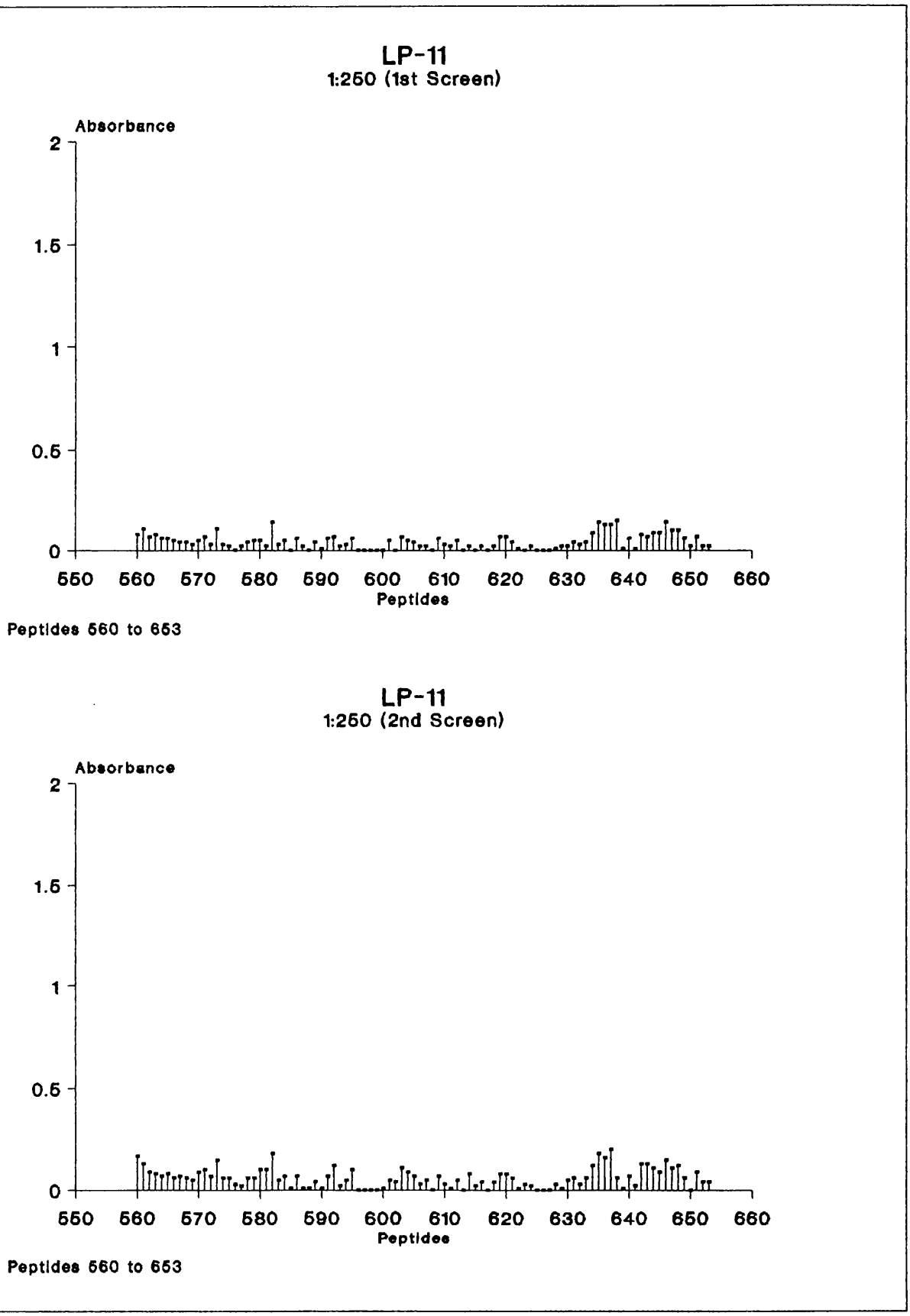


Fig 31. Reactivity of LP-11 antibody with synthetic hexapeptides. 560-653

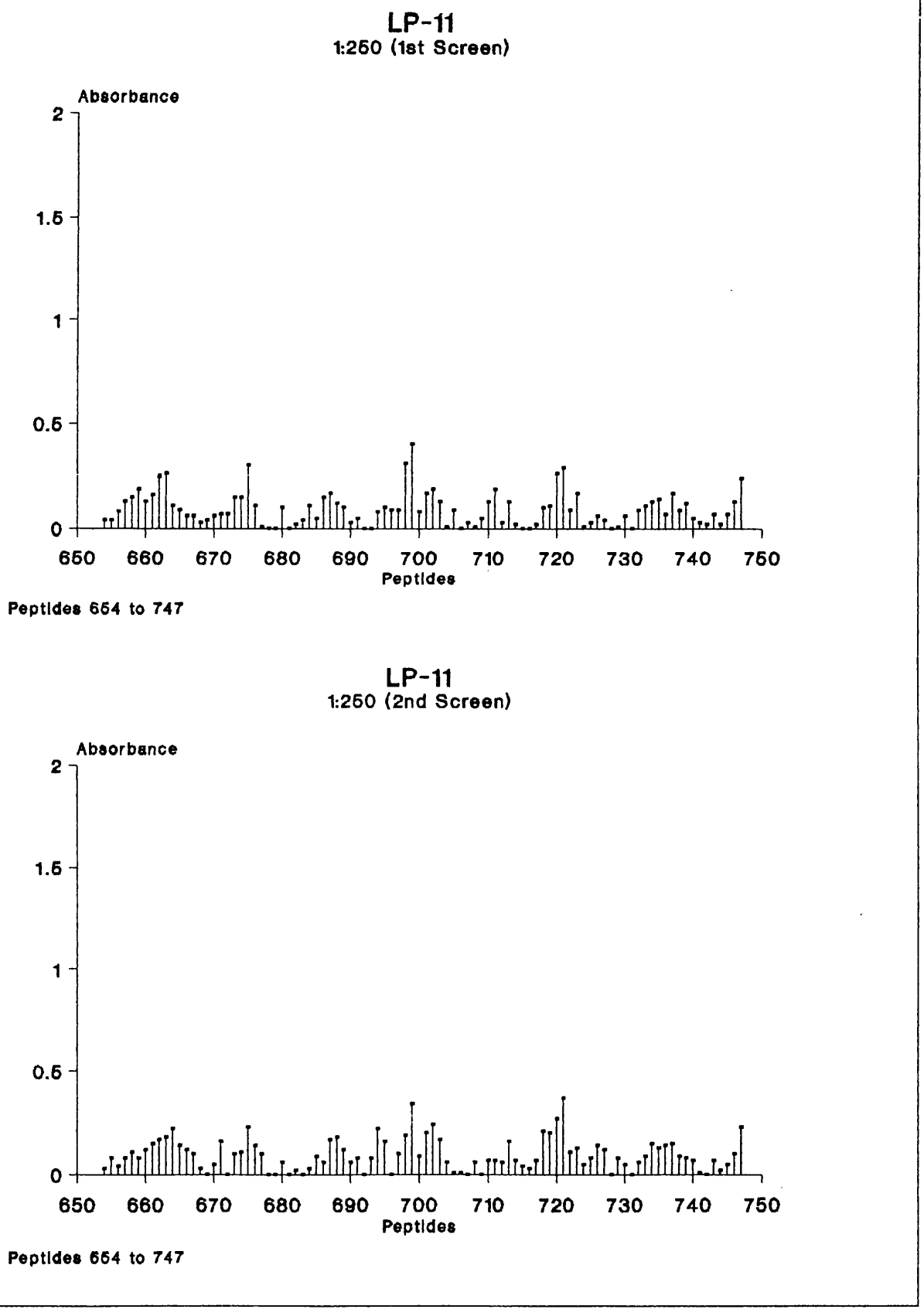
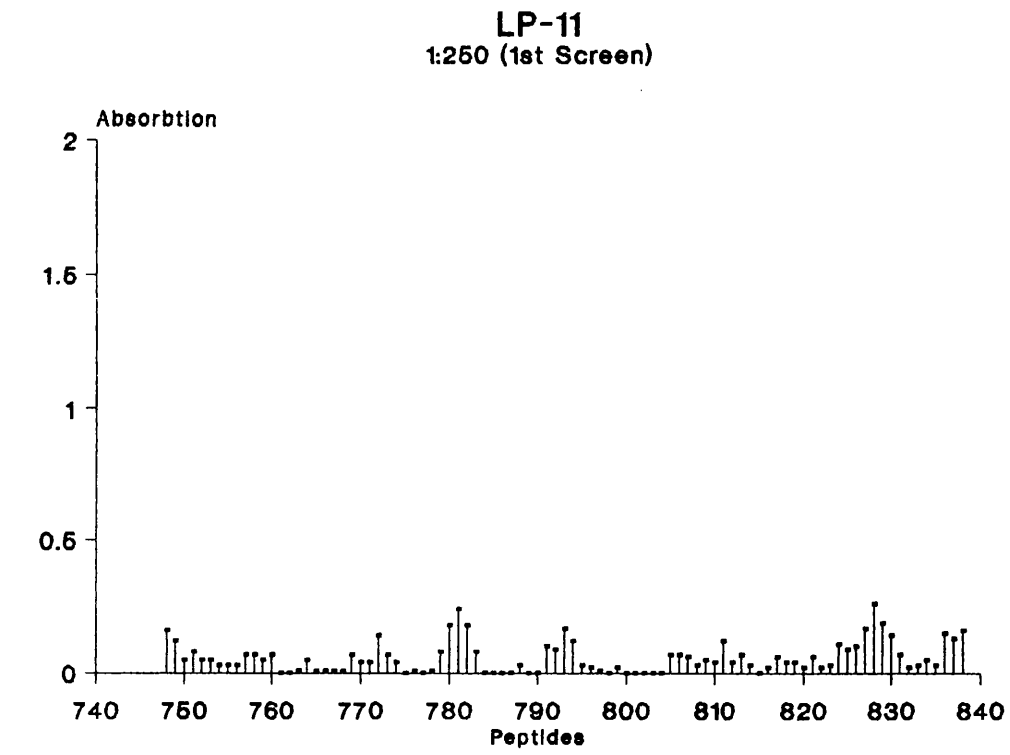


Fig 32. Reactivity of LP-11 antibody with synthetic hexapeptides. 654-747



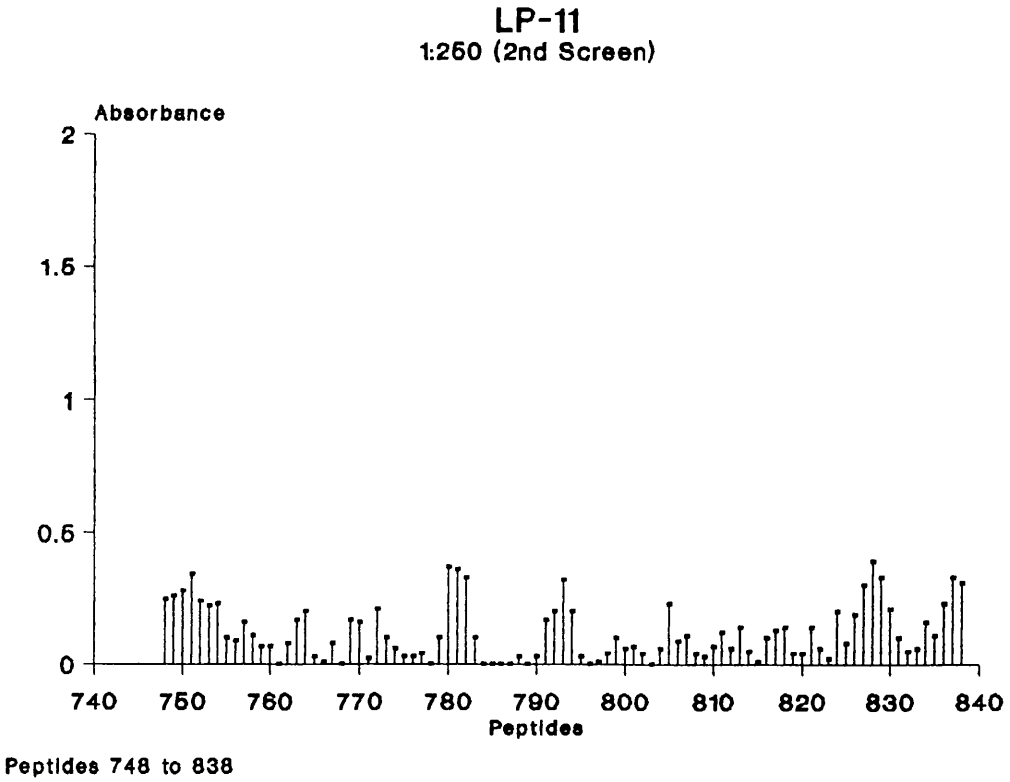


Fig 33. Reactivity of LP-11 antibody with synthetic hexapeptides. 748-838

4.5 Mapping with TrpE-gH fusion protein polyclonal antibodies

Mapping with TrpE-gH rabbit polyclonal serum at a dilution of 1:500 was carried out in duplicate as previously described (4.3), and the results are illustrated in Figs 34-42.

The following peptides were adjudged most reactive with TrpE-gH serum antibodies.

D T P P P R 196

F M G L V I 276

V P A G Q T 294
A G Q T L D 296
G Q T L D R 297

E N P P G A 309

S G D A G A 370

K T L F W L 582

A A S L R F 595

P S T A L L 780
S T A L L L 781
T A L L L F 782

I H L L A F 793
H L L A F D 794

G I L K V L 827
I L K V L R 828
L K V L R T 829

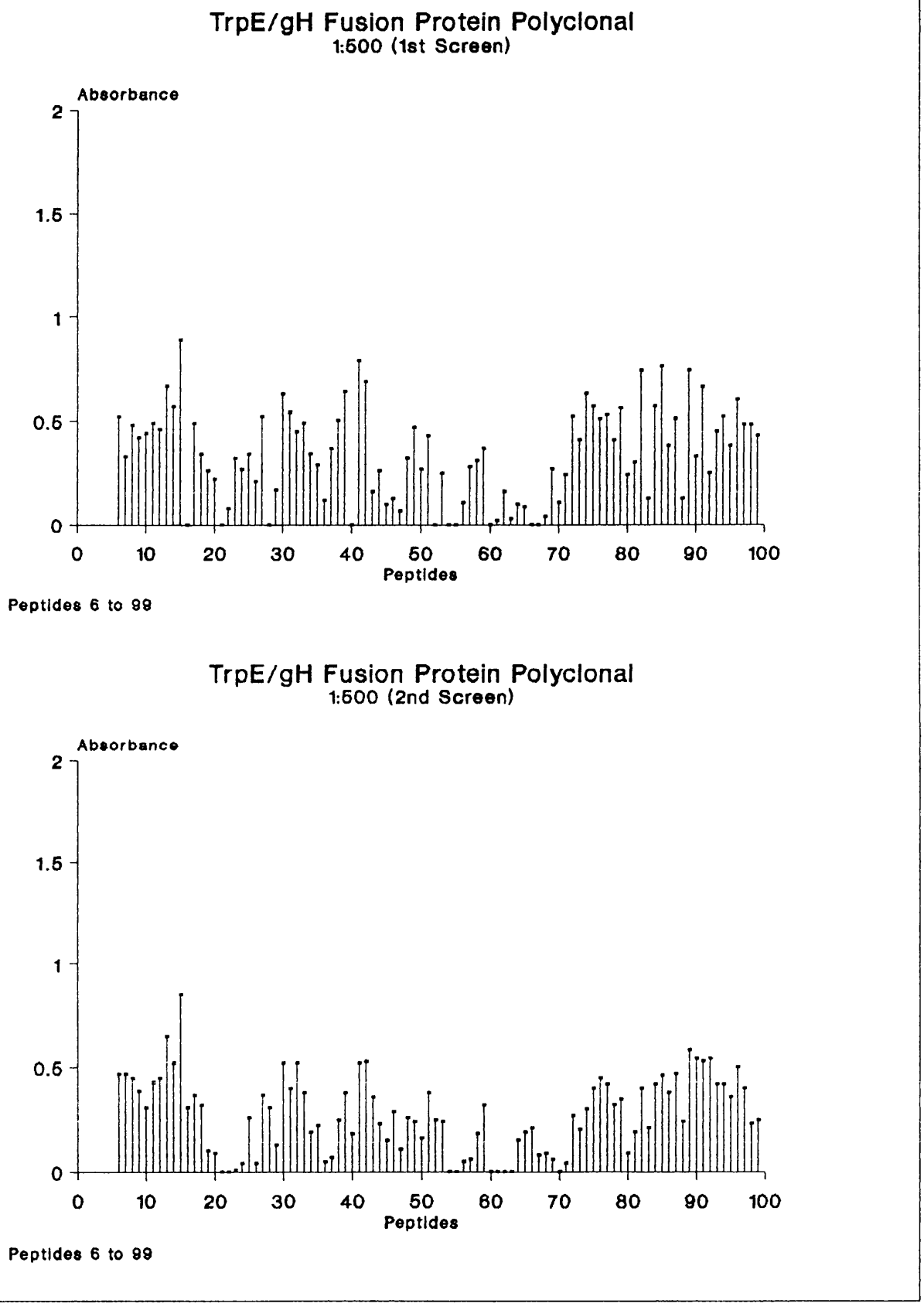


Fig 34. Reactivity of TrpE/gH-1 antibodies with synthetic hexapeptides. 6-99

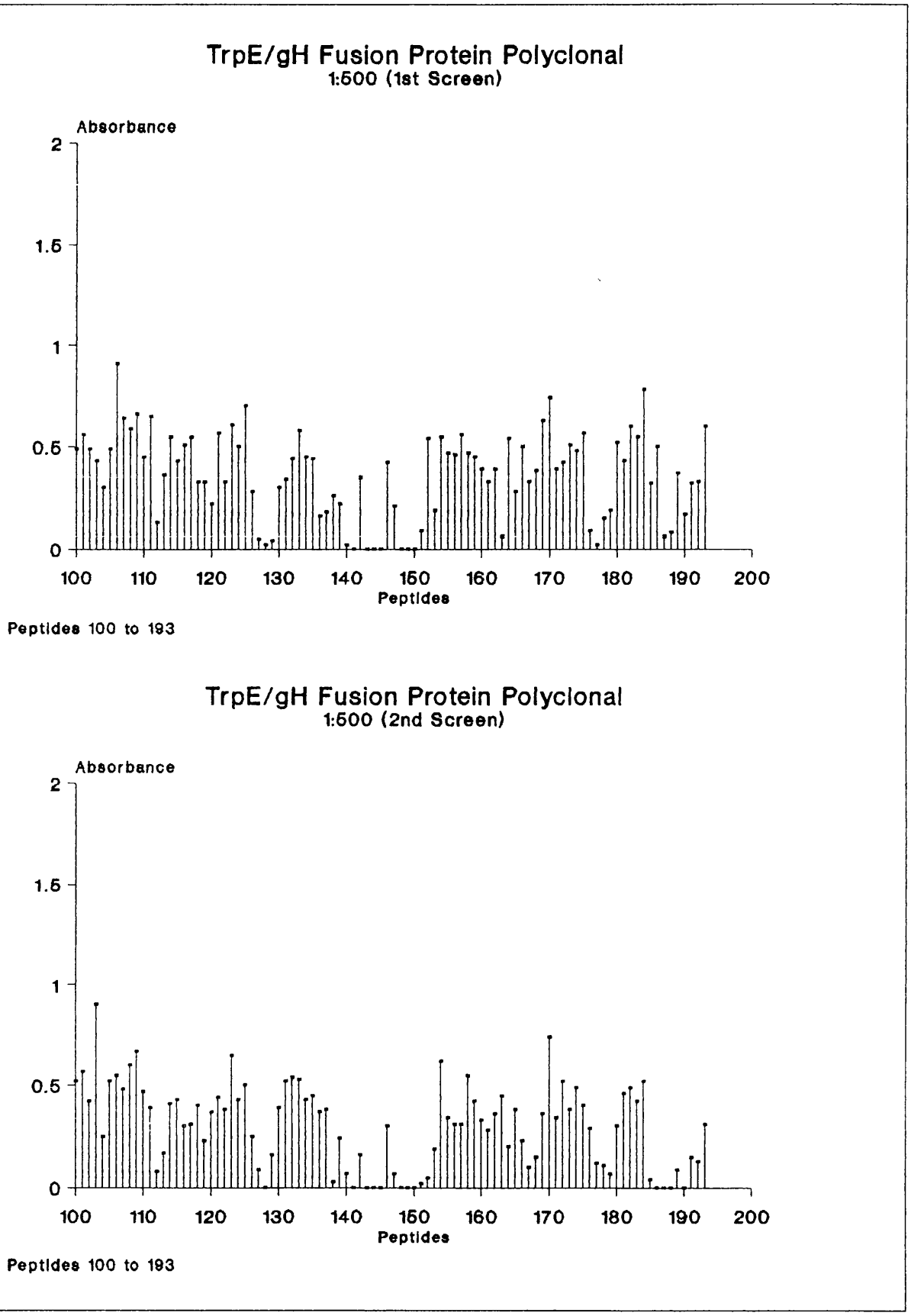
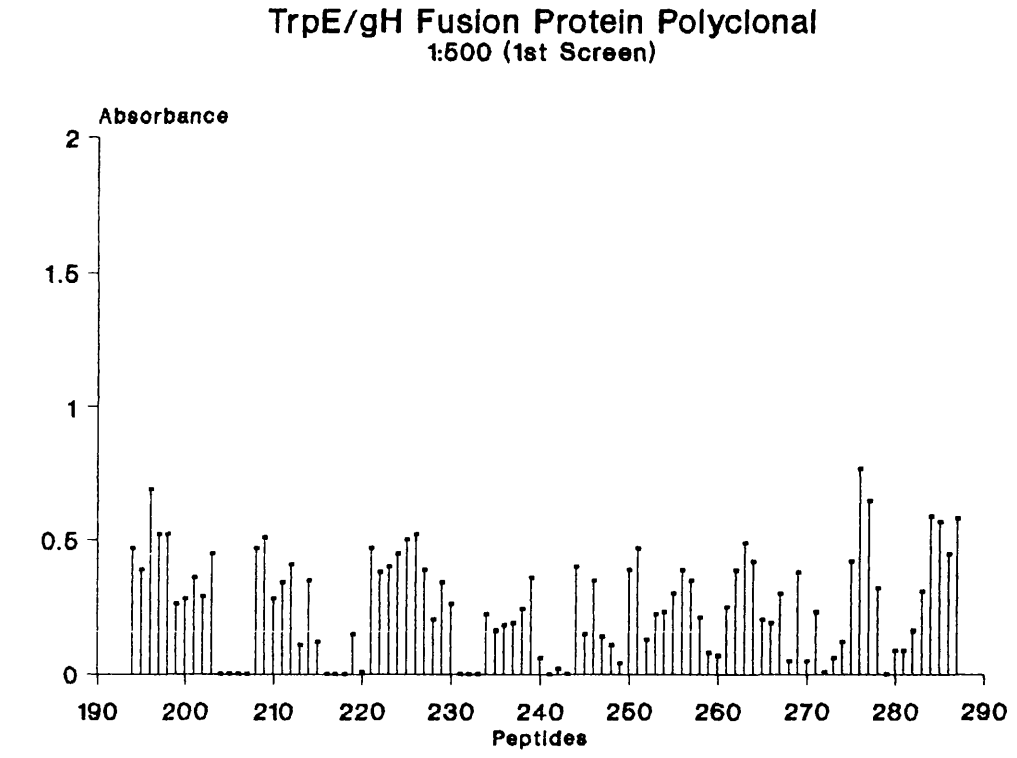


Fig 35. Reactivity of TrpE/gH-1 antibodies with synthetic hexapeptides. 100-193



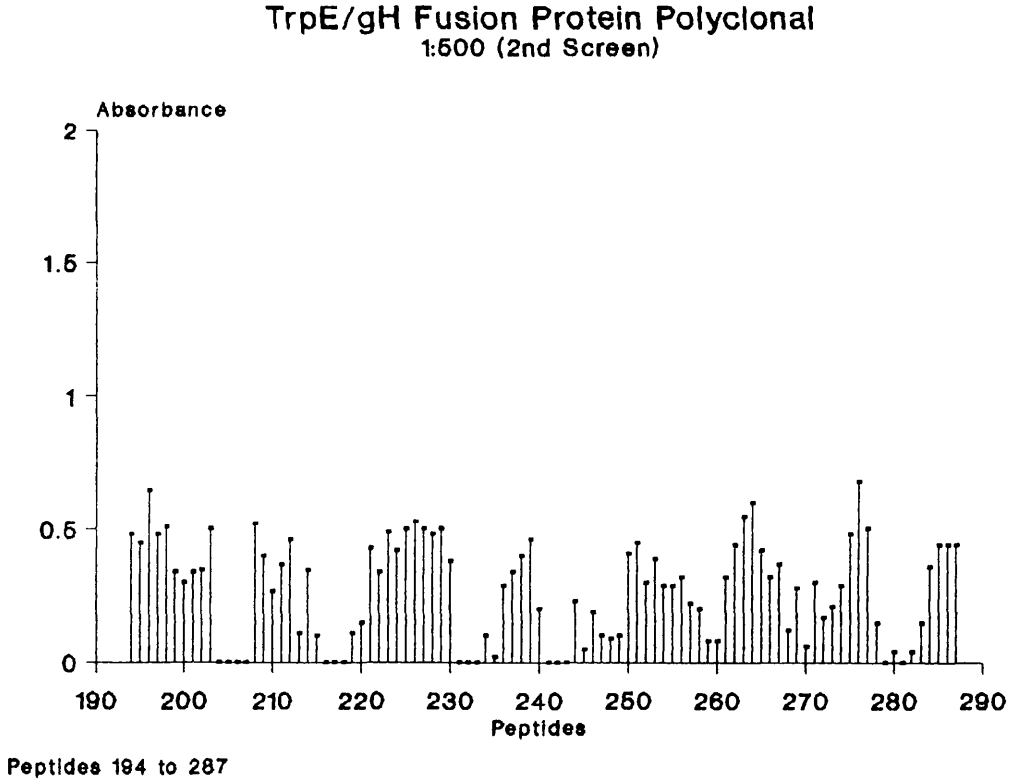


Fig 36. Reactivity of TrpE/gH-1 antibodies with synthetic hexapeptides. 194-287

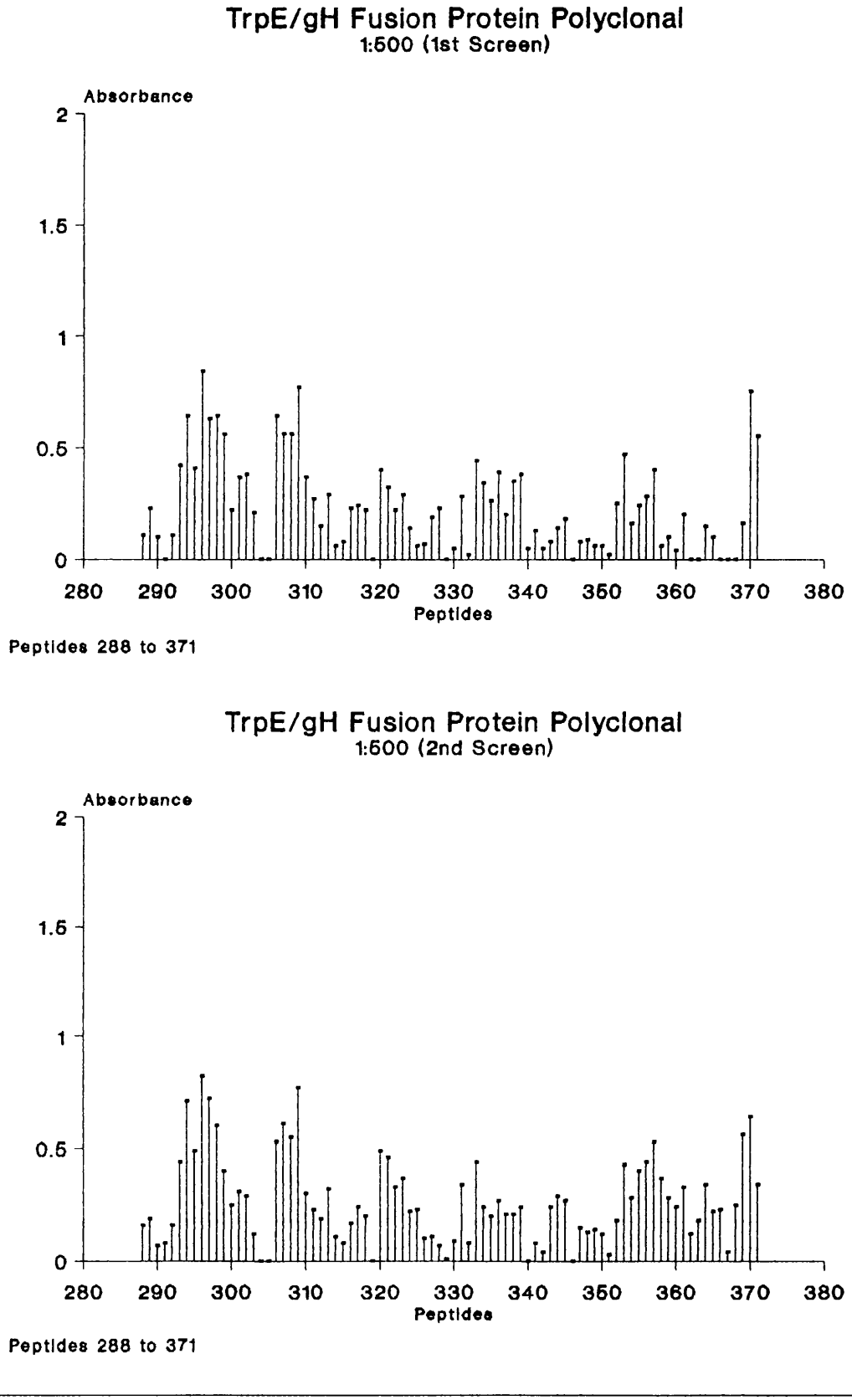


Fig 37. Reactivity of TrpE/gH-1 antibodies with synthetic hexapeptides. 288-371

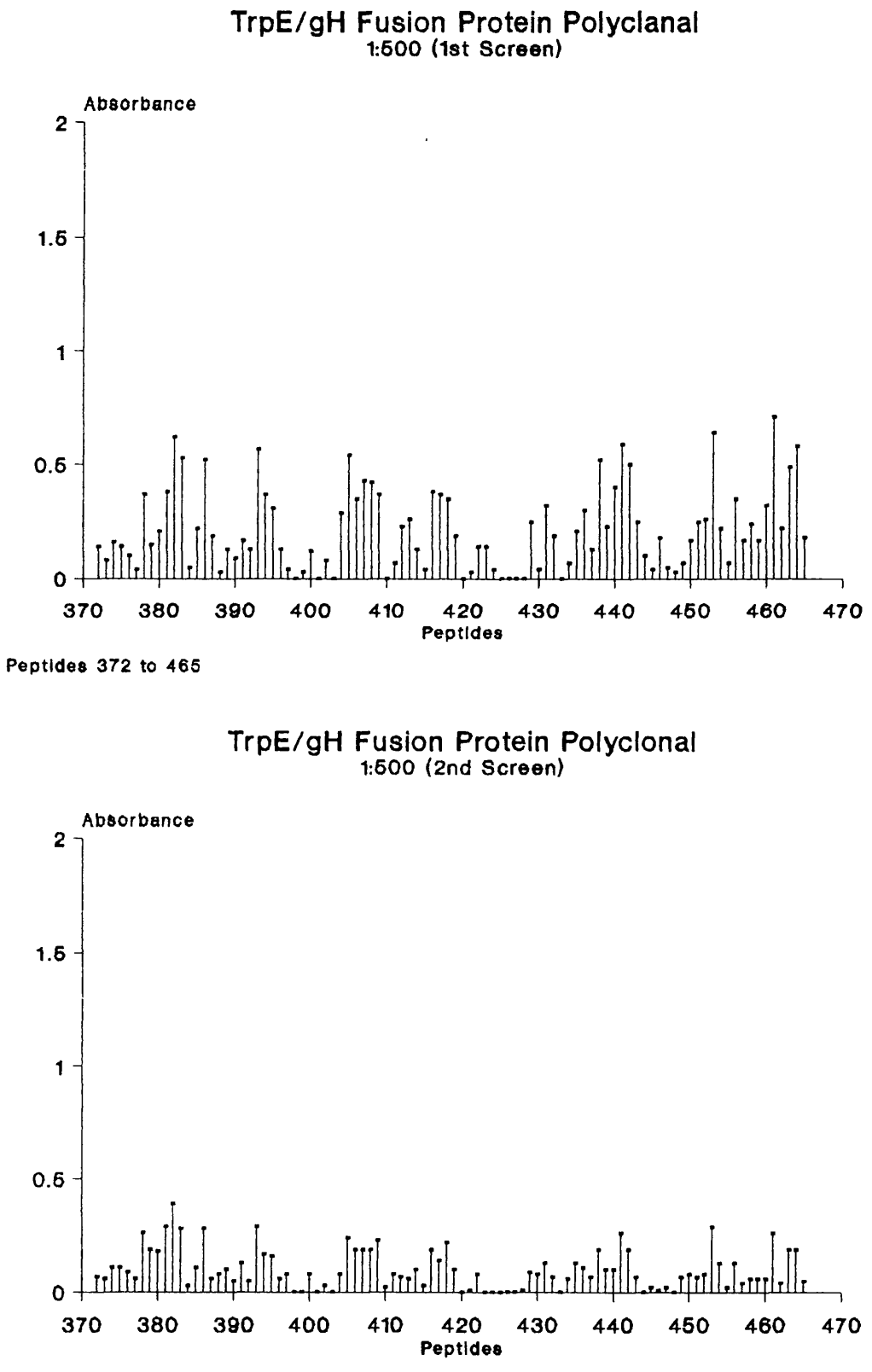


Fig 38. Reactivity of TrpE/gH-1 antibodies with synthetic hexapeptides. 372-465

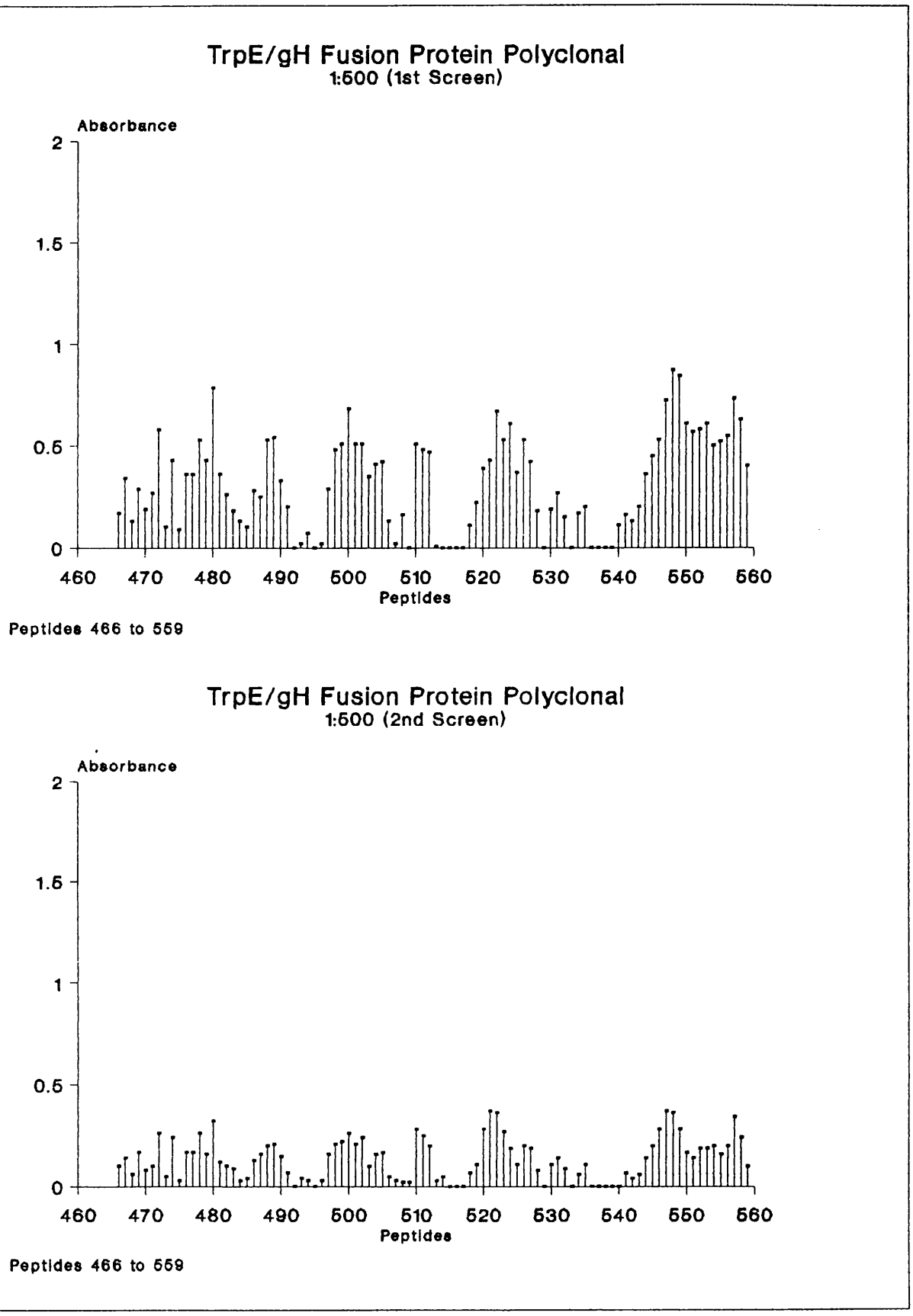


Fig 39. Reactivity of TrpE/gH-1 antibodies with synthetic hexapeptides. 466-559

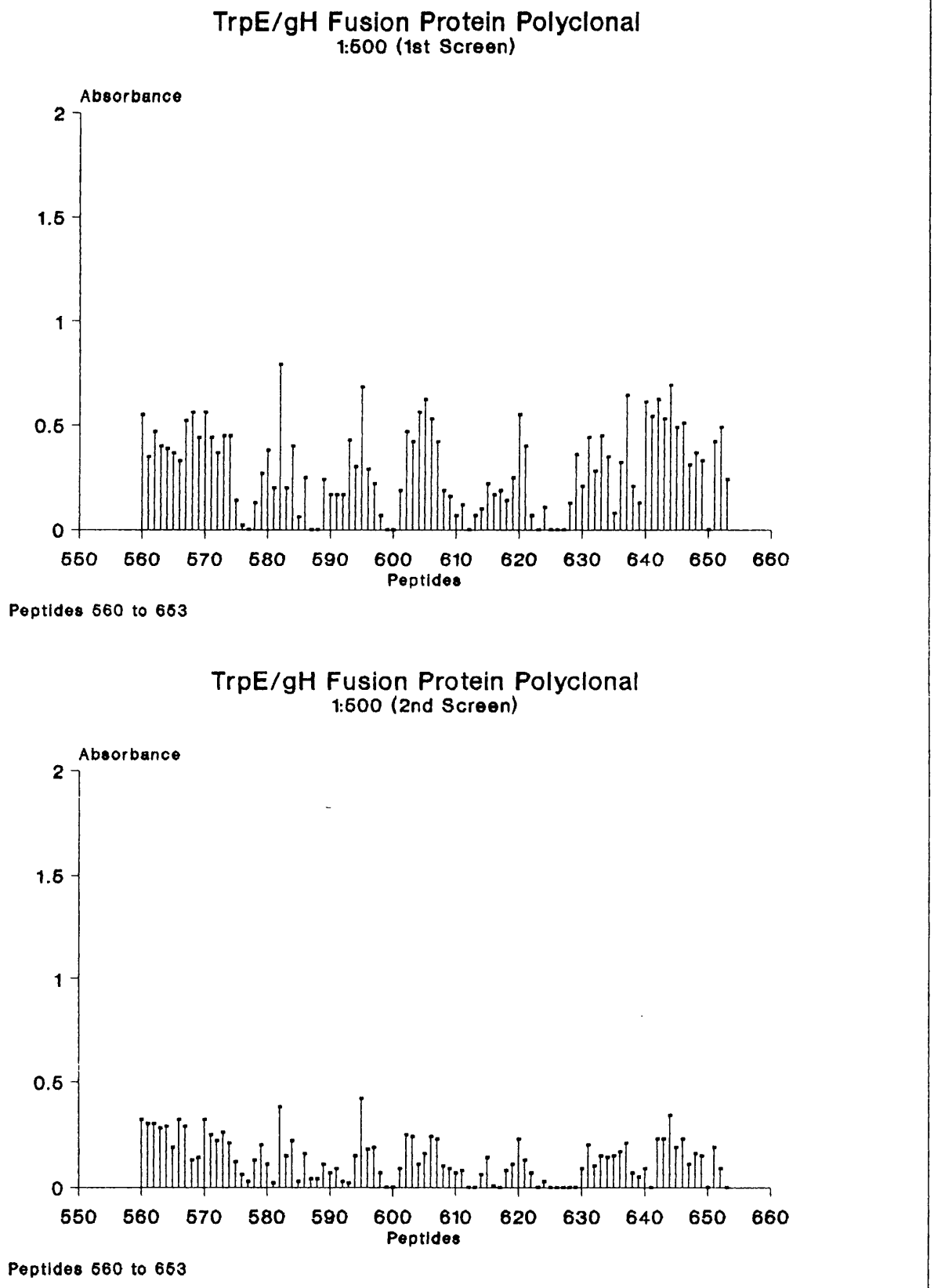


Fig 40. Reactivity of TrpE/gH-1 antibodies with synthetic hexapeptides. 560-653

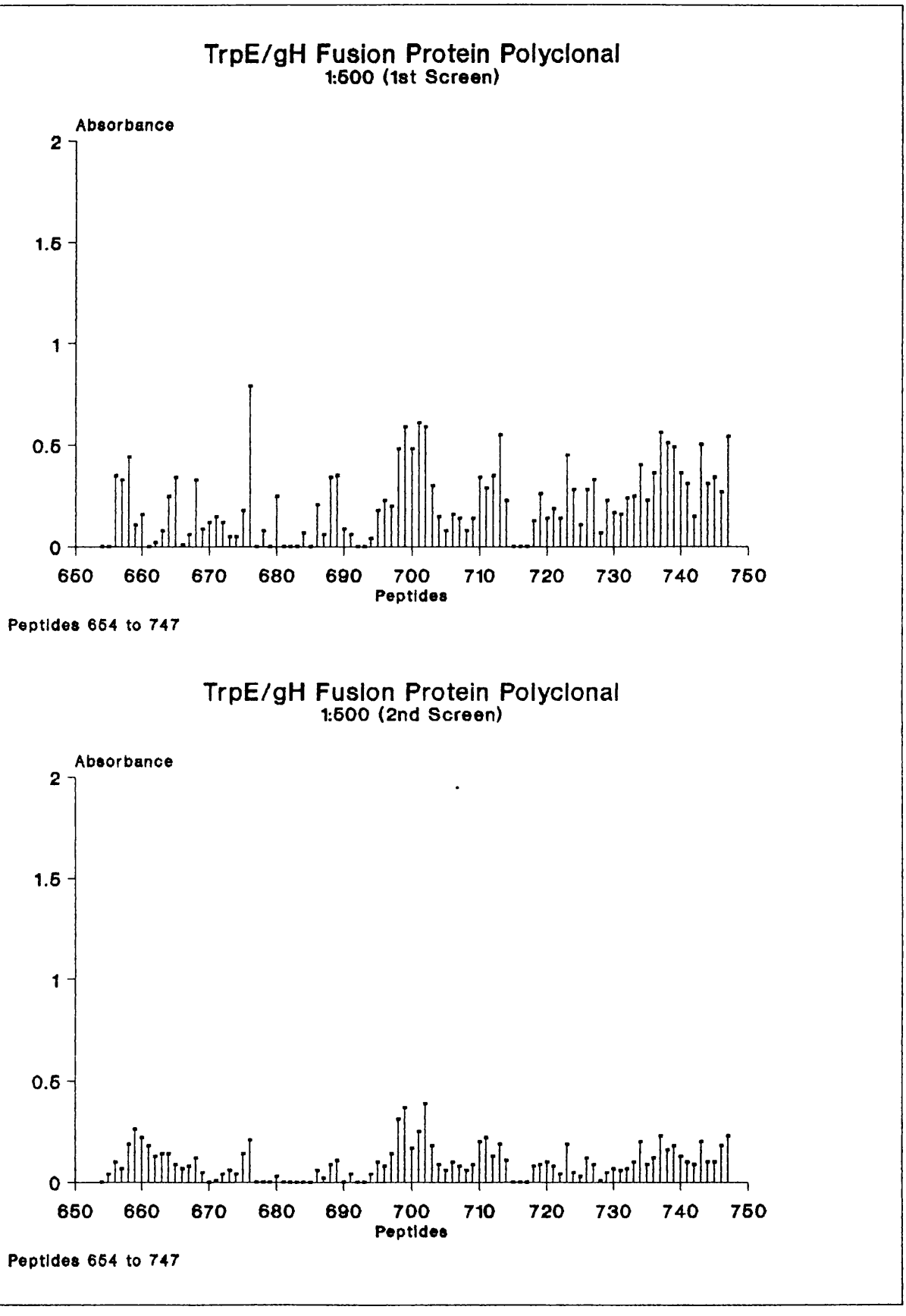
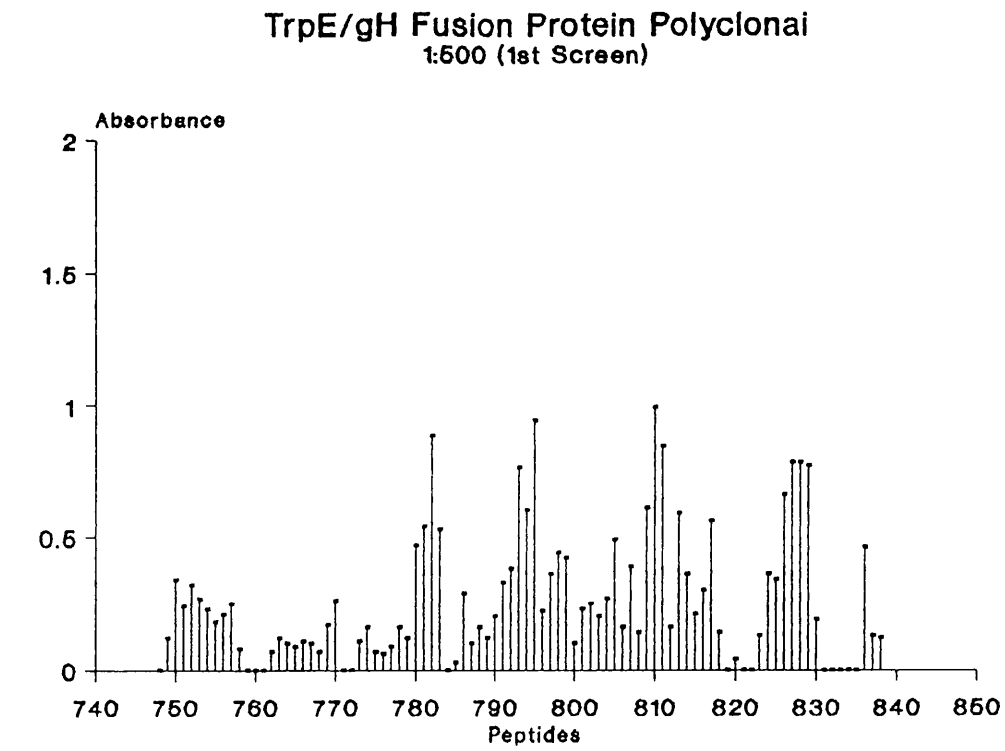


Fig 41. Reactivity of TrpE/gH-1 antibodies with synthetic hexapeptides. 654-747



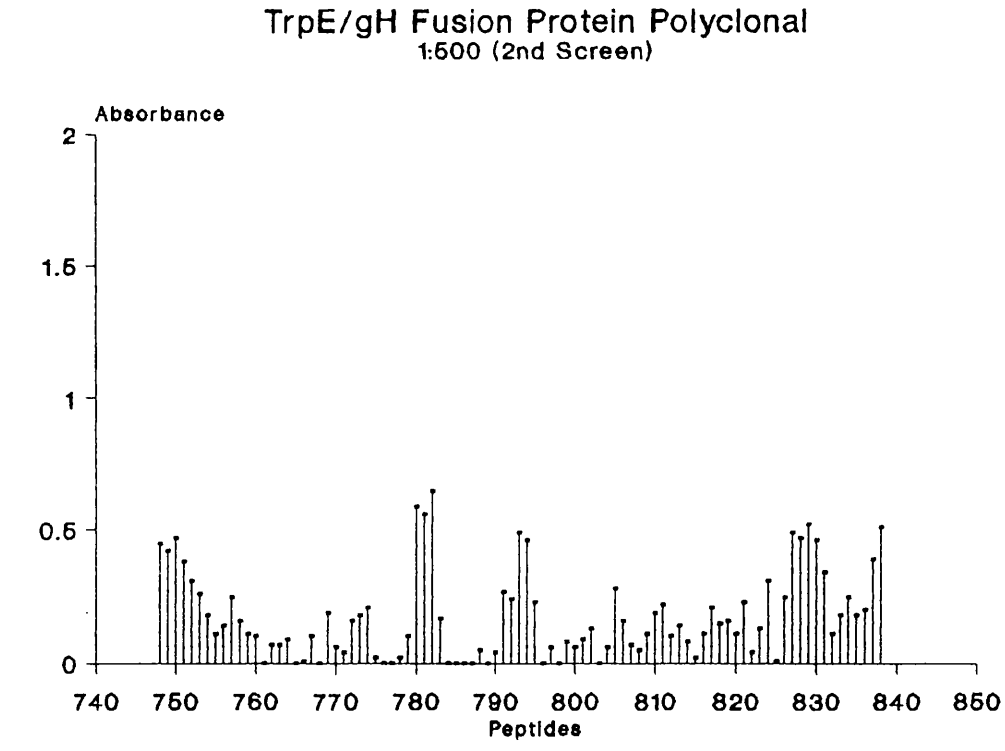


Fig 42. Reactivity of TrpE/gH-1 antibodies with synthetic hexapeptides. 748-838

4.6 Mapping with 52S and 53S

Mapping of the gH-1 peptide series with monoclonal antibodies 52S and 53S supplied as tissue culture supernatant was carried out at dilutions 1:50 and 1:10. Absorbance readings were consistently low in these assays indicating an absence of antibody-peptide interaction. Controls confirmed assay conditions and peptides to be in order.

4.7 Control antibodies

In addition to the controls already described the peptide series was screened with normal rabbit serum at a dilution of 1:500 and a control monoclonal antibody supplied as ascitic fluid and diluted at 1:250. Nonspecific peptide recognition was absent.

Chapter 5

PROPERTIES OF ANTI PEPTIDE ANTIBODIES

Table 5 **Peptides synthesised for Conjugation to KLH on the basis of Antibody Reactivity with Pin Hexapeptides**

Reactive Antibody	Pin Hexapaptides	Derived Antigenic Peptide
R3a	185 190 HPSGPR	183 192 ASHPSGPRDT
R3a	188 193 GPRDTP 189 194 PRDTPP 190 195 RDTPPP 191 196 DTPPPR	188 197 GPRDTPPPRP
LP-11	292 297 GQDLDR 297 302 RVGDPA	290 300 PAGQTLDRVGD
LP-11	303 308 DENPPG 304 309 ENPPGA	302 311 ADENPPGALP
LP-11	328 333 TRADNG	326 335 SLTRADNGSA
LP-11	364 369 FSGDAG	363 372 FFSGDAGAEQ

5.1 Antigenic peptides

A total of twelve peptides were synthesised, conjugated to carrier molecules (KLH) and used to elicit antibodies. (See Appendix II and III). Several criteria influenced peptide selection and the rationale for this choice is outlined below.

The peptide screening results described in Chapter 4 had identified several potential epitopes and the question now was how could these potential epitopes be validated. The approach adopted was to raise and characterise antipeptide antibodies. Recognition of gH-1 by antipeptide antibodies would validate a potential epitope. Six peptides were selected for synthesis solely on the basis of strong R3a or LP-11 reactivity with pin hexapeptide (see Appendix III). The recognising antibody, corresponding hexapeptide and related antigenic peptide are listed in Table 5.

Three peptides were selected because of their association at or close to known AA point mutations responsible for conferring resistance to neutralising monoclonal antibodies LP-11 or 52S. The lack of LP-11 and 52S reactivity with pin hexapeptides corresponding to these point mutations suggests these residues are not incorporated into epitopes which can be represented as linear peptides. Antibodies raised to peptides encompassing these point mutations would allow this hypothesis to be tested further. Peptide 81-90 encompassed position 86, a point mutation implicated in viral resistance to LP-11 neutralisation (Gompels, 1990). Peptide 92-102 was synthesised on the basis of slightly elevated levels to hexapeptide 102, perhaps indicating the presence of an epitope with close proximity to AA.86. The suggestion is that AA substitution here may confer neutralisation resistance by influencing nearby secondary structure and subsequent loss of epitope. Similarly peptide 543-554 reflected slightly raised LP-11 antibody binding at hexapeptides 547 and 548 as well as close proximity to point mutation at AA 536 and 537 which confer neutralisation resistance.

Peptide 373-383 was synthesised solely on the basis of the predictive algorithums incorporated into the computer programme devised by Krchnak and colleagues (1987). The programme uses the algorithms of Hopp and Woods (1981) and Chou-Fasman (1978) to predict hydrophilicity and probability of ß turn occurrence and expresses the overall ß turn potential (p) as a numerical value. Krchnak and colleagues used the programme to evaluate peptides from various studies which had elicited protein reactive antibodies and found 96% contained sequences in which the potential to form a beta turn structure exceeded 1.5×10^{-4} (Krchnak). To put this predictive success to the test peptide 373 - 383 was synthesised which incorporates the tetrapeptide ^{373} GPRP ^{376} of beta turn potential value 2.07×10^{-4}.

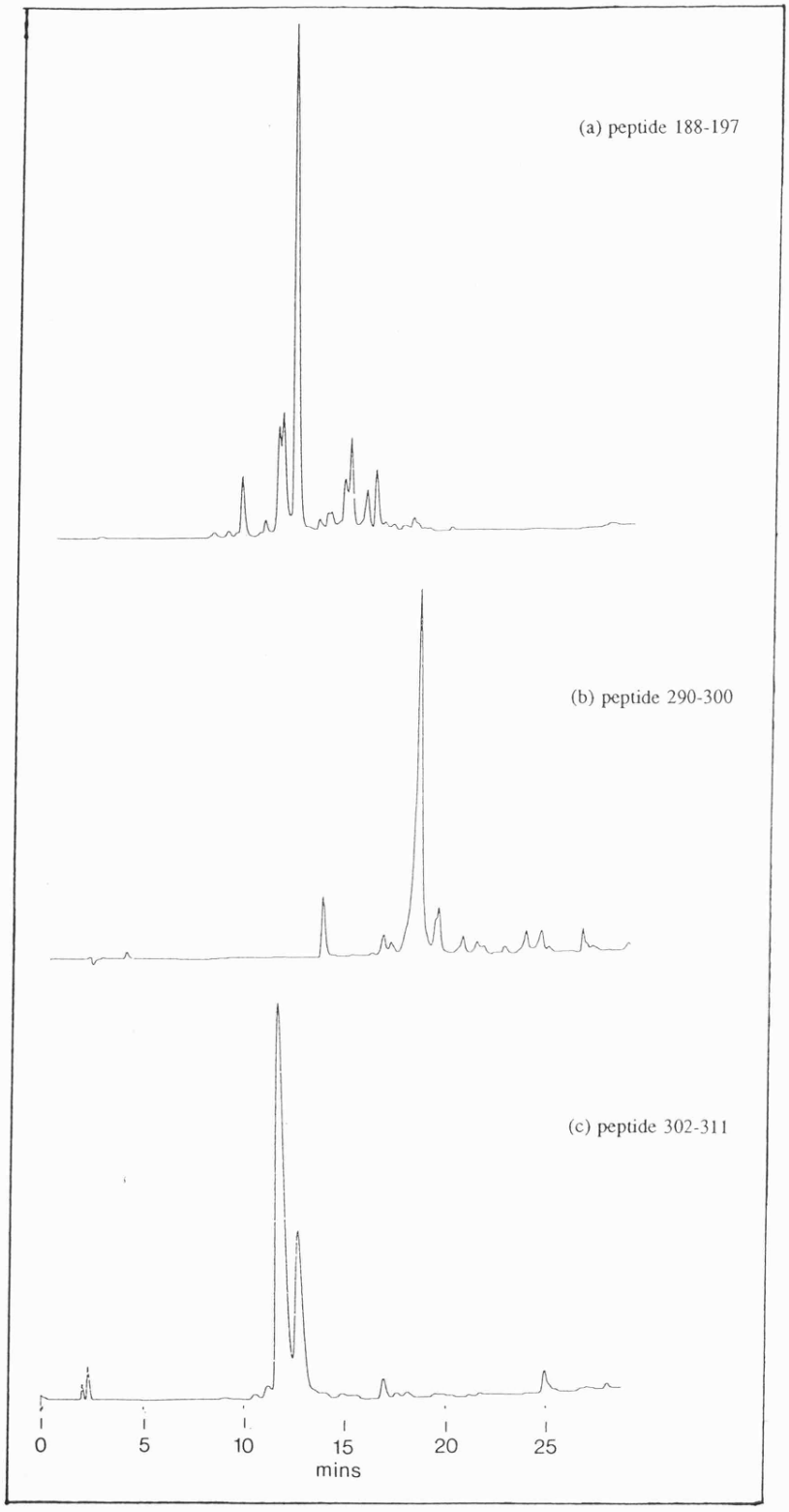


Fig 43. Typical HPLC profile of synthesised free peptides

Peptide 632-642 was synthesised as a negative control, after indications from hexapeptide screening, point mutation data, and computer algorithm (Krchnack 1987) all pointed to an inert sequence.

Peptide 829-838 originates from the cytoplasmic tail of the primary AA sequence. The characterisation of the antipeptide serum generated by this peptide is included although the reagent was designed primarily for use in unrelated experiments (not reported here) concerning the orientation of the gH molecule within the plasma membrane.

Purity of synthesised free peptides was assessed as greater than 80% following AA analysis (CRB) together with HPLC analysis showing a single main peak.

Typical HPLC profiles are shown in Fig. 43.

5.2 ELISA of Peptides Immobilized on Microtitre Plates

The titre of antipeptide antibodies contained in each serum was assessed using homologous peptide immobilized on microtitre plates (as previously described). In all cases serum proved capable of eliciting a strong signal compared to pre-bleed serum and normal rabbit serum controls.

Titration of antipeptide antibodies began at an initial serum dilution of 1:500 and the results of these experiments are presented graphically in Figs 44 to 55. The binding curve for each antipeptide serum can be seen to have moved to the left in all cases indicating the presence of reactive antibodies in serum. Endpoint dilutions are summarised in Table 6 below.

Antigenic Peptide	End Point Dilution (Reciprocal x 1000)
81-90	16
92-102	256
183-192	64
188-197	64
290-300	64
302-311	8
326-335	2
363-372	16
373-383	64
543-554	2
632-642	128
329-838	32

Table 6.

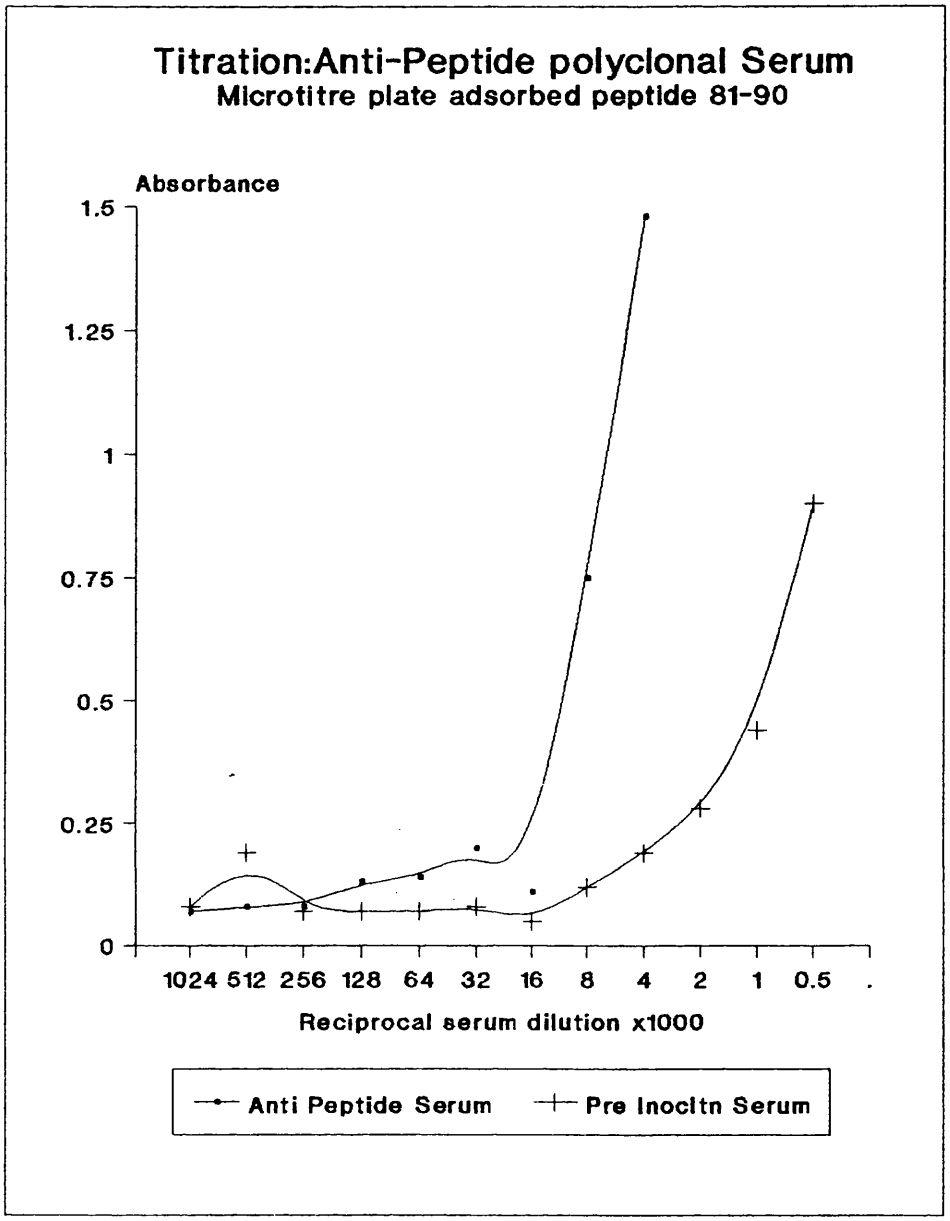


Fig 44. Antipeptide 81-90 antibody titration.

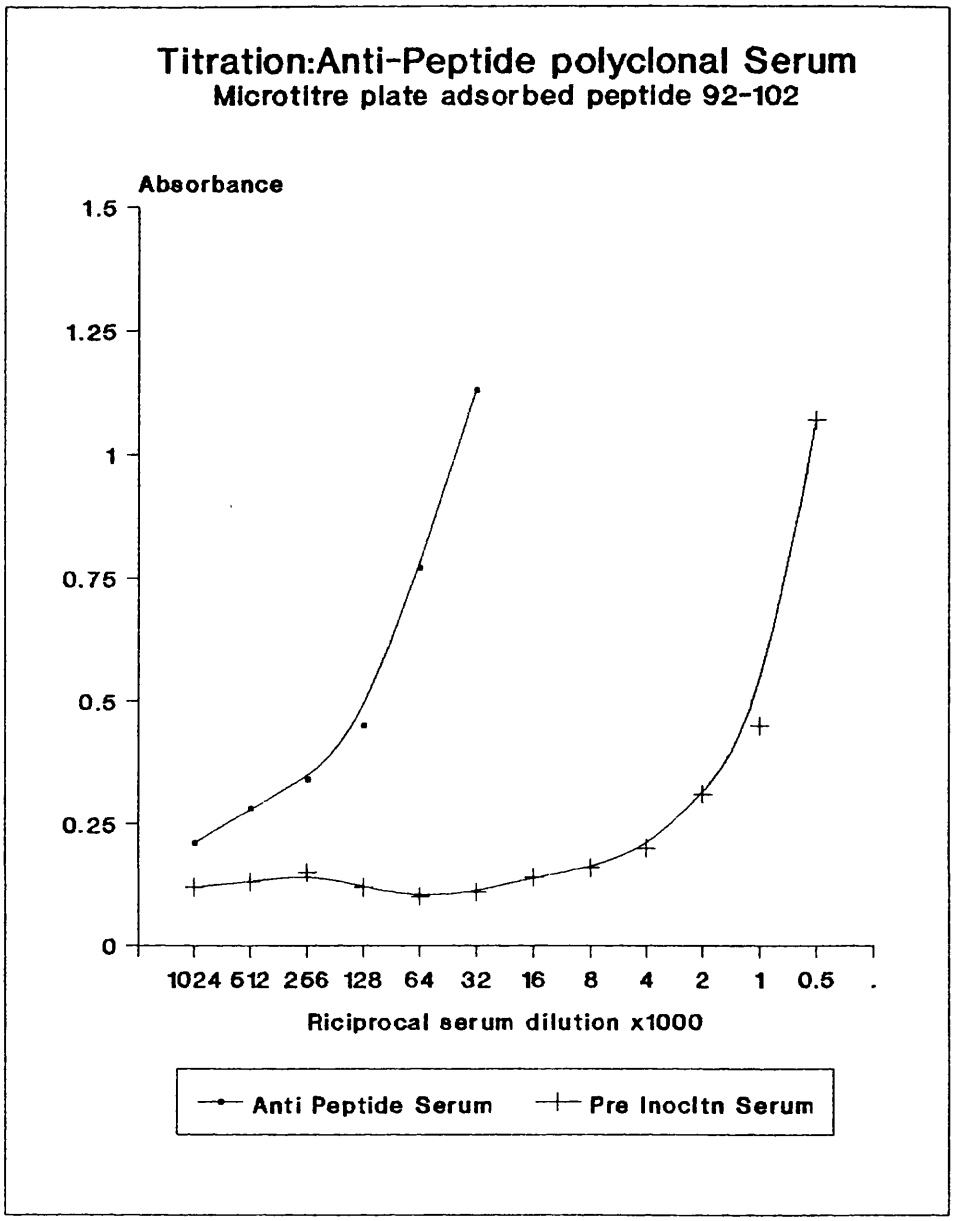


Fig 45. Antipeptide 92-102 antibody titration .

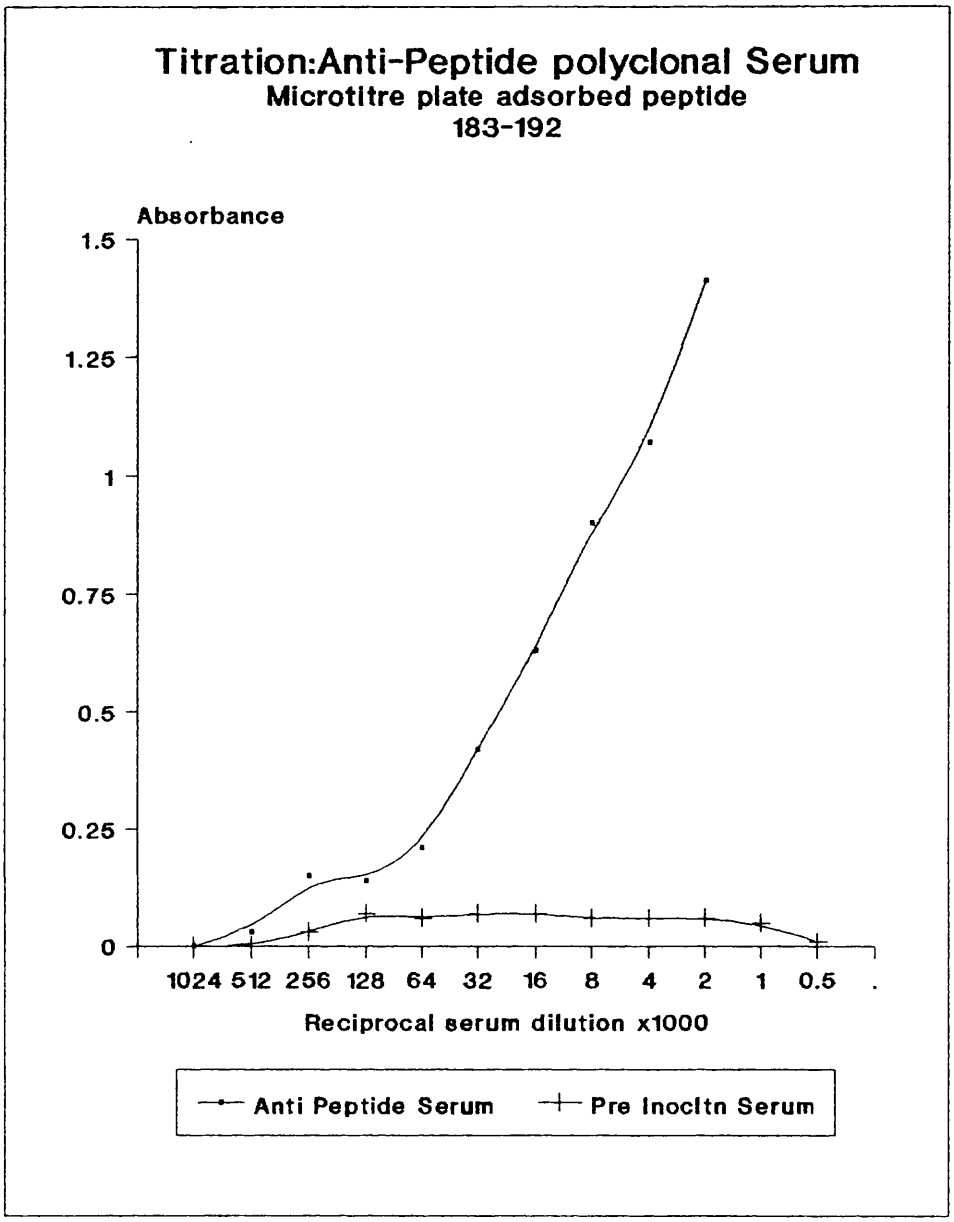


Fig 46. Antipeptide 183-192 antibody titration.

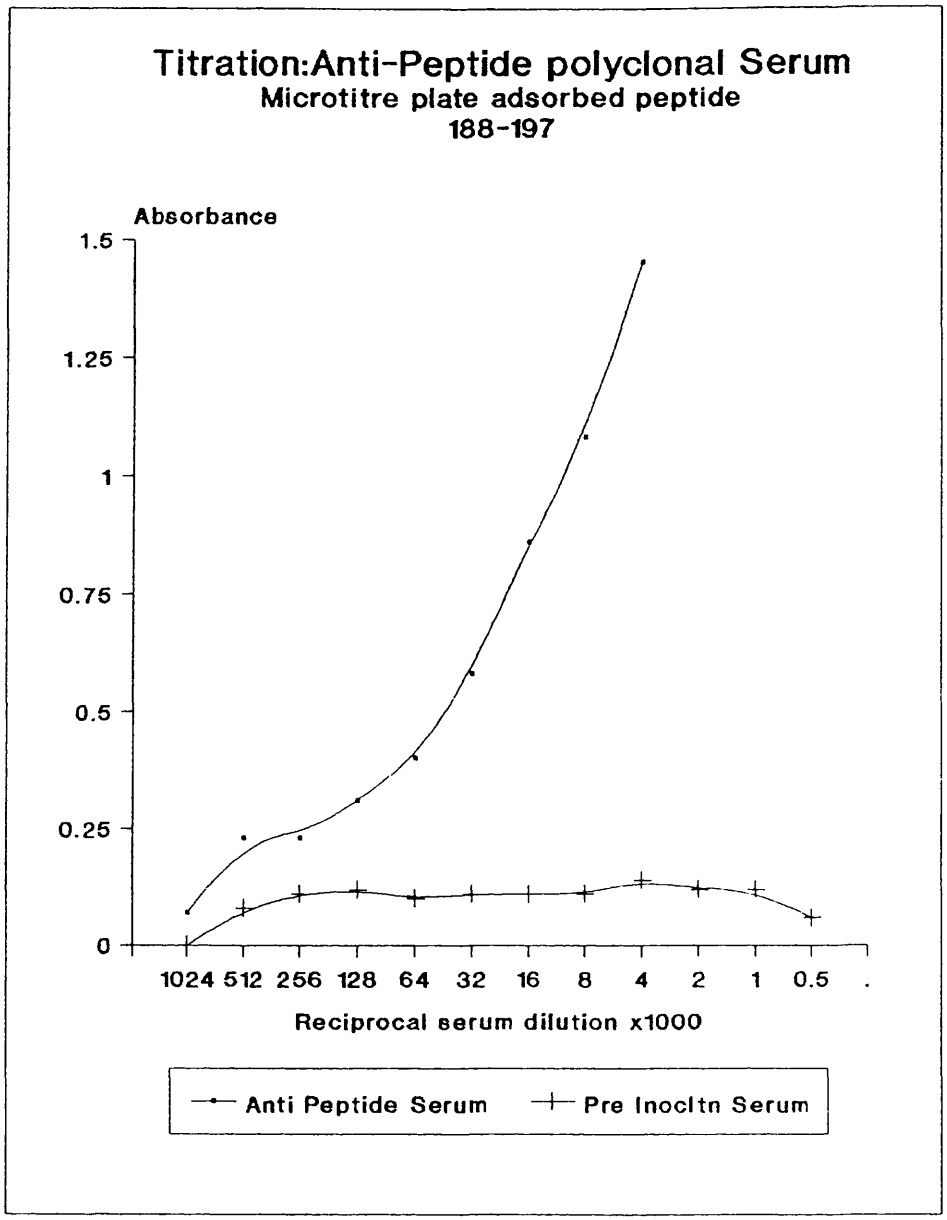


Fig 47. Antipeptide 188-197 antibody titration.

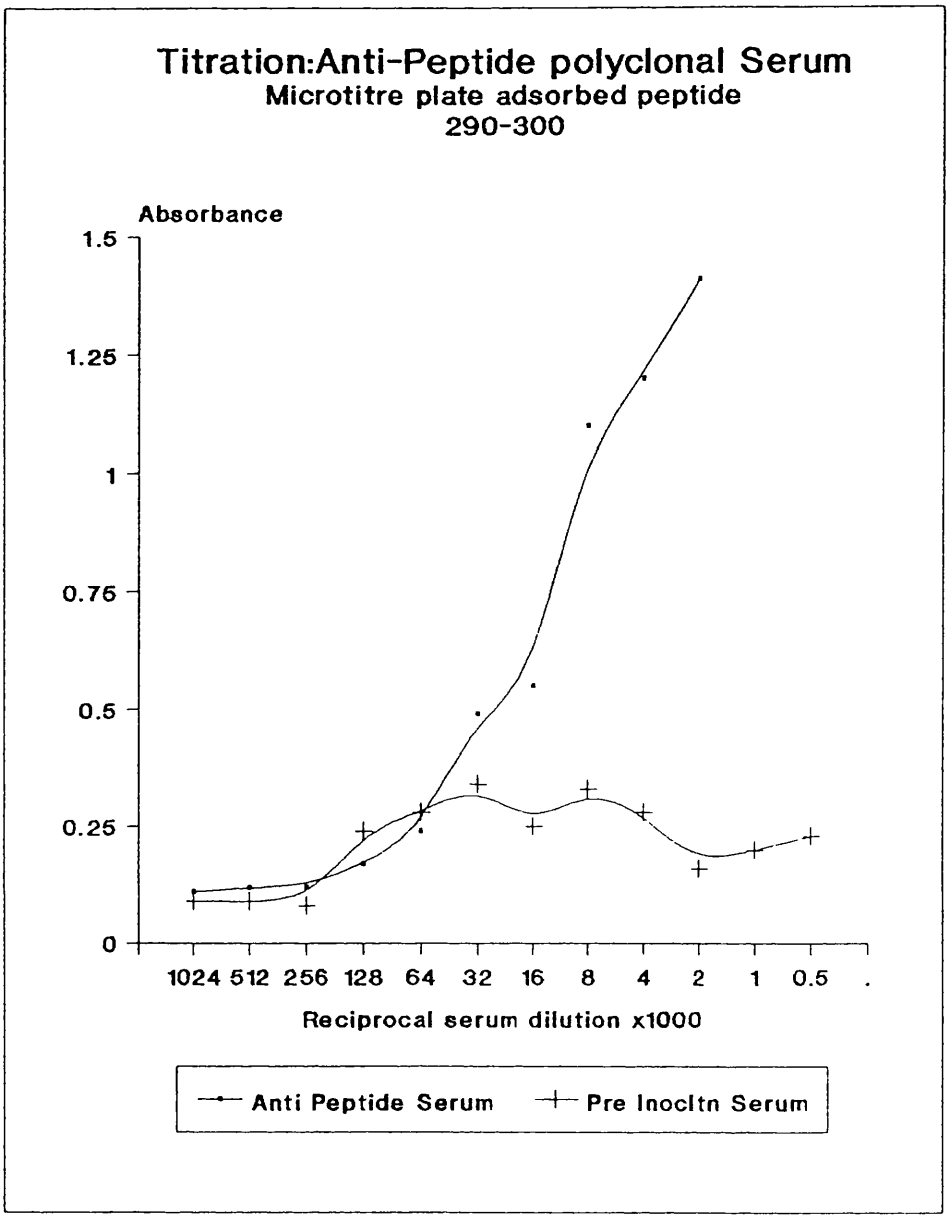


Fig 48. Antipeptide 290-300 antibody titration.

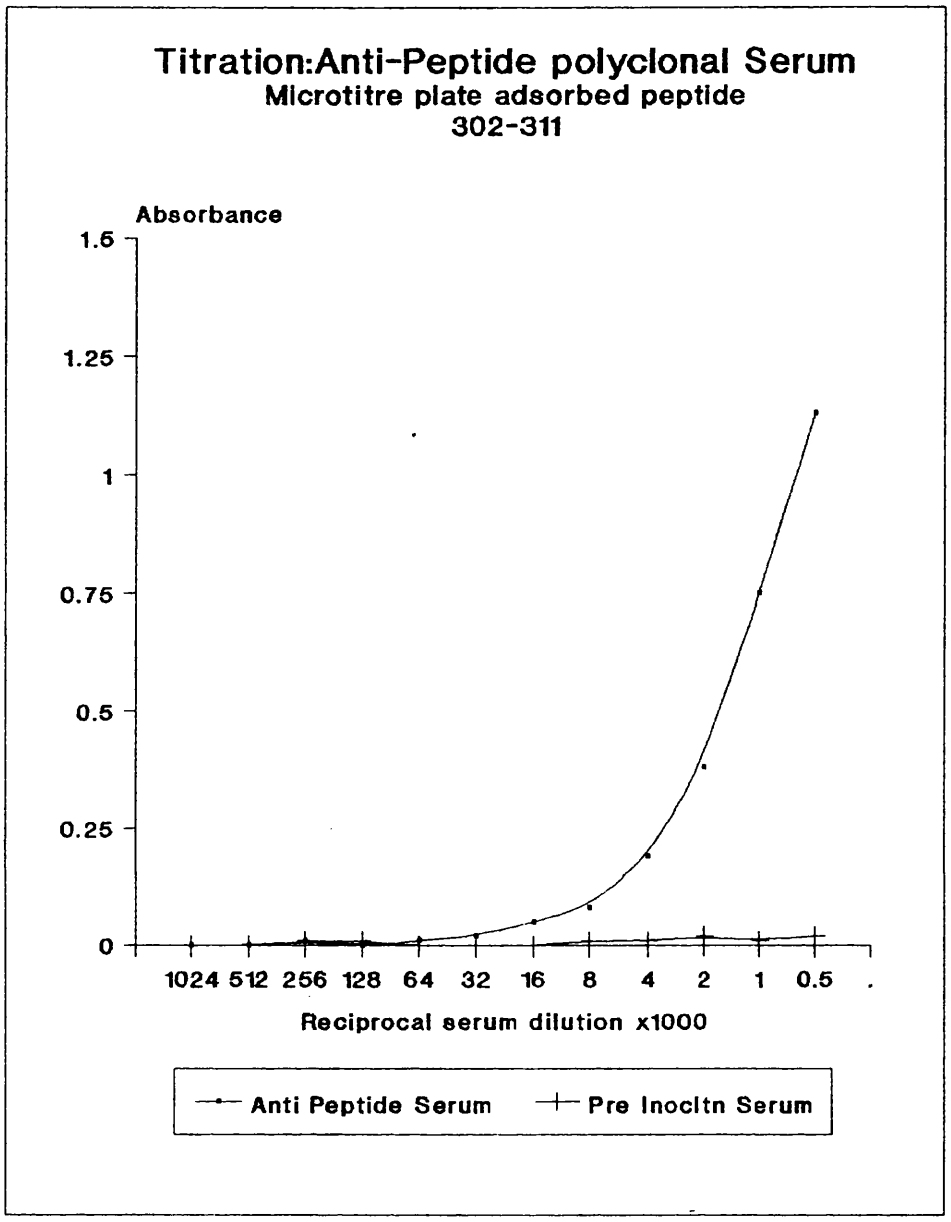


Fig 49. Antipeptide 302-311 antibody titration.

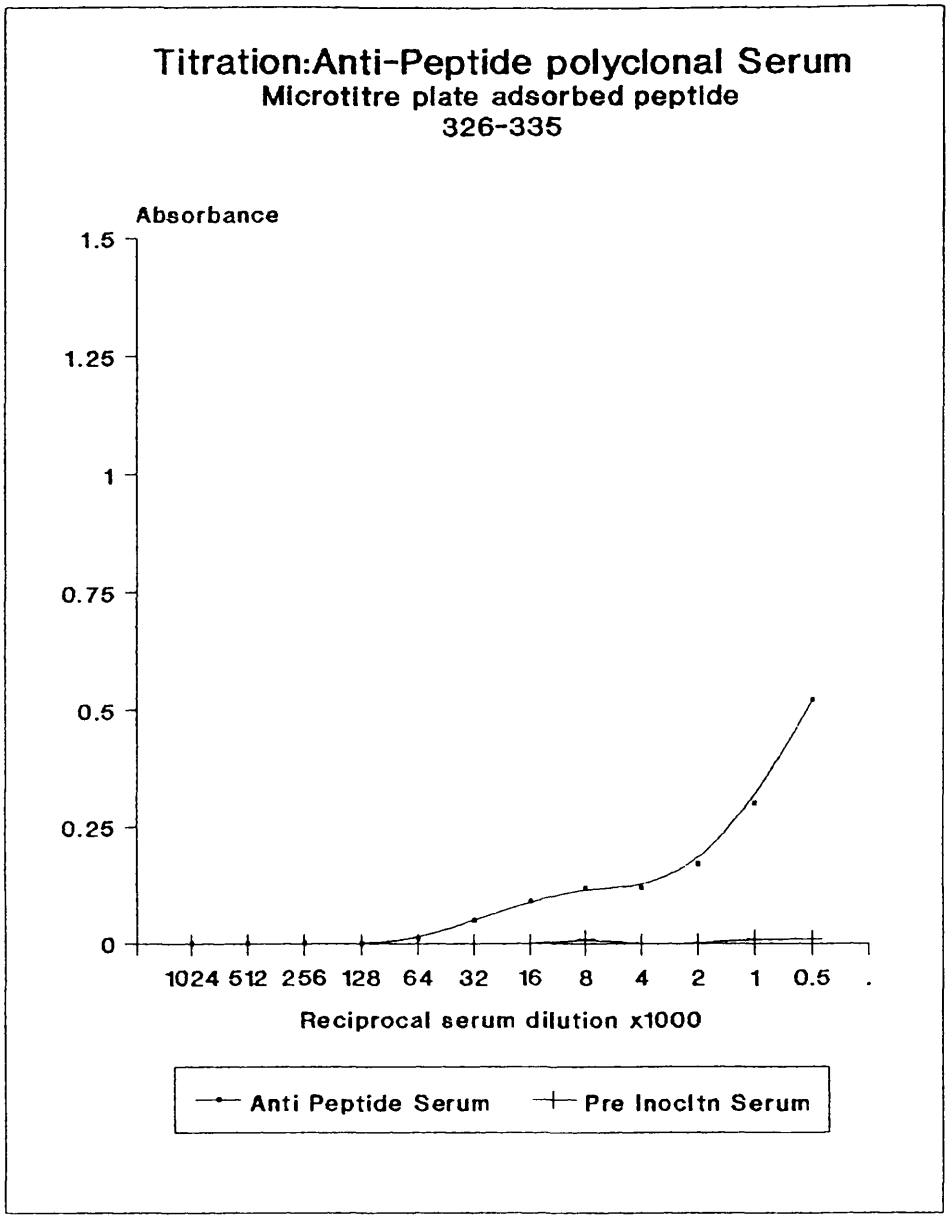


Fig 50. Antipeptide 326-335 antibody titration.

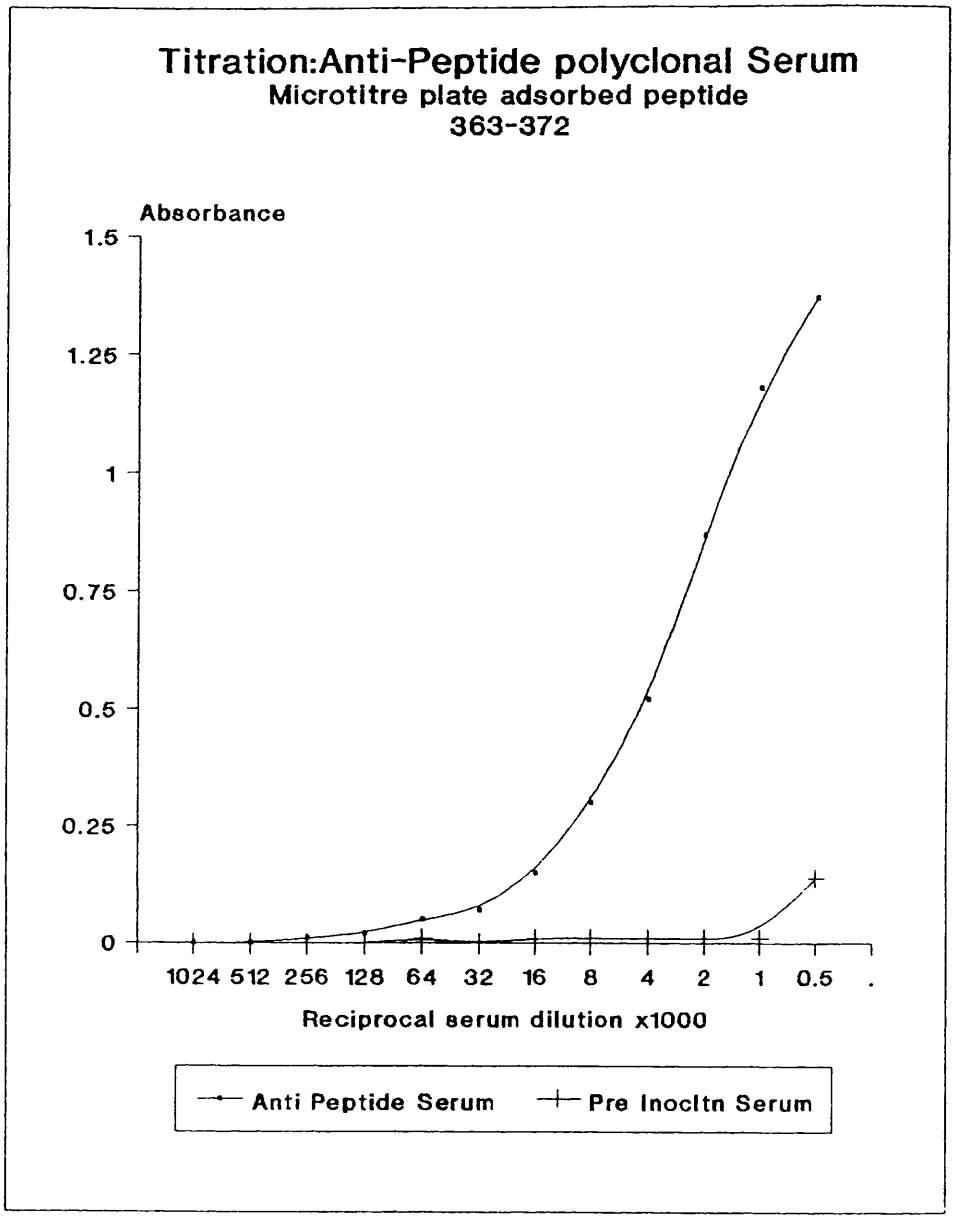


Fig 51. Antipeptide 363-372 antibody titration.

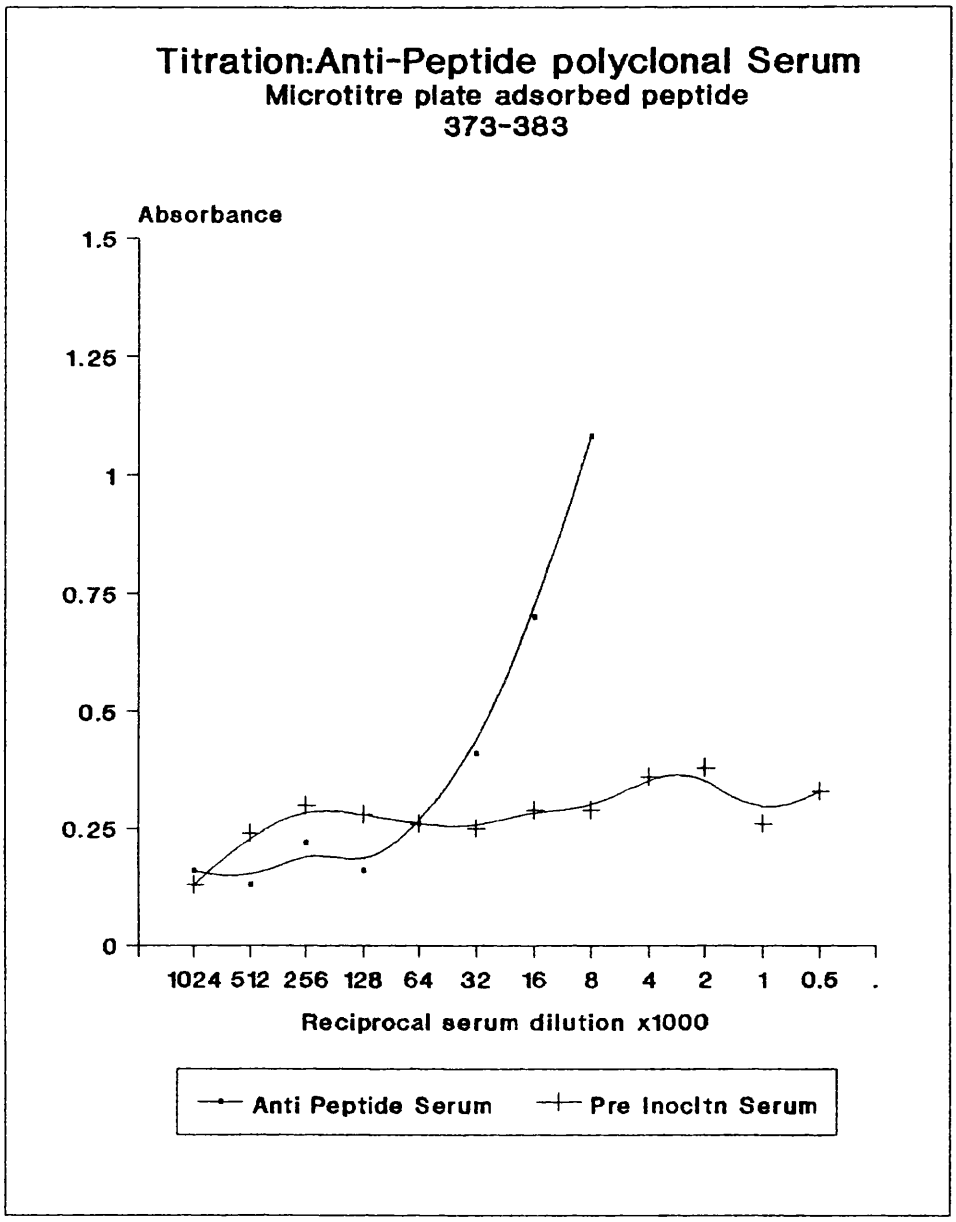


Fig 52. Antipeptide 373-383 antibody titration.

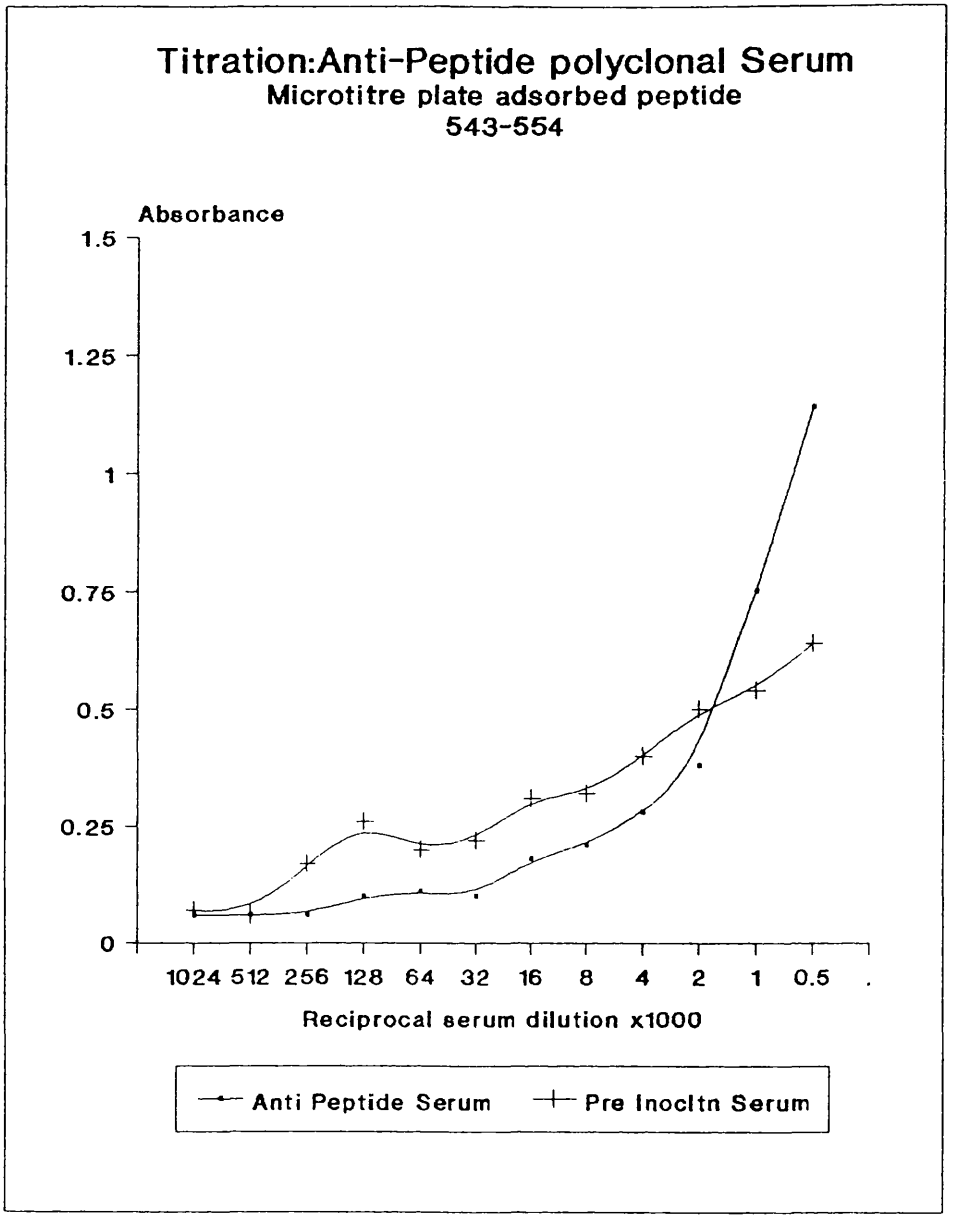


Fig 53. Antipeptide 543-554 antibody titration.

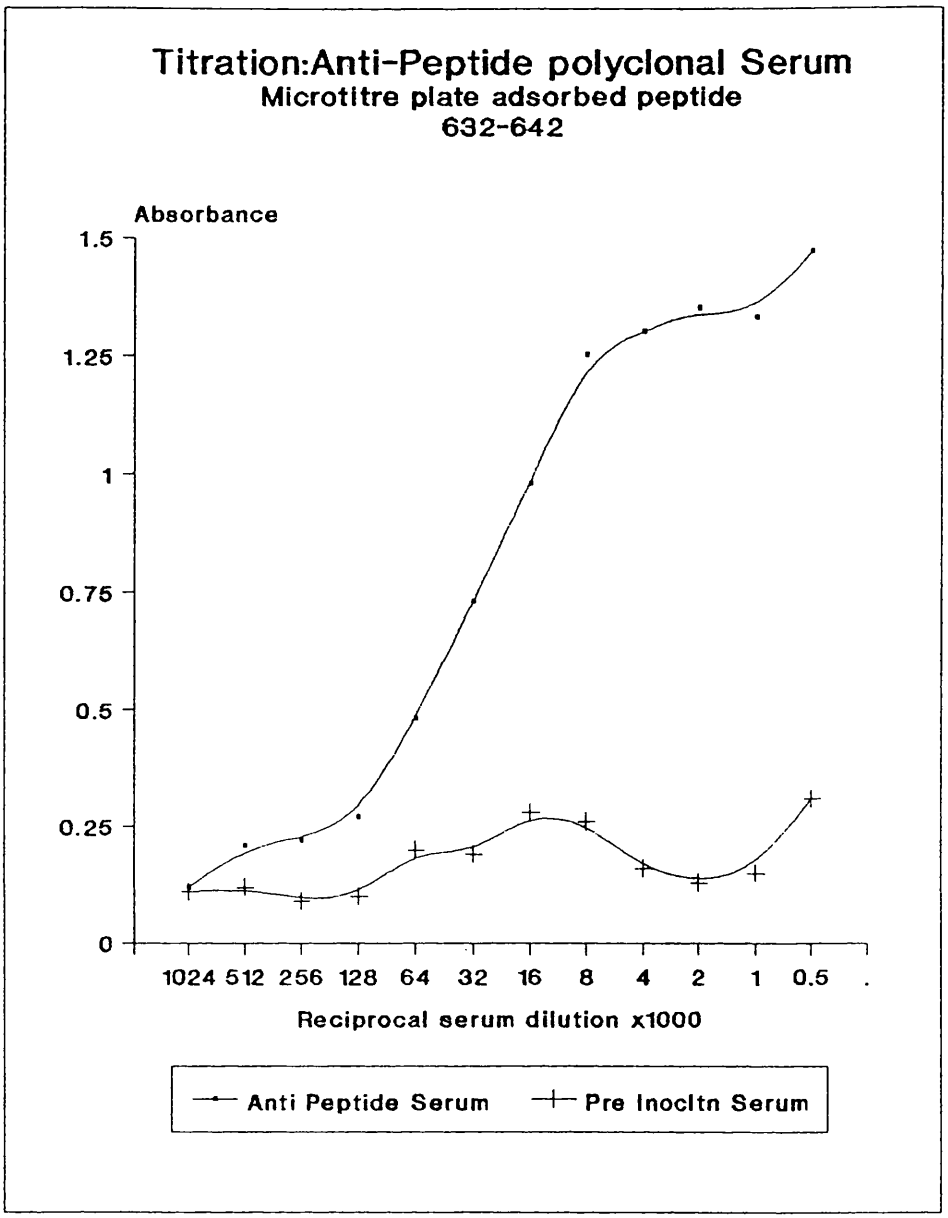


Fig 54. Antipeptide 632-642 antibody titration.

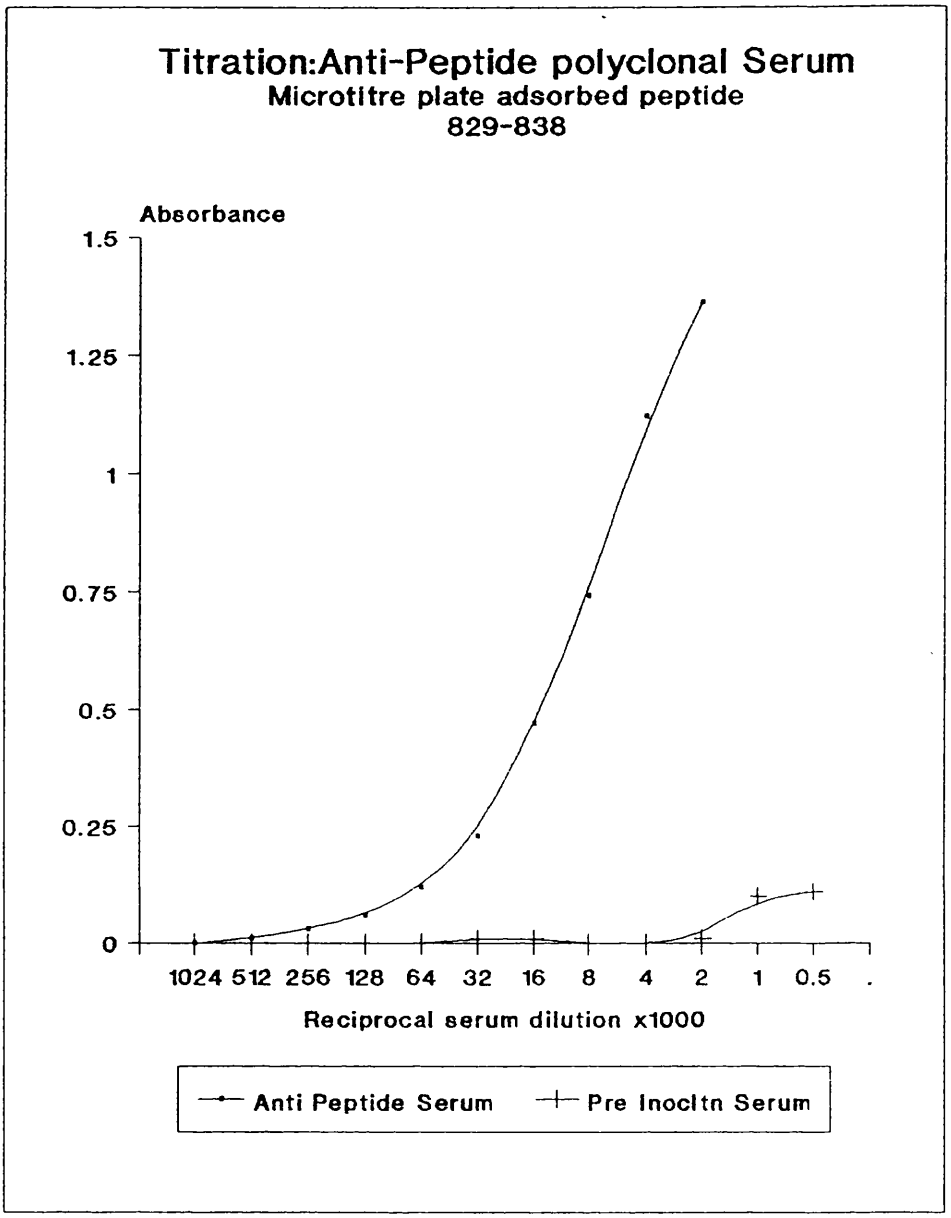
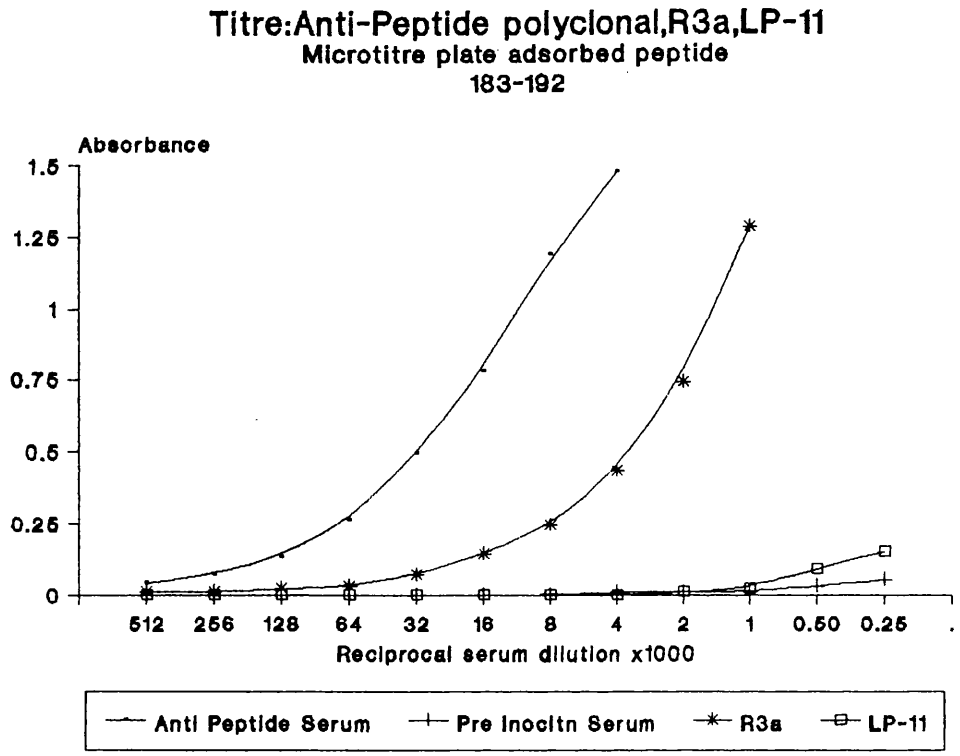


Fig 55. Antipeptide 829-838 antibody titration.



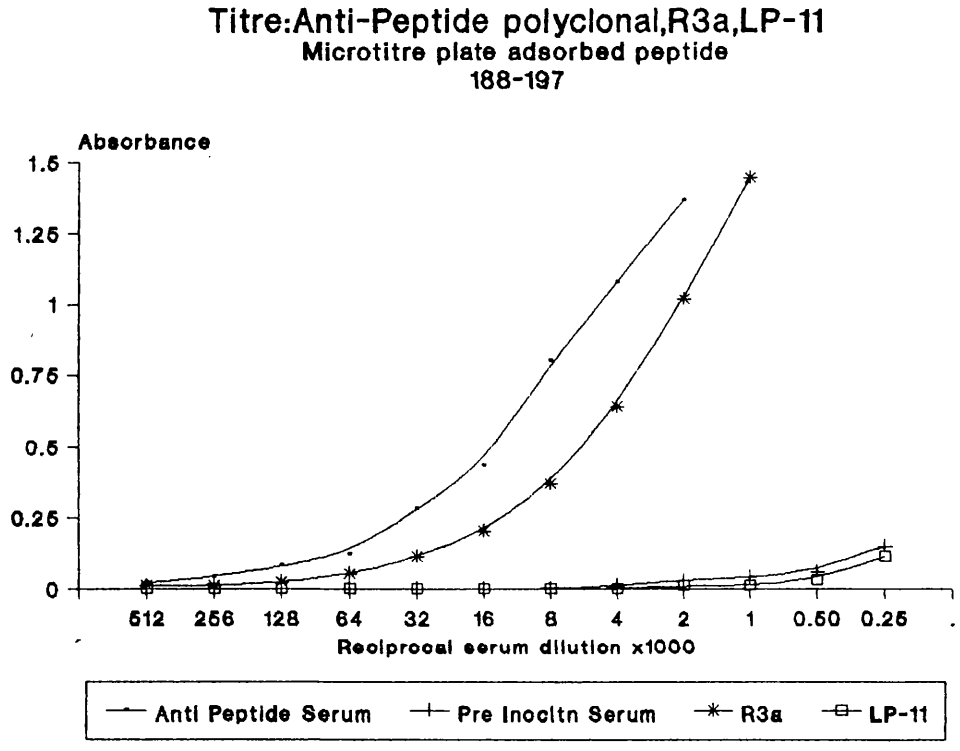


Fig 56. Reactivity of R3a and LP-11 antibodies with peptides immobilized on Micro titre Plates - see text 5.3.

5.3 R3a and LP-11 Reactivity with Immobilized Peptides.

A Series of experiments were performed in which R3a and LP-11 were tested for reactivity with peptides immobilized on microtitre plates.

The results shown in Fig. 56 indicate R3a antibodies reactive with peptides 183 - 192 and 188 - 197, reinforcing the choice of these peptides for synthesis, made originally on the basis of strong R3a interaction with related pin hexapeptides.

Reactivity of immobilized peptides with LP-11 was absent.

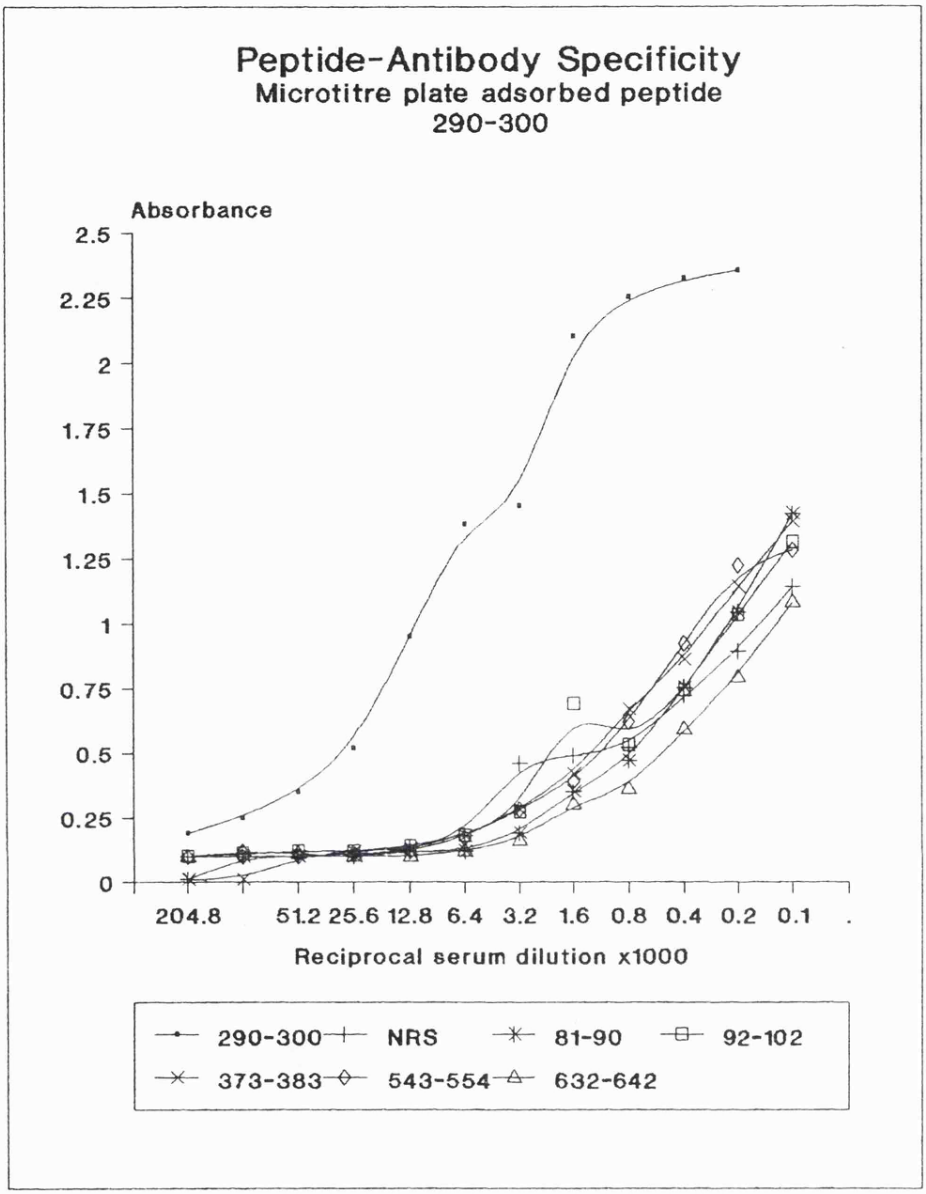


Fig 57. Anti Peptide antibody specificity - see text 5.4.

5.4 Antipeptide-Antibody Specificity

To investigate the specificity of the antibody responses a series of experiments were performed in which six randomly chosen antipeptide sera were examined for reactivity against five other unrelated peptides adsorbed to microtitre plates.

The results, an example of which is presented in Fig. 57, indicated in all cases antipeptide antibodies bound only to their corresponding peptide confirming the specificity of the antibody response.

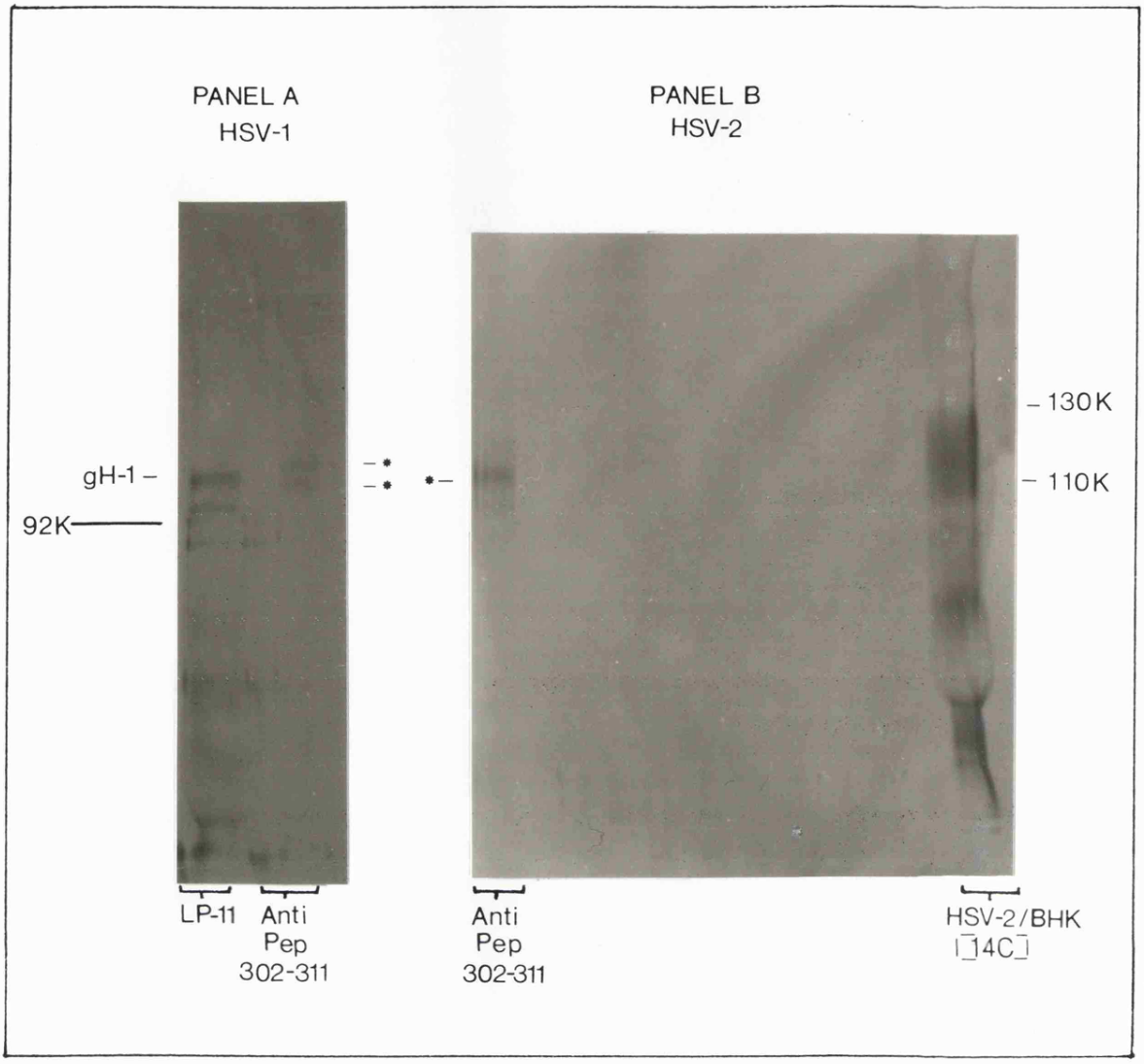


Fig 58. Immunoprecipitation properties of Anti-peptide Antibodies - see text 5.5.

5.5 Immunoprecipitation

The ability of antipeptide antibodies to immunoprecipitate [14C] radiolabelled gH was assessed using solubilized material derived from BHK-21 cells infected with either HSV-1 (Troisbel) or HSV-2 (25766) in the presence of [14C] glucosamine hydrochloride.

Antibodies elicited by peptide ^{302}ADENPPGALP^{311} immunoprecipitated protein species from both HSV-1 and HSV-2 derived samples (Fig. 58).

Glycoprotein H-1 immunoprecipitated by LP-11 was evident in autoradiograph (Fig. 58, Panel A) as a closely spaced doublet with Mr of approximately 110K above a faint lower band of approximately 95K, presumed to be precursor in which glycosylation is incomplete. The protein species immunoprecipitated from HSV-1 infected cell by the antipeptide antibodies exhibited a different pattern and was seen as two distinct bands, one with an apparent molecular ratio slightly higher than 110K, while the other was slightly lower.

The protein species immunoprecipitated from solubilized HSV-2 infected cells produced a banding pattern in autoradiograph very similar to HSV-1 gH immunoprecipitated by LP-11 (Fig. Panel, B). The shortage of HSV-2 specific reagents made it impossible to confirm the identity of these HSV-2 species by immunological methods.

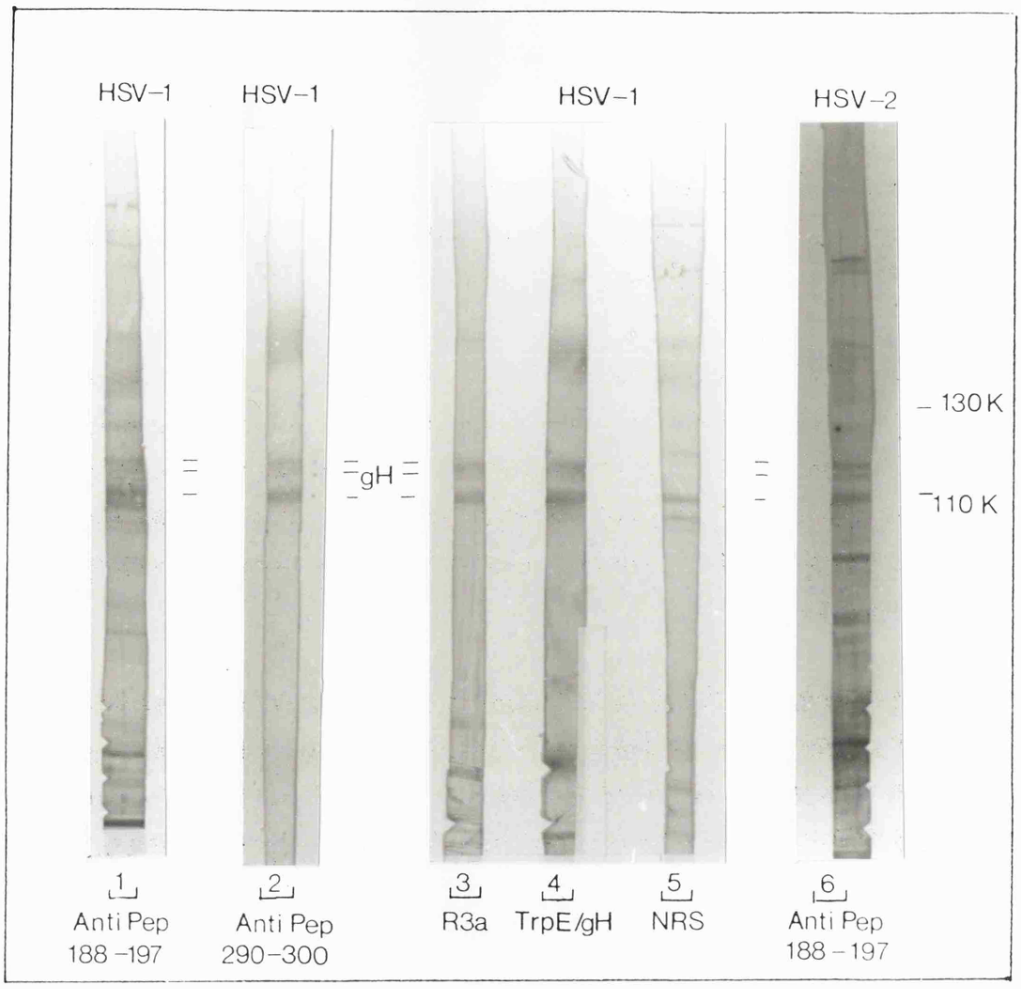


Fig 59. Antipeptide antibody reactivity against Western transferred [14C] radiolabelled HSV-1 and HSV-2 infected cell proteins - see text 5.6.

5.6 Western Blotting

The reactivity of antipeptide antibodies following Western transfer was investigated using HSV-1 or HSV-2 infected BHK-21 cells. Cells were infected in the presence of [14C] glucosamine hydrochloride.

The results of probing HSV-1 infected cell proteins following electrophoretic transfer to nitrocellulose are illustrated in Fig. 59. All nitrocellulose strips originate from a single nitrocellulose sheet, allowing accurate band pattern comparisons.

The characteristic binding pattern exhibited by anti Trp E/gH-1 fusion protein polyclonal antibodies and R3a antibodies are clearly illustrated. Antibodies elicited by peptide 188-197 and peptide 290-300 proved reactive with infected cell proteins which correlate precisely with the gH-1 bands identified by the anti Trp E/gH-1 fusion protein polyclonal antibodies and R3a antibodies. Autoradiography of these blots (not shown) confirmed these bands to be correctly situated relative to glycoprotein region of Mr 110-130K.

Similar screening of western transferred HSV-2 infected cell proteins revealed binding of anti peptide 188-197 antibodies to protein bands exhibiting a pattern similar to that seen following probing of gH-1 based western blots using anti Trp E/gH-1 fusion protein antibodies and R3a antibodies. Autoradiography of this blot showed these bands to align with glycosylated proteins of Mr circa 110-130K. The lack of HSV-2 specific reagents prevented further investigation of these protein species.

5.7 Neutralisation

None of the antipeptide sera proved capable of neutralising infectious virus during *in vitro* plaque assays over a range of dilutions in the presence and absence of complement.

5.8 Reactivity of Homologous Decapeptides

The peptide mapping results outlined in Chapter 4 were dependent on pin hexapeptides adopting limited conformation similar to that present in the glycoprotein. The opportunity to synthesise pin decapeptides, retrospectively allowed two questions to be addressed. First would the structures adopted by pin hexapeptides, which were reactive with R3a and LP-11, be maintained when these hexapeptide sequences were incorporated into pin decapeptides? Second do the antibodies raised against free peptide recognise closely related pin decapeptide?

Decapeptides were synthesised in duplicate according to the methods of Geysen. The resultant binding characteristics of pin hexapeptides with antipeptide antibodies, R3a, LP-11, and a control ascitic fluid are shown in Figs 60-74. In figures the sequence of pin decapeptides is depicted in single letter amino acid code with n-terminal amino acids at the top of each column. Residues shown at the base of each column represent amino acids incorporated into free peptides but not pin decapeptides.

In all, seven of twelve antipeptide serum antibodies proved reactive with pin decapeptides (Figs 60, 61, 62, 63, 67, 69, 70) and this reactivity did not relate to end point titres of antipeptide antiserum determined using peptide immobilized on microtitre plates (Table 6).

R3a was reactive with only two decapeptides which incorporated hexapeptides highlighted by mapping studies using R3a antiserum, (Fig 72) and reinforces their choice as potential epitopes. Antipeptide antibodies raised on the basis of R3a pin hexapeptide reactivity also recognised their corresponding pin decapeptides.

In contrast LP-11 failed to recognise any pin decapeptides incorporating original LP-11 reactive hexapeptides (Fig 73). In addition antipeptide antibodies raised of the basis of LP-11 pin hexapeptide reactivity did not recognise corresponding pin decapeptitides. (Figs 64, 65, 66, 67 and Appendix II).

The results indicate that in some cases the limited conformation present in hexapeptides is maintained when their residues are incorporated into larger peptides and that antibodies raised aginst free peptide may not recognise homologous pin peptides.

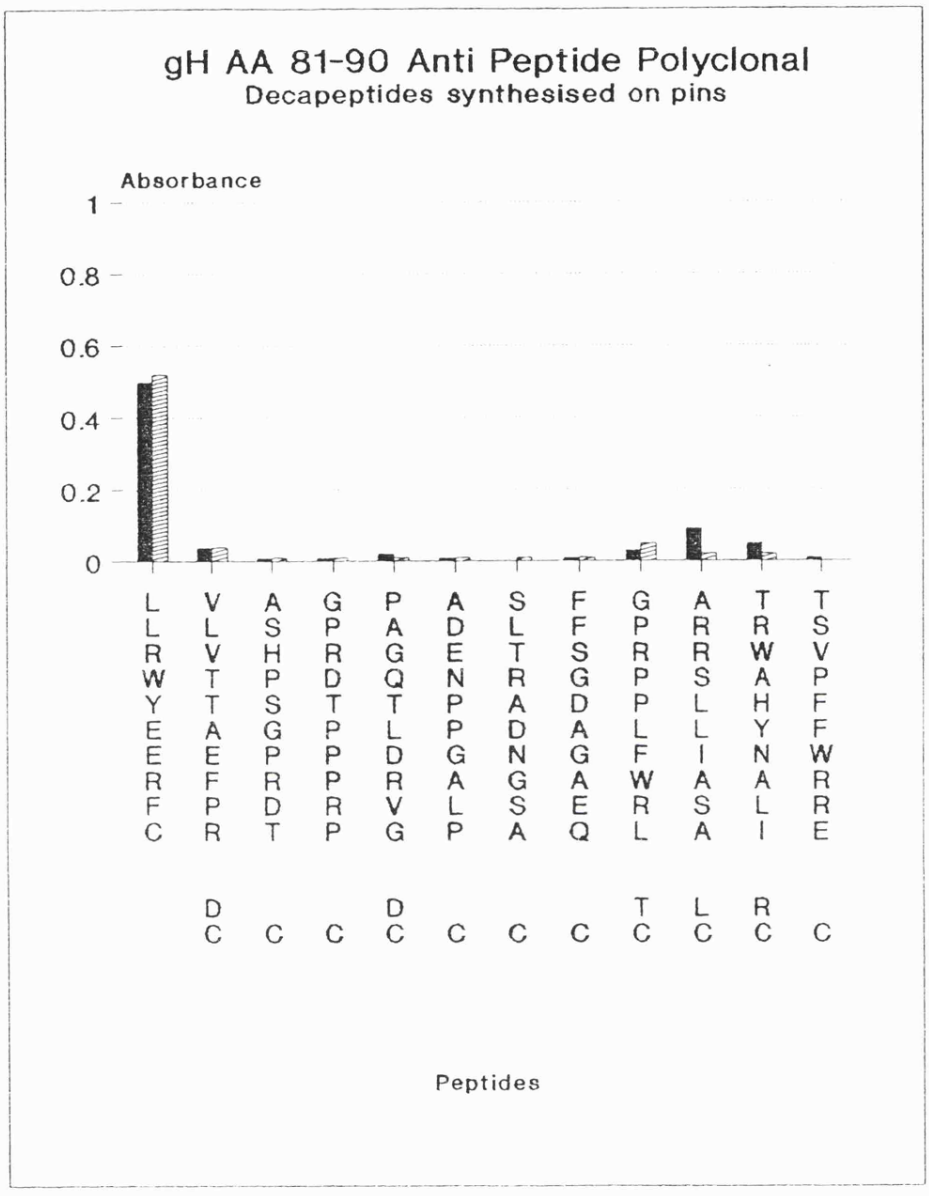


Fig 60. Reactivity of antipeptide 81-90 antibodies with Pin Decapeptides.

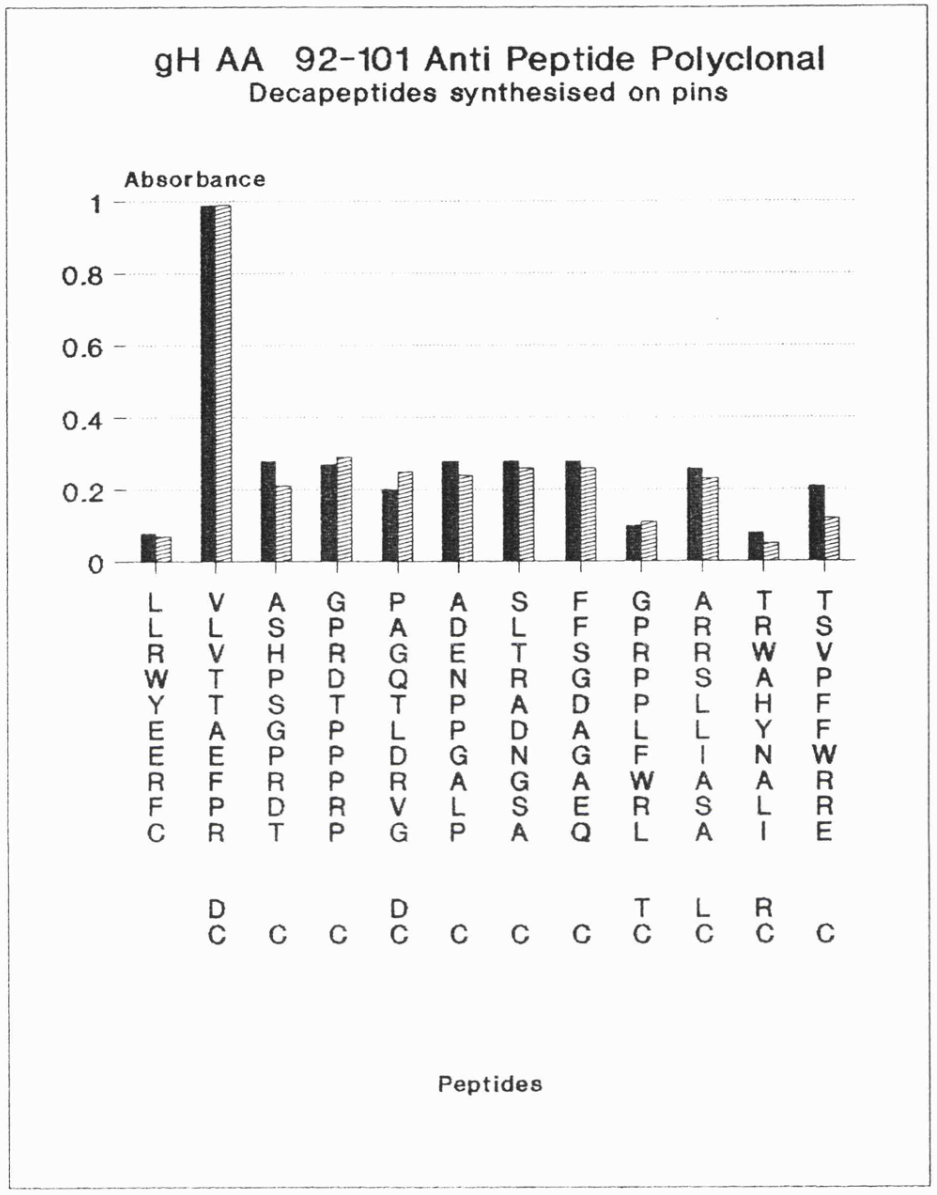


Fig 61. Reactivity of antipeptide 92-102 antibodies with Pin Decapeptides.

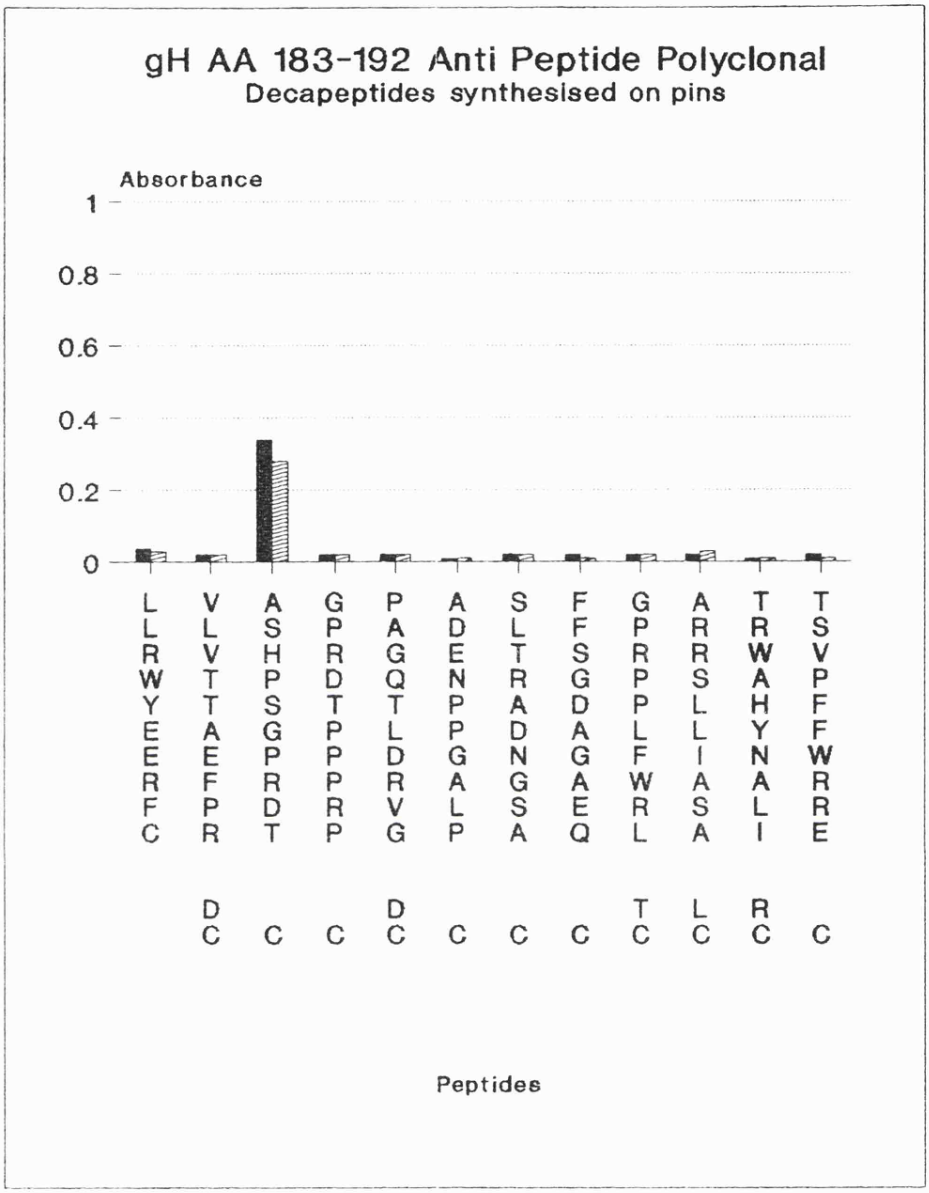


Fig 62. Reactivity of antipeptide 183-192 antibodies with Pin Decapeptides.

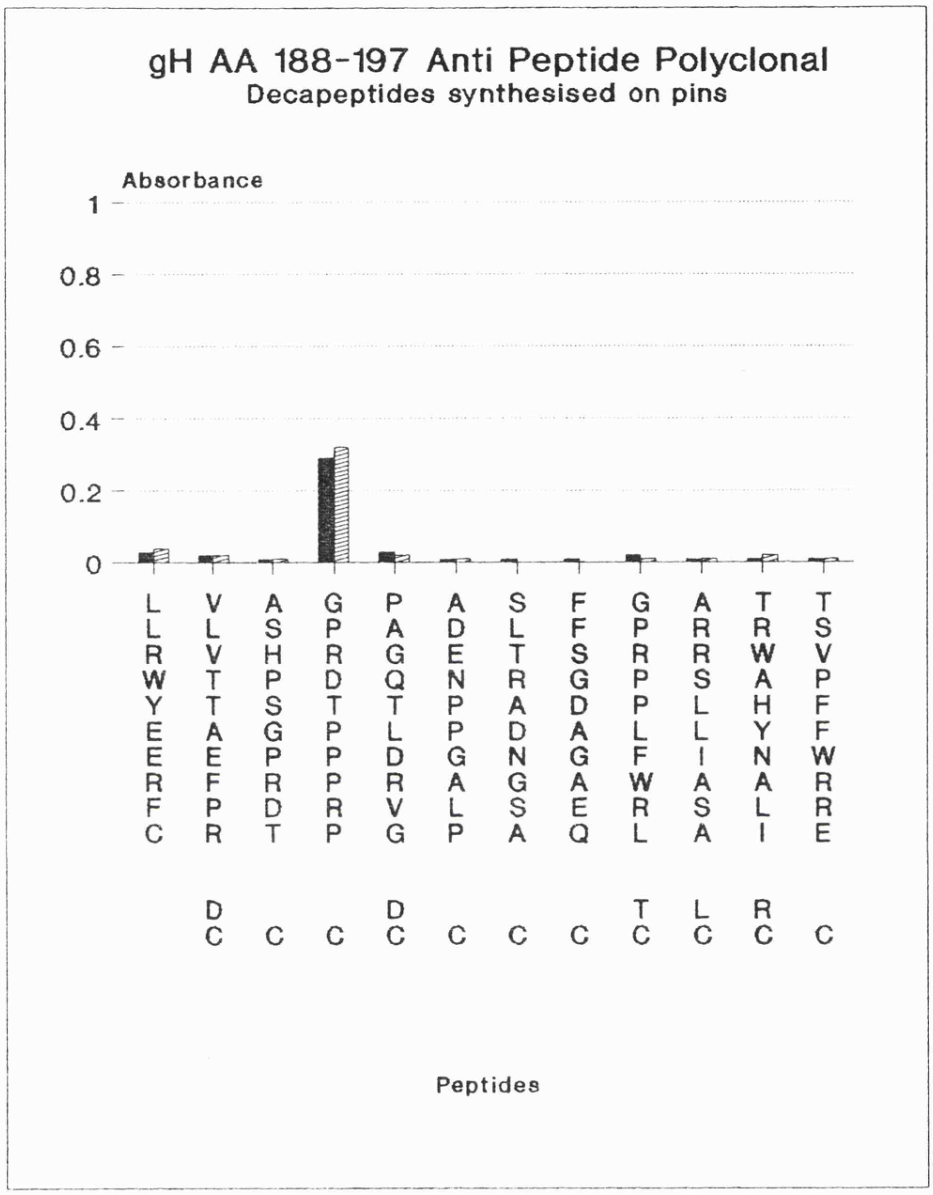


Fig 63. Reactivity of antipeptide 188-197 antibodies with Pin Decapeptides.

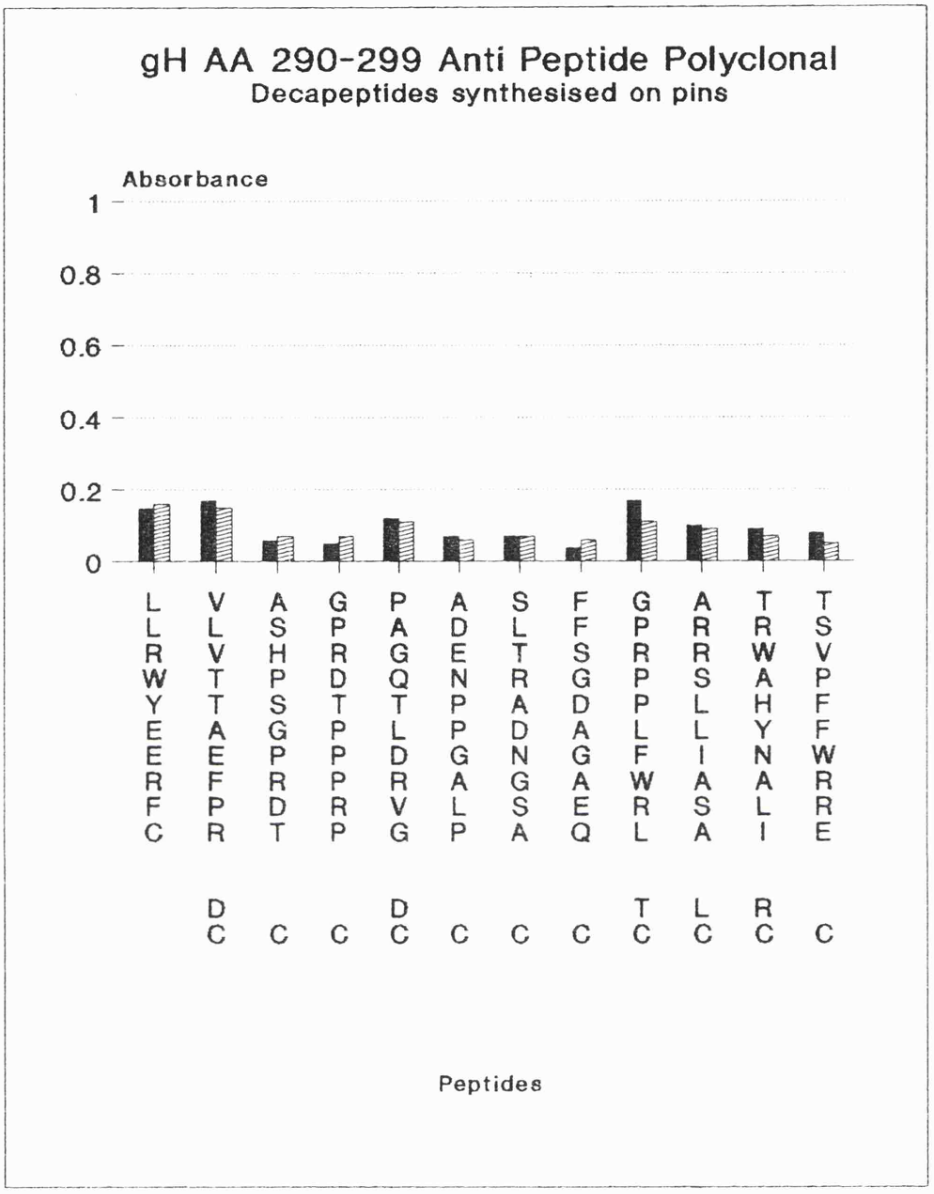


Fig 64. Reactivity of antipeptide 290-300 antibodies with Pin Decapeptides.

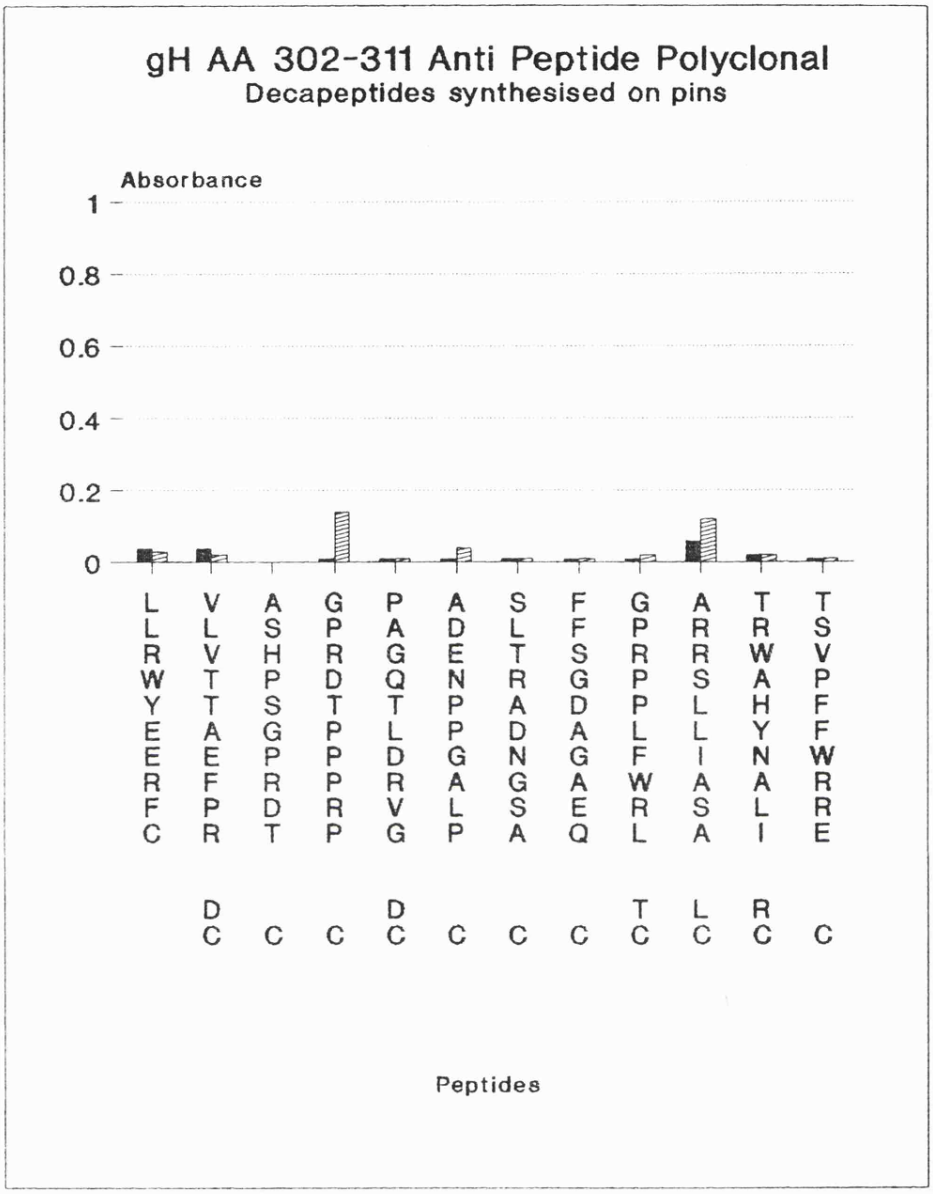


Fig 65. Reactivity of antipeptide 302-311 antibodies with Pin Decapeptides.

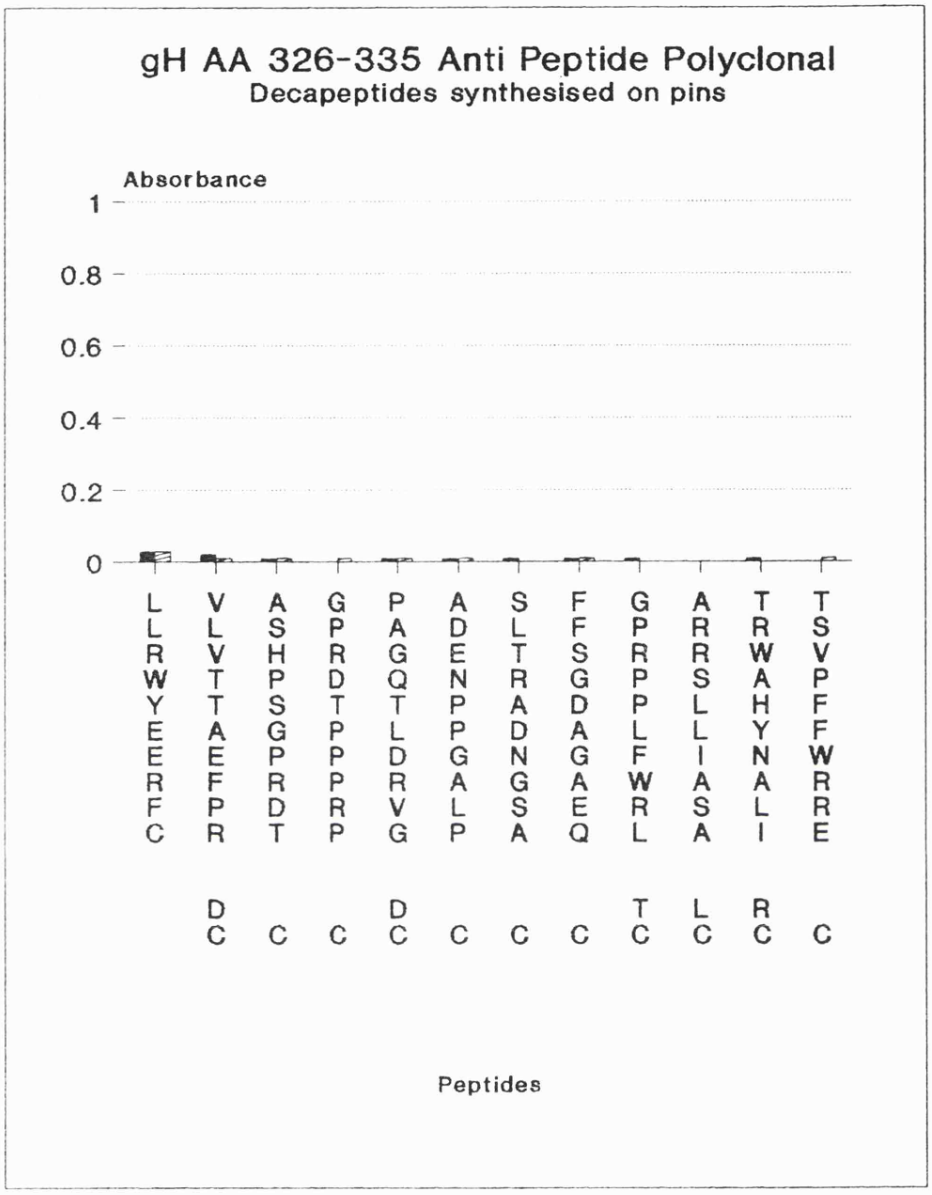


Fig 66. Reactivity of antipeptide 326-335 antibodies with Pin Decapeptides.

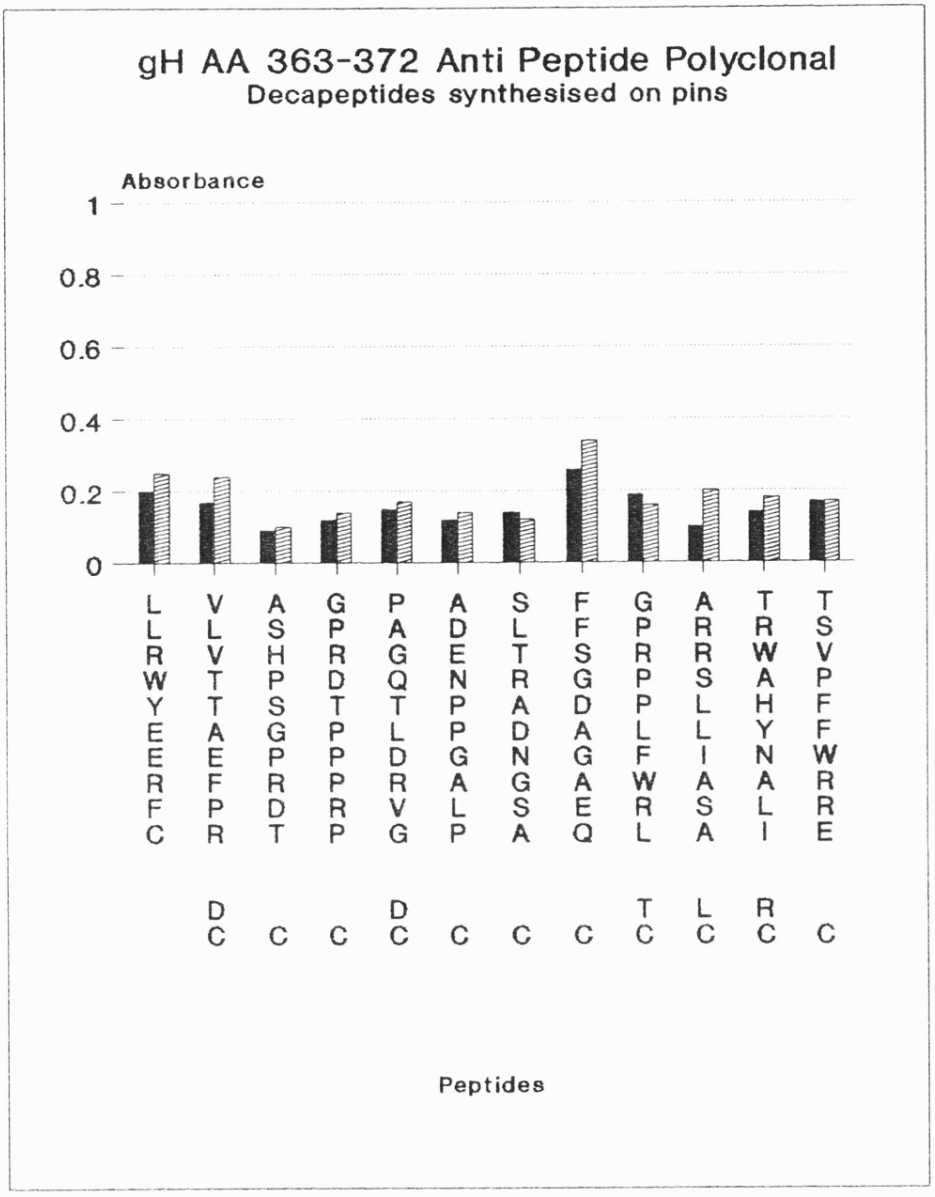


Fig 67. Reactivity of antipeptide 363-372 antibodies with Pin Decapeptides.

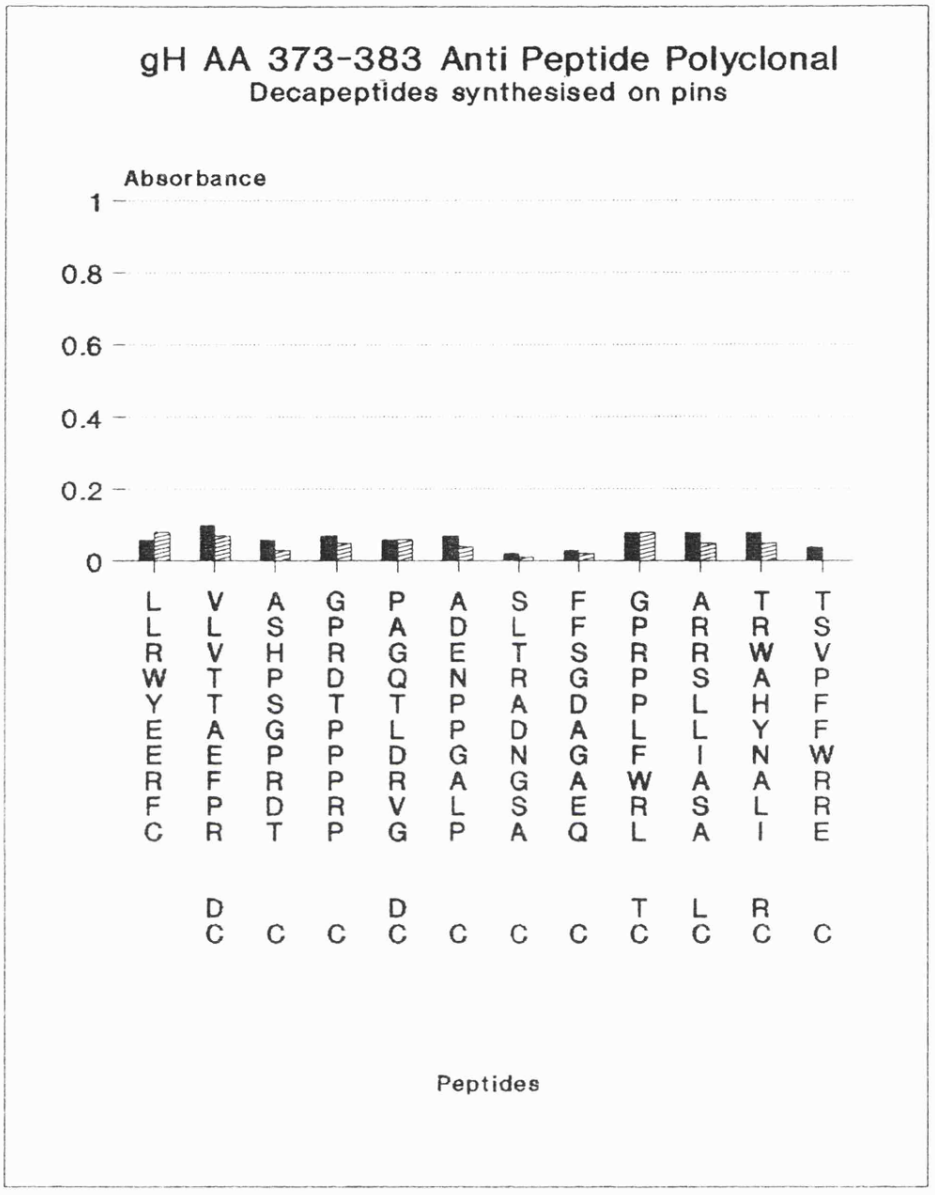


Fig 68. Reactivity of antipeptide 373-383 antibodies with Pin Decapeptides.

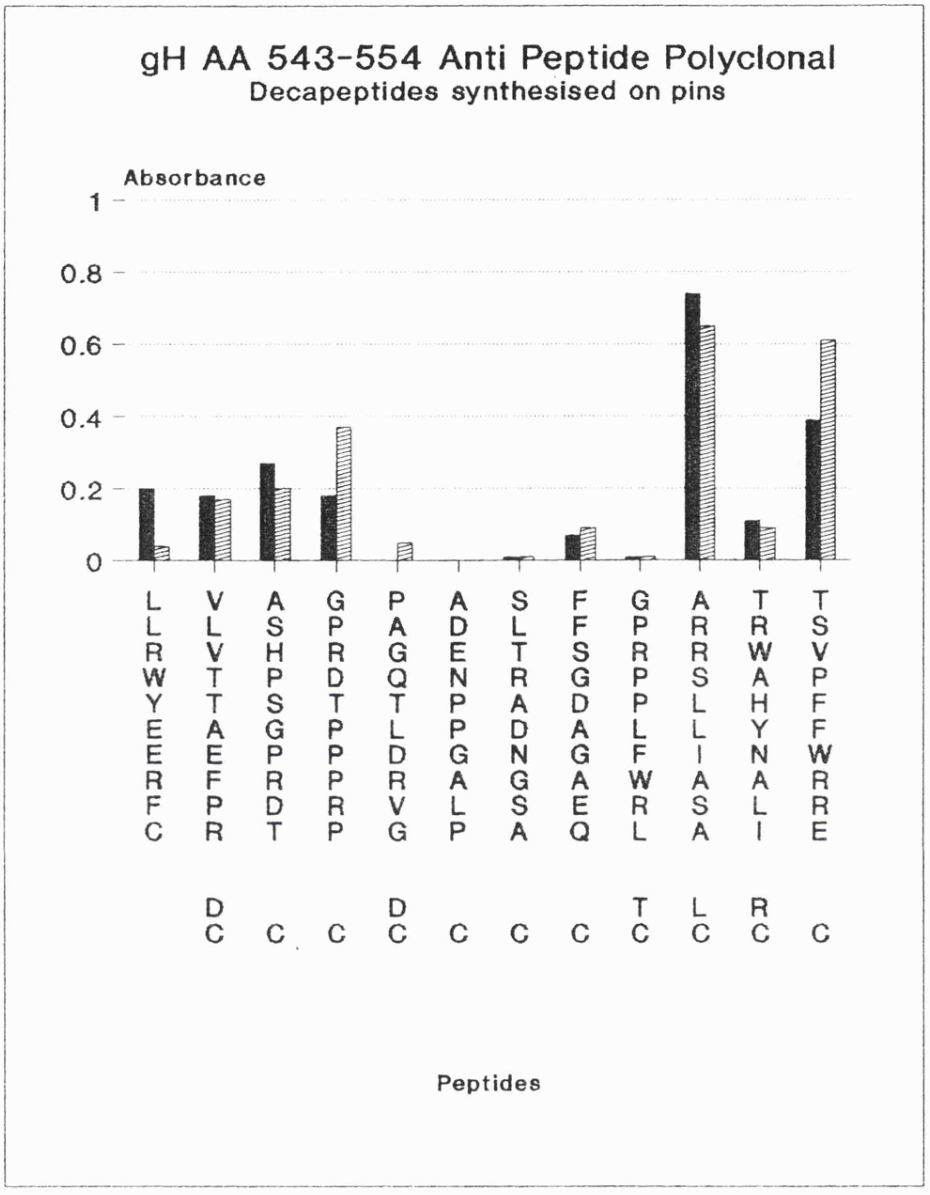


Fig 69. Reactivity of antipeptide 543-554 antibodies with Pin Decapeptides.

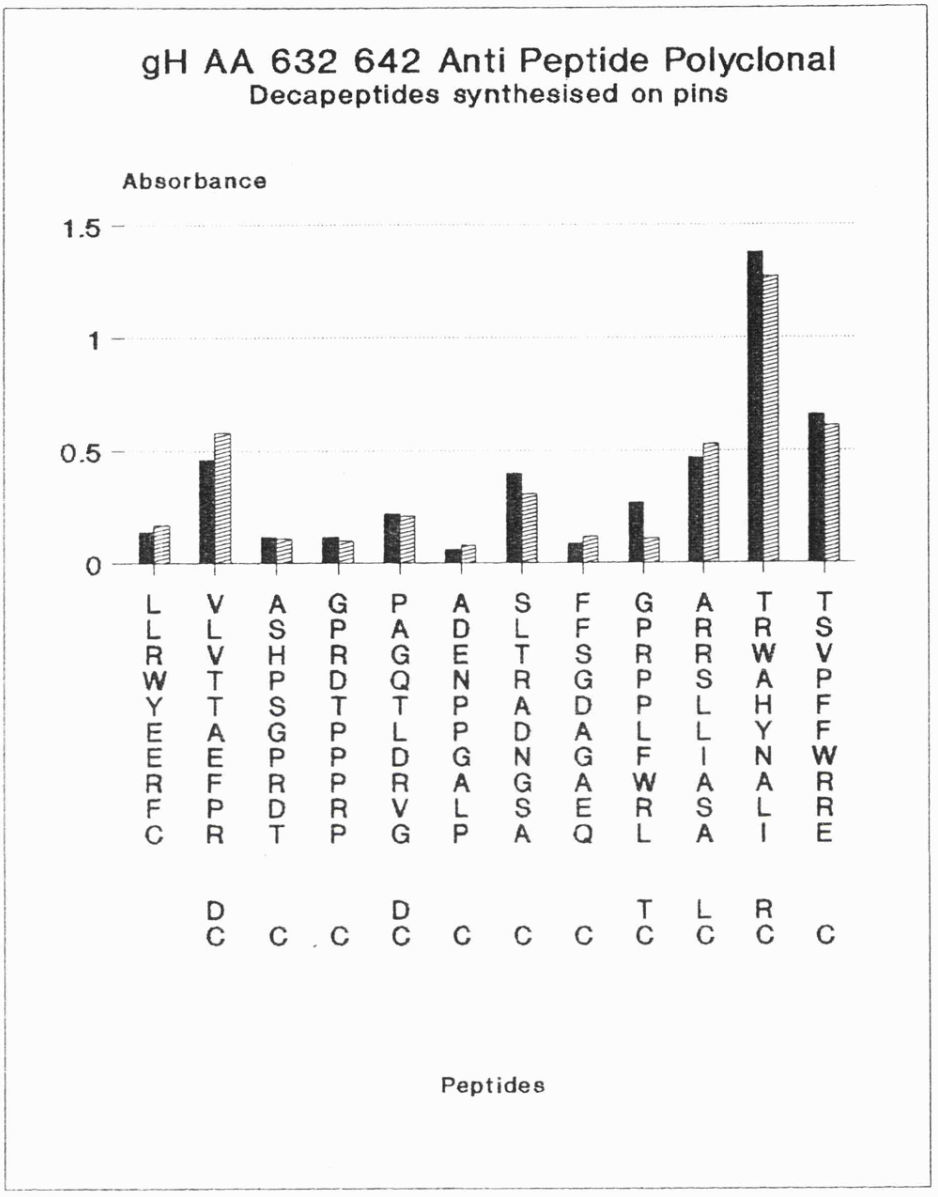


Fig 70. Reactivity of antipeptide 632-642 antibodies with Pin Decapeptides.

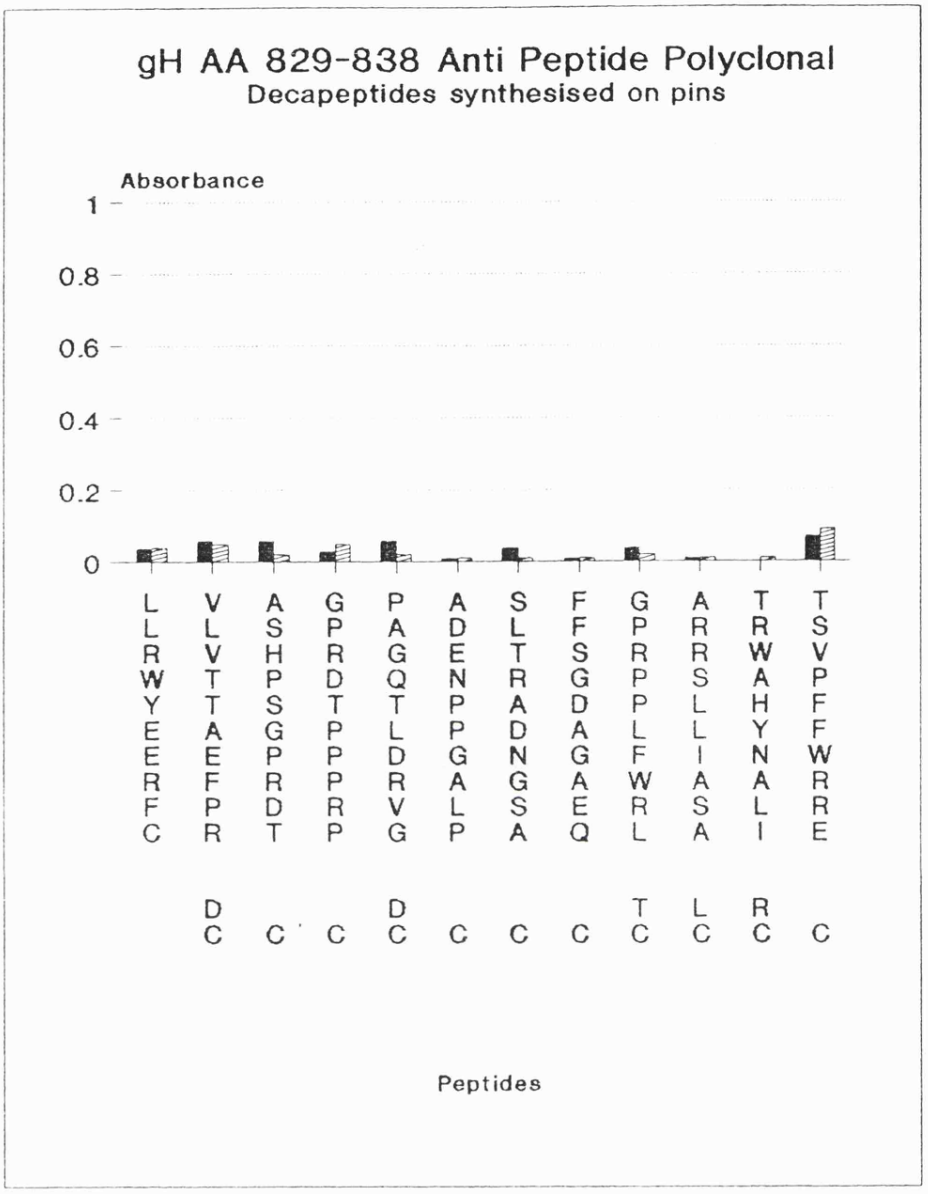


Fig 71. Reactivity of antipeptide 829-838 antibodies with Pin Decapeptides.

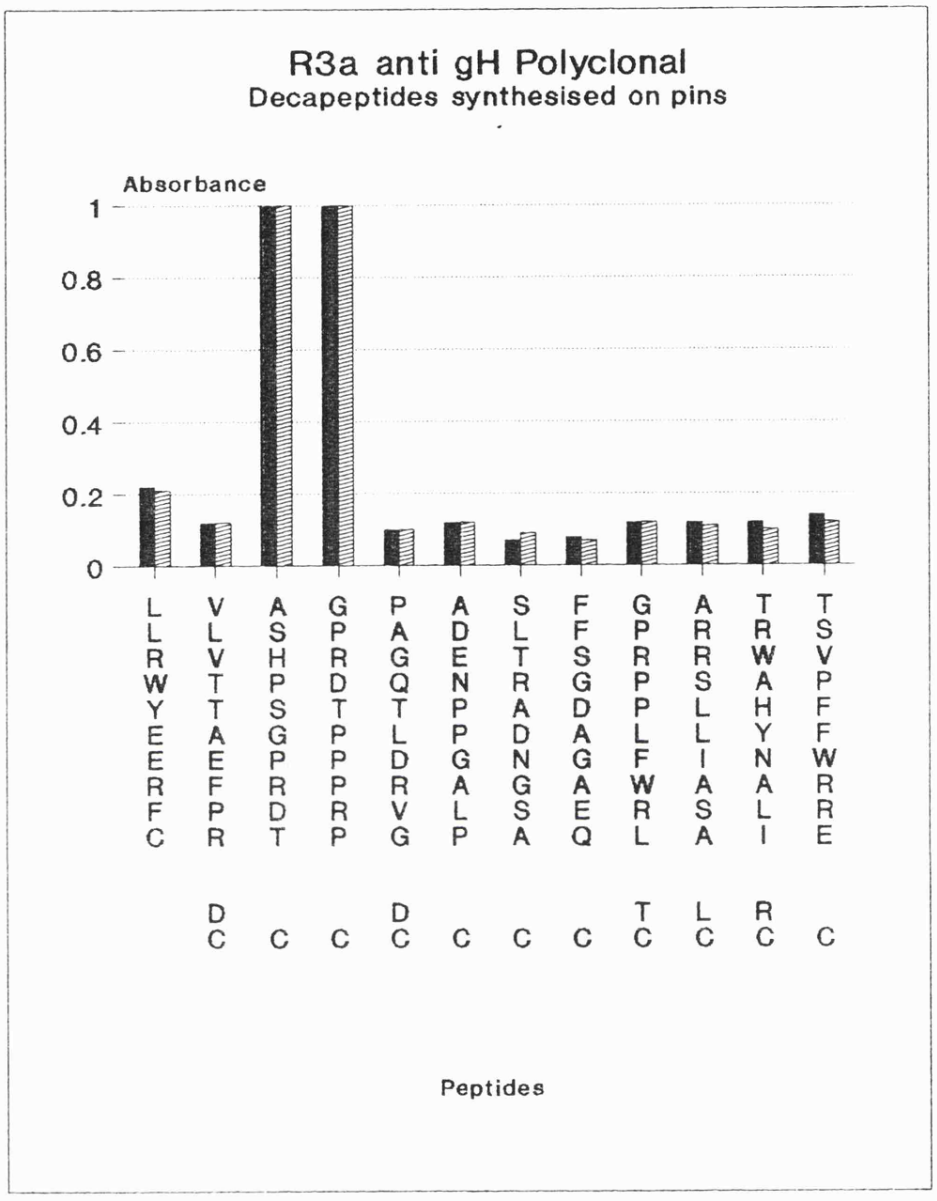


Fig 72. Reactivity of R3a antibodies with Pin Decapeptides.

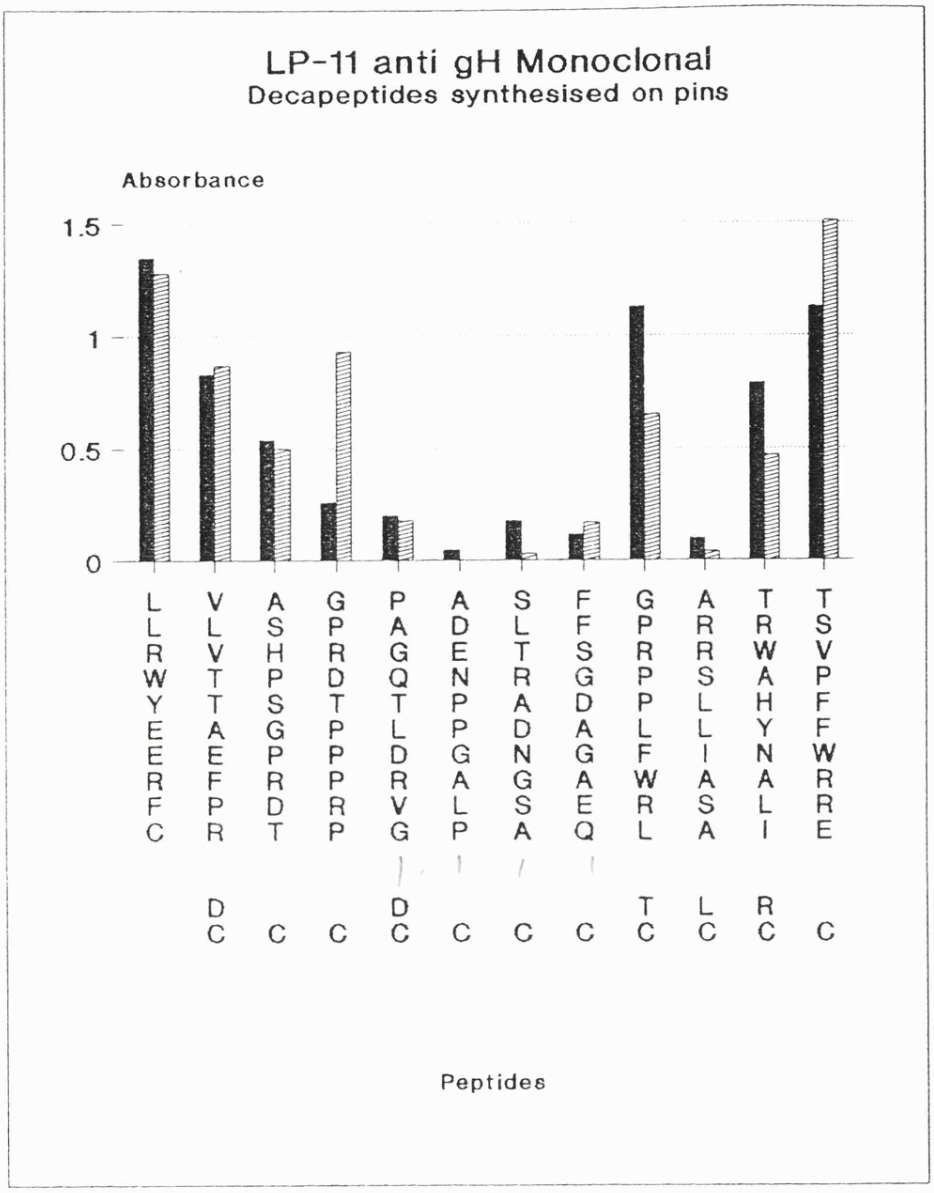


Fig 73. Reactivity of LP-11 antibodies with Pin Decapeptides.

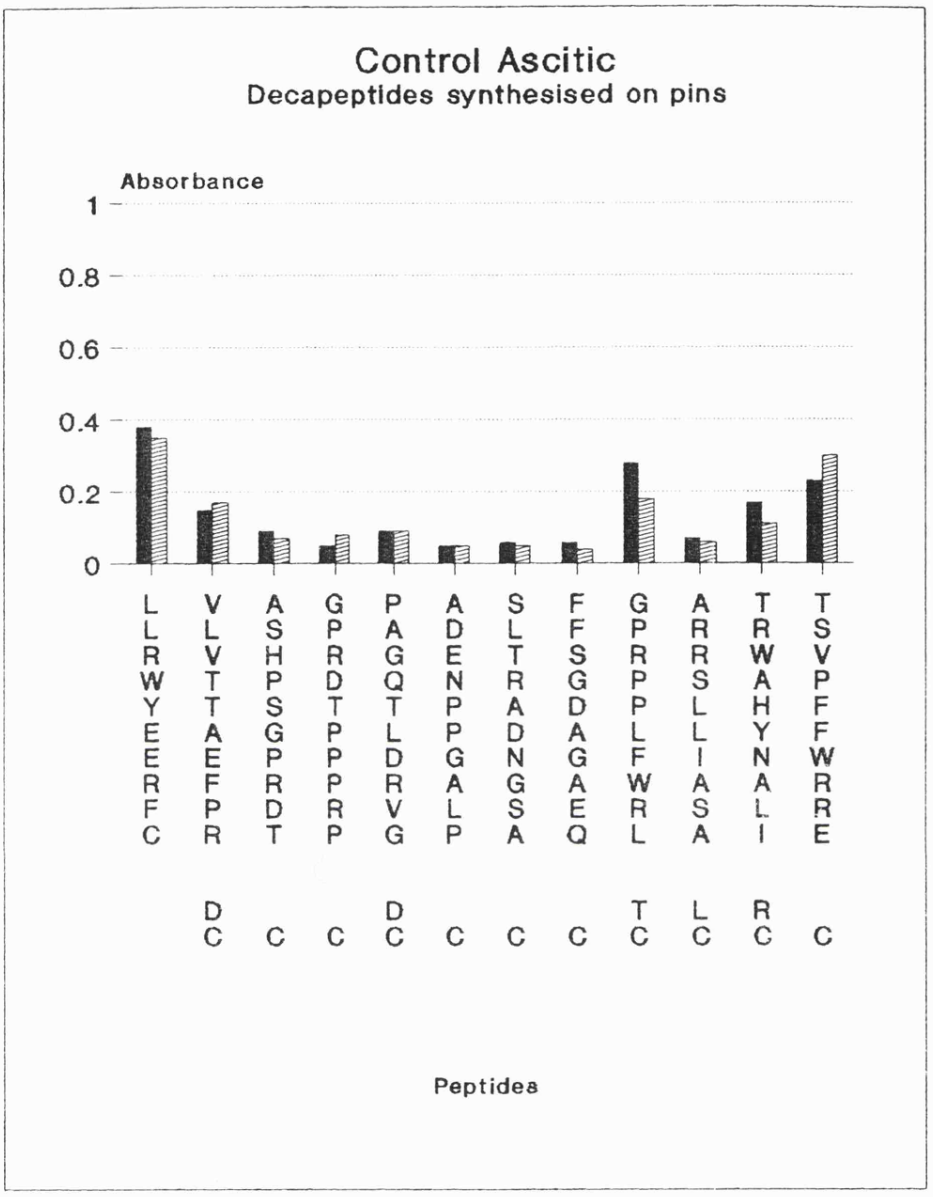


Fig 74. Reactivity of Control Ascitic antibodies with Pin Decapeptides.

Chapter 6

DISCUSSION

Discussion

Although glycoprotein H is one of three glycoproteins essential for HSV-1 infectivity it is the least well studied. The relative shortage of specific antibodies has been a contributory factor hindering progress. Considering the numerous attempts at generating antibodies against the spectrum of HSV proteins the scarcity of gH specific reagents is puzzling. Many have used whole infectious virus or crude viral protein preparations as an antigen source and it may be that B lymphocyte responses against quantitatively abundant components predominate over those directed toward minor constituents such as gH. It is also possible that gH is an intrinsically poor antigen. Attempts at increasing the odds in favour of producing gH specific reagents by expressing the gH-1 gene as part of a fusion protein or in a vaccinia vector for example have encountered problems. Antibodies generated using these methods have proved incapable of recognising the non-denatured native protein, suggesting subtle processing requirements before this glycoprotein adopts the correct conformation. These suspicions have been supported by the finding (Gompels and Minson 1989) that even when using a transient cell expression system noted for its correct processing of other viral glycoproteins, the gH gene product did not present the same epitopic repertoire as native non-denatured protein.

Reports describing the purification of gH or any other minor glycoprotein are not available and there was therefore an absence of guiding precedent concerning the most suitable method of purification. At the outset the particularly fickle nature of recombinant gH epitopes were just beginning to be revealed and due consideration was given to the nature of the antigen likely to be provided by the chosen method of purification. To some degree the properties of antibodies are dependent on the nature of the antigen, and for this reason purified gH-1 in its native form would be desirable, although the properties of antibodies raised against denatured antigen could be equally interesting.
The hybridoma LP-11 has been characterised as secreting immunoglobulin IgG 2a, a type which readily binds Protein A. Thus an immunoaffinity method of purification based on LP-11-protein A sepharose interaction was theoretically possible. Experiments illustrated [14C] gH-1 could be readily immunoprecipitated using these reagents, and the adoption of a similar approach on a larger scale for purification of quantities of antigen suitable for immunization proved successful.

The approach was relatively straight forward, with gH immunoprecipitated from LSAP and batch bound to Protein A sepharose. Elution at this stage using relatively mild pH conditions would probably have yielded antigen close to its native state. Unfortunately this approach would also have produced an inoculum containing mouse antibody which could lead to future cross reactive problems.

To circumvent this potential problem antigen-antibody complexes were separated by PAGE and gH eluted from appropriate gel sections. A modification of this technique using Western transferred gH as an alternative antigen source was also investigated.

A drawback with these methods was the probable denaturation of antigen due to the robust procedures involved. Experiments indicated that although eluted gH-1 exhibited a molecular weight consistent with that of the native molecule it was not amenable to re-immunoprecipitation by LP-11. Clearly, even in conditions designed to promote protein renaturation following elution (ie. removal of SDS, detergent, and return to physiological pH) the discontinuous epitopes recognised by LP-11 were lost.

Three LSAP's were prepared, two of which proceeded via the electroelution route and one utilized Western transferred antigen. If these had failed an immunogenic preparation containing immune complexes eluted directly from protein A sepharose would have been used as an antigen source.

According to estimates approximately 220ug of gH would be immunoprecipitated from each LSAP. Pessimistically assuming that antigen recovery following purification was at best 15% of that initially precipitated this would provide in the region of 33 ug. The quantity of gH required to induce an immune response was unknown, but since antibodies have been raised successfully against 0.25ug quantities of protein (Goding, 1986) it was considered that 33ug would prove adequate.

It transpired that gH specific rabbit polyclonal antibodies were successfully generated using antigen prepared by immunoaffinity electroelution purification. Glycoprotein H-1 Western transferred to nitrocellulose failed to elicit antibodies which would recognise [14C] gH by immunoprecipitation or Western blot analysis even though others have reported using this procedure with success.
Examination of the nitrocellulose autoradiograph (Fig. 9) clearly shows an excellent correlation between the position of marker [14C] gH-1 and the strip used as the basis for inoculum. There seems no reason why unlabelled gH would not be included in the excised nitrocellulose strip.
Solubilizing the membrane with DMSO may have been a factor in destroying antigenicity although DMSO is generally thought not to unduly effect antigens on nitrocellulose and is relatively non cytotoxic compared to some other organic solvents. Others have reported successfully using this solvent in similar systems (Aboud-Zeid, *et al.*, 1987, Knudson, 1985). Similarly, one electroeluted gH-1 preparation also failed and the reasons for this are uncertain.

Rabbit polyclonal serum R3a proved capable of immunoprecipitating [14C] gH-1 and recognition after Western transfer. Confirmation that the correct protein species had been recognised was provided by LP-11 immunoprecipitated gH-1 bound by R3a following Western transfer.

The fact that R3a antibodies would work in immunoprecipitation and Western blot suggests two possibilities concerning the recognised epitopes. Either the serum contains antibodies which interact with a continuous epitope present on both the native (immunoprecipitated) and denatured (Western transferred) protein, or the serum contains several clonal antibody sub-populations which recognise different epitopes. Thus an epitope involved in precipitation may differ from others responsible for recognition in westerns. The inability of R3a to neutralise virus indicates that these epitopes differ from those recognised by neutralising antibodies LP-11, 52S and 53S.

Generation of R3a specific polyclonal serum presents the opportunity for further gH purification by immune affinity methods without the problems associated with mouse immunoglobulin. In addition, the generation of this gH specific polyclonal also made possible experiments designed to identify antigenic determinants on the molecule using the latest peptide technology.

The peptide approach to epitope mapping has limitations and there is no certainty that all continuous epitopes of 6 residues or less will be detected. It is well known that peptides randomly attached to a solid phase do not always display similar conformation in solution. Thus antibodies capable of interacting with a peptide in solution may not react to the same peptide adsorbed to microtitre plates. An ideal peptide screening system would allow peptides to adopt the conformation seen in solution while being attached to a solid phase. The peptide synthesis method devised by Geysen and colleagues presently offers the closest approximation to this ideal, for large numbers of synthesised peptides. Even so objections can be levelled against it. For example it might be argued that a peptide free at its N-terminus but attached at its carboxy terminus might behave differently were this situation to be reversed.

Nevertheless experimental evidence has shown that this strategy can lead to the identification of important epitopes (Conlan *et al.* 1988, Wang *et al.* 1990, Langedijk *et al.* 1990). Peptides representing the entire gH-1 primary amino acid sequence were synthesised based on the gene sequence of HSV-1 strain 17 determined by McGeoch and Davison (1986). Polyclonal serum R3a and LP-11 were based on strain Troisbel and strain HFEM respectively.

It might be anticipated that a polyclonal monospecific serum raised against a large antigen would contain antibodies of different clonal origin capable of recognising a range of epitopes. It follows that were such antibodies to be used for screening purposes a relatively large number of potential epitopic peptides might be highlighted compared to those expected from a monoclonal antibody serum. Polyclonal serum R3a bound strongly to 18 peptides, some isolated in the series, some grouped together. A feature of the binding pattern was the span of approx 300 AA from peptide 202 to 504 in which no interaction was observed.

Peptides recognised in sequential groups may define an epitope core. This is illustrated by the following three peptides bound by R3a.

26 31
Q T D P W F

27 32
T D P W F L

28 33
D P W F L D

Assuming these peptides are bound by antibodies derived from a single clonal origin then the residues common to all three peptides, D P W F, would form the basis of an epitope core. It is known that an epitope can be represented by as few as four residues (Van Regenmortel, 1989) and this is confirmed by the control peptide PLAQ. Furthermore a single amino acid change (GLAQ) will result in loss of reactivity with antibody.

This interpretation runs into difficulty when more than three consecutive peptides are included in a group.

A group of four peptides reactive with R3a such as those below

188
G P R D T P

189
P R D T P P

190
R D T P P T

191
D T P P T R

would yield an epitope core of only three residues [D T P].

While this may be feasible, an epitope of 3 residues probably approaches the lower limit of specificity, with the likelihood that any particular three residues will randomly occur in a sequence increased compared with a 4 residue sequence. However, the tripeptide RGD sequence of residues has been implicated as a common functional motif in proteins involved in cell attachment (Ruoslahti and Pierschbacher, 1987, Fox *et al.* 1989) suggesting that in terms of protein-protein interaction three resides will suffice. It may be noted that the HSV-1 gH sequence contains an RGD sequence not implicated in the epitopes identified by this study.

An alternative explanation for the recognition of 4 or more sequential peptides is that such groups reflect an antigenic region or site containing within it more than one epitope. Taken to its extreme this view would contend that every peptide in a group has the potential to represent an epitope recognised by an antibody of distinct clonal origin.

Cohen and colleagues (1985) refer to a similar problem when sorting gD monoclonal antibodies into groups according to their binding characteristics, and point out that monoclonal antibodies included within a group may not react with precisely the same epitope but may react with neighbouring epitopes which make up an antigenic site. As a consequence of this idea, an antigenic site is represented by a widening span of residues, in contrast to the view, previously mentioned, which condensed and defined an epitope core. The antigenic core hypothesis could be tested by screening smaller synthetic peptides for antibody binding.

Assuming that binding to individual peptides, pairs, or groups of up to 3 adjacent hexapeptides represent epitopes, whereas binding to larger clusters of 4 or more adjacent hexapeptides points to a larger antigenic site, then polyclonal serum R3a identifies 9 potential epitopes between 4 and 6 AA in length and a single larger determinant spanning AA 193 to 196. This concept of a larger antigenic region is given support by the observation that antibodies from R3a bind the nearby peptides 190, 201 and 202.

It might be anticipated that the pattern and number of peptides recognised by a monoclonal antibody would differ from those of a polyclonal serum with the numbers of interactive peptides reduced.

This proved to be the case for LP-11 with strongest binding at 6 sites, 4 individual peptides and 2 pairs of peptides, being consistently highly reactive with LP-11. This interaction tended to be concentrated over a relatively narrow range from peptides 297 to 369. Interaction with LP-11 was a surprising finding since all indications pointed to LP-11 epitopes being non-sequential in nature and these findings also raise the question of how antibody derived from a single clonal origin could interact with 6 potential binding sites. One possibility is that some of these peptides represent mimotopes, peptides differing in AA composition which present similar conformational structure to actual epitopes, and are thus cross-reactive with antibody.

Additionally the peptides recognised by LP-11 could represent small continuous parts of a longer discontinuous epitope a view also extolled by Van-Regenmortel (1989). Assuming that each of the three complementary determining regions (CDR) available on each chain of an IgG molecule has the potential to interact with a small peptide then in its simplest form (one CDR, binds one peptide) this model could account for the binding of up to 6 peptides. Naively assuming an average of three residues to be the minimum recognition requirement for each CDR binding site, then the number of AA required for a complete non-sequential epitope would be 18. This aligns reasonably well with findings from X-Ray crystallography studies of antigen-antibody complexes which have indicated the antibody contact surface of an antigen to be comprised of approximately 16 AA (Amit *et al.* 1986, Sheriff *et al.* 1987). However it is probably unlikely that all six components of a non-sequential epitope had been identified, although the concentration of LP-11 reactivity over a span of 70 AA was intriguing.

Probing the hexapeptide series with antibodies raised against the TrpE/gH fusion protein proved inconclusive. Since only amino acids 270-660 of the gH sequence was incorporated into the fusion product (Minson-pers comm) it was anticipated this would be reflected by reactivity with pin hexapeptides incorporating amino acids within this range. Results were disappointing with background levels elevated and the distinct reactivity patterns observed with R3a and LP-11 absent, although normal rabbit serum antibodies were unreactive. A possible explanation for these results maybe that following approximately 25 antibody screens the quality of pin hexapeptides may have deteriorated although assays suggested the four residue control peptides remained reactive.

All twelve antipeptide antibodies tested were reactive with their corresponding peptide adsorbed to microtitre plates. The variation in antibody-peptide reactivity between systems was illustrated by the finding that only seven antipeptide antibodies would bind to homologous decapeptides synthesised on pins.

Peptides synthesised on the basis of their proximity to known antibody resistant gH gene point mutations proved incapable of eliciting antipeptide antibodies reactive with the glycoprotein. It therefore seems unlikely that gH mutant viruses escape antibody neutralisation because of amino acid substitution bringing about changes within continuous neutralising epitopes. In other words these point mutations bring about escape from neutralisation because of their influence on tertiary structure. Computer algorithm indications (Krchnak, 1987) failed to highlight any tendancy for ß turn structure from tetrapeptides contained within these sequences.

The peptide 373-383, predicted from algorithm data to encompass a tetrapeptide with high probability of a beta turn structure also failed to elicit antibodies reactive with the glycoprotein.

By contrast, three of the six peptides chosen on the basis of R3a and LP-11 interaction with pin-hexapeptides elicited antibodies which appeared reactive with the glycoprotein.

The properties of antibodies raised against peptides 183-192 and 188-197, chosen because of R3a reactivity proved interesting, in that these two peptides overlapped by five residues and both encompassed tetrapeptides with a high probability of a beta turn structure according to computer algorithm predictions (Krchnak, 1987). In addition both antipeptide antibodies were reactive against homologous peptide adsorbed to microtitre plates and pin decapeptides. R3a antibodies also proved reactive both with peptide on plate and pin decapeptides. This reinforced the validity of the initial hexapeptide reactivity, with all indications pointing to potential epitopes within these sequences. However, only antibodies to peptide 188-197 proved reactive with the glycoprotein, and capable of

binding to the molecule following Western transfer, and thus for the first time confirming the position of a continuous epitope on the glycoprotein.
The amino acid sequence ^{188}G P R D T ^{192} was common to both peptides, and seems to infer residues downstream of this sequence are important for this epitope.

LP-11 reactivity with hexapeptides led to the synthesis of 4 peptides eliciting four antipeptide antisera. At least one and probably two of these sera appeared reactive with the glycoprotein.

Antibodies from only one of these sera proved reactive with homologous decapeptides synthesised on pins according to the Geysen method, but they proved unreactive with the glycoprotein. LP-11 was itself unreactive with pin decapeptides or peptide adsorbed to microtitre plate.

Peptide 290-300 elicited non neutralising antibodies reactive with gH-1 following Western transfer confirming the position of a second continuous epitope. There were contraindications concerning the anticipated properties of this antipeptide serum since both neutralising LP-11 and non-neutralising anti Trp E/gH fusion protein appeared to bind to hexapeptide 297. The limitations of computer algorithm prediction of epitopes was also well illustrated by this result. Only two of the LP-11 related peptides used to elicit antibodies would have been chosen on the basis of Krchnaks (1987) algorithm and peptide 290 -300 would not have been included.

Antipeptide 302-311 antibodies immunoprecipitated two glycoprotein bands with apparent Mr's close to gH-1. If these precipitated species are confirmed as gH related, by probing Western transferred precipitated glycoprotein for example, the position of a third continuous epitope present on the native non-denatured molecule will have been identified.

One question raised by these results is why antipeptide antibodies are non-neutralising although selected on the basis of neutralising LP-11 activity with the pin hexapeptide series? In other words, how can a peptide (continuous epitope) be involved in the binding of both neutralising and non-neutralising antibodies?

An explanation, touched upon earlier, is that these peptides represent only part (a sub-epitope?) of a larger discontinuous epitope. A hypothesis suggested by this author is that antigenic regions of particularly important proteins may offer a selection of sub epitopes and the particular combination of sub-epitopes selected (bound) will dictate an antibodies biological properties.
Antibody commitment to a non neutralising selection could work in favour of the parasite with ineffectual selection obstructing further potential lethal interaction. This hypothesis could be crudely

tested using a cocktail of dissociated Fab light and heavy chains which might prove neutralising, whereas a whole antibody cocktail would not.

Unfortunately these results tell us little about gH structure-function relationships and protein-protein interactive sites other than the antibodies reactive with continuous epitopes defined in this study are alone insufficient to bring about neutralisation. It could be that the mixture of these antibodies would prove more effective. The indications are that the relationship between gH-1 epitopes identified in this study and the biological properties of their corresponding antibodies is complex. Based on the results of R3a and LP-11 presented here it seems that gH antibodies of dissimilar biological properties are reactive with peptides originating from different regions of the primary amino acid sequence.

The relatively restricted span of hexapeptides reactive with LP-11 and the closeness of two peptides both capable of eliciting glycoprotein reactive antibodies is striking and deserves further investigation as do the several other potential epitopes identified by probing pin hexapeptides with R3a. Other avenues of exploration might therefore include further generation and characterisation of antipeptide antibodies, and continuation of mapping studies utilising larger pin peptides and novel antibodies raised against non-denatured gH-1 immuno-affinity purified using R3a. Further characterisation of the identified epitopes in terms of essential residues could also be determined. In addition the appearance of two bands following immunoprecipitation from HSV-1 infected cells with anti-peptide antibody will require explanation.

The strategy overall proved remarkably successful. In the short term novel polyclonal antibodies were generated against purified gH-1 which proved capable of reacting in immunoprecipitation and Western transfer. The use of synthetic peptide technology led directly to the synthesis of six peptides thought to have potential homology with epitopes of the glycoprotein.
Two and possibly a third peptide elicited antisera which were reactive with the glycoprotein and for the first time confirmed the identity of gH-1 B lymphocyte epitopes.

APPENDIX I

Appendix I Synthetic Peptide Series.

1	6	31	36	61	66
	M G N G L W		F L D G L G		P P R G F L
2	7	32	37	62	67
	G N G L W F		L D G L G M		P R G F L A
3	8	33	38	63	68
	N G L W F V		D G L G M D		R G F L A P
4	9	34	39	64	69
	G L W F V G		G L G M D R		G F L A P P
5	10	35	40	65	70
	L W F V G V		L G M D R M		F L A P P D
6	11	36	41	66	71
	W F V G V I		G M D R M Y		L A P P D E
7	12	37	42	67	72
	F V G V I I		M D R M Y W		A P P D E L
8	13	38	43	68	73
	V G V I I L		D R M Y W R		P P D E L N
9	14	39	44	69	74
	G V I I L G		R M Y W R D		P D E L N L
10	15	40	45	70	75
	V I I L G V		M Y W R D T		D E L N L T
11	16	41	46	71	76
	I I L G V A		Y W R D T N		E L N L T T
12	17	42	47	72	77
	I L G V A W		W R D T N T		L N L T T A
13	18	43	48	73	78
	L G V A W G		R D T N T G		N L T T A S
14	19	44	49	74	79
	G V A W G Q		D T N T G R		L T T A S L
15	20	45	50	75	80
	V A W G Q V		T N T G R L		T T A S L P
16	21	46	51	76	81
	A W G Q V H		N T G R L W		T A S L P L
17	22	47	52	77	82
	W G Q V H D		T G R L W L		A S L P L L
18	23	48	53	78	83
	G Q V H D W		G R L W L P		S L P L L R
19	24	49	54	79	84
	Q V H D W T		R L W L P N		L P L L R W
20	25	50	55	80	85
	V H D W T E		L W L P N T		P L L R W Y
21	26	51	56	81	86
	H D W T E Q		W L P N T P		L L R W Y E
22	27	52	57	82	87
	D W T E Q T		L P N T P D		L R W Y E E
23	28	53	58	83	88
	W T E Q T D		P N T P D P		R W Y E E R
24	29	54	59	84	89
	T E Q T D P		N T P D P Q		W Y E E R F
25	30	55	60	85	90
	E Q T D P W		T P D P Q K		Y E E R F C
26	31	56	61	86	91
	Q T D P W F		P D P Q K P		E E R F C F
27	32	57	62	87	92
	T D P W F L		D P Q K P P		E R F C F V
28	33	58	63	88	93
	D P W F L D		P Q K P P R		R F C F V L
29	34	59	64	89	94
	P W F L D G		Q K P P R G		F C F V L V
30	35	60	65	90	95
	W F L D G L		K P P R G F		C F V L V T

91 96
FVLVTT
92 97
VLVTTA
93 98
LVTTAE
94 99
VTTAEF
95 100
TTAEFP
96 101
TAEFPR
97 102
AEFPRD
98 103
EFPRDP
99 104
FPRDPG
100 105
PRDPGQ
101 106
RDPGQL
102 107
DPGQLL
103 108
PGQLLY
104 109
GQLLYI
105 110
QLLYIP
106 111
LLYIPK
107 112
LYIPKT
108 113
YIPKTY
109 114
IPKTYL
110 115
PKTYLL
111 116
KTYLLG
112 117
TYLLGR
113 118
YLLGRP
114 119
LLGRPP
115 120
LGRPPN
116 121
GRPPNA
117 122
RPPNAS
118 123
PPNASL
119 124
PNASLP
120 125
NASLPA
121 126
ASLPAP
122 127
SLPAPT
123 128
LPAPTT
124 129
PAPTTV
125 130
APTTVE
126 131
PTTVEP
127 132
TTVEPT
128 133
TVEPTA
129 134
VEPTAQ
130 135
EPTAQP
131 136
PTAQPP
132 137
TAQPPP
133 138
AQPPPS
134 139
QPPPSV
135 140
PPPSVA
136 141
PPSVAP
137 142
PSVAPL
138 143
SVAPLK
139 144
VAPLKG
140 145
APLKGL
141 146
PLKGLL
142 147
LKGLLH
143 148
KGLLHN
144 149
GLLHNP
145 150
LLHNPA
146 151
LHNPAA
147 152
HNPAAS
148 153
NPAASV
149 154
PAASVL
150 155
AASVLL
151 156
ASVLLR
152 157
SVLLRS
153 158
VLLRSR
154 159
LLRSRA
155 160
LRSRAW
156 161
RSRAWV
157 162
SRAWVT
158 163
RAWVTF
159 164
AWVTFS
160 165
WVTFSA
161 166
VTFSAV
162 167
TFSAVP
163 168
FSAVPD
164 169
SAVPDP
165 170
AVPDPE
166 171
VPDPEA
167 172
PDPEAL
168 173
DPEALT
169 174
PEALTF
170 175
EALTFP
171 176
ALTFPR
172 177
LTFPRG
173 178
TFPRGD
174 179
FPRGDN
175 180
PRGDNV
176 181
RGDNVA
177 182
GDNVAT
178 183
DNVATA
179 184
NVATAS
180 185
VATASH

181 186
A T A S H P
182 187
T A S H P S
183 188
A S H P S G
184 189
S H P S G P
185 190
H P S G P R
186 191
P S G P R D
187 192
S G P R D T
188 193
G P R D T P
189 194
P R D T P P
190 195
R D T P P P
191 196
D T P P P R
192 197
T P P P R P
193 198
P P P R P P
194 199
P P R P P V
195 200
P R P P V G
196 201
R P P V G A
197 202
P P V G A R
198 203
P V G A R R
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202 207
R R H P T T
203 208
R H P T T E
204 209
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205 210
P T T E L D
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E L D I T H
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L D I T H L
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D I T H L H
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L H N A S T
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G L L R S P
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L L R S P G
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L R S P G R
229 234
R S P G R Y
230 235
S P G R Y V
231 236
P G R Y V Y
232 237
G R Y V Y F
233 238
R Y V Y F S
234 239
Y V Y F S P
235 240
V Y F S P S
236 241
Y F S P S A
237 242
F S P S A S
238 243
S P S A S T
239 244
P S A S T W
240 245
S A S T W P
241 246
A S T W P V
242 247
S T W P V G
243 248
T W P V G I
244 249
W P V G I W
245 250
P V G I W T
246 251
V G I W T T
247 252
G I W T T G
248 253
I W T T G E
249 254
W T T G E L
250 255
T T G E L V
251 256
T G E L V L
252 257
G E L V L G
253 258
E L V L G C
254 259
L V L G C D
255 260
V L G C D A
256 261
L G C D A A
257 262
G C D A A L
258 263
C D A A L V
259 264
D A A L V R
260 265
A A L V R A
261 266
A L V R A R
262 267
L V R A R Y
263 268
V R A R Y G
264 269
R A R Y G R
265 270
A R Y G R E
266 271
R Y G R E F
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Y G R E F M
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G R E F M G
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R E F M G L
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282 287
P P V E V M
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P V E V M V
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V M V V P A
287 292
M V V P A G
288 293
V V P A G Q
289 294
V P A G Q T
290 295
P A G Q T L
291 296
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292 297
G Q T L D R
293 298
Q T L D R V
294 299
T L D R V G
295 300
L D R V G D
296 301
D R V G D P
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R V G D P A
298 303
V G D P A D
299 304
G D P A D E
300 305
D P A D E N
301 306
P A D E N P
302 307
A D E N P P
303 308
D E N P P G
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G A L P G P
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G P R Y R V
317 322
P R Y R V F
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F V L G S L
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R A D N G S
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332 337
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333 338
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334 339
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337 342
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A L R R V G
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343 348
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Y A E F F S

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362 367
EFFSGD
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FFSGDA
364 369
FSGDAG
365 370
SGDAGA
366 371
GDAGAE
367 372
DAGAEQ
368 373
AGAEQG
369 374
GAEQGP
370 375
AEQGPR
371 376
EQGPRP
372 377
QGPRPP
373 378
GPRPPL
374 379
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RLTGLL
382 387
LTGLLA
383 388
TGLLAT
384 389
GLLATS
385 390
LLATSG
386 391
LATSGF
387 392
ATSGFA
388 393
TSGFAF
389 394
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390 395
GFAFVN
391 396
FAFVNA
392 397
AFVNAA
393 398
FVNAAH
394 399
VNAAHA
395 400
NAAHAN
396 401
AAHANG
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AHANGA
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HANGAV
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ANGAVC
400 405
NGAVCL
401 406
GAVCLS
402 407
AVCLSD
403 408
VCLSDL
404 409
CLSDLL
405 410
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DLLGFL
408 413
LLGFLA
409 414
LGFLAH
410 415
GFLAHS
411 416
FLAHSR
412 417
LAHSRA
413 418
AHSRAL
414 419
HSRALA
415 420
SRALAG
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417 422
ALAGLA
418 423
LAGLAA
419 424
AGLAAR
420 425
GLAARG
421 426
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427 432
AGCAAD
428 433
GCAADS
429 434
CAADSV
430 435
AADSVF
431 436
ADSVFF
432 437
DSVFFN
433 438
SVFFNV
434 439
VFFNVS
435 440
FFNVSV
436 441
FNVSVL
437 442
NVSVLD
438 443
VSVLDP
439 444
SVLDPT
440 445
VLDPTA
441 446
LDPTAR
442 447
DPTARL
443 448
PTARLQ
444 449
TARLQL
445 450
ARLQLE
446 451
RLQLEA
447 452
LQLEAR
448 453
QLEARL
449 454
LEARLQ
450 455
EARLQH

451 456
A R L Q H L

452 457
R L Q H L V

453 458
L Q H L V A

454 459
Q H L V A E

455 460
H L V A E I

456 461
L V A E I L

457 462
V A E I L E

458 463
A E I L E R

459 464
E I L E R E

460 465
I L E R E Q

461 466
L E R E Q S

462 467
E R E Q S L

463 468
R E Q S L A

464 469
E Q S L A L

465 470
Q S L A L H

466 471
S L A L H A

467 472
L A L H A L

468 473
A L H A L G

469 474
L H A L G Y

470 475
H A L G Y Q

471 476
A L G Y Q L

472 477
L G Y Q L A

473 478
G Y Q L A F

474 479
Y Q L A F V

475 480
Q L A F V L

476 481
L A F V L D

477 482
A F V L D S

478 483
F V L D S P

479 484
V L D S P S

480 485
L D S P S A

481 486
D S P S A Y

482 487
S P S A Y D

483 488
P S A Y D A

484 489
S A Y D A V

485 490
A Y D A V A

486 491
Y D A V A P

487 492
D A V A P S

488 493
A V A P S A

489 494
V A P S A A

490 495
A P S A A H

491 496
P S A A H L

492 497
S A A H L I

493 498
A A H L I D

494 499
A H L I D A

495 500
H L I D A L

496 501
L I D A L Y

497 502
I D A L Y A

498 503
D A L Y A E

499 504
A L Y A E F

500 505
L Y A E F L

501 506
Y A E F L G

502 507
A E F L G G

503 508
E F L G G R

504 509
F L G G R V

505 510
L G G R V L

506 511
G G R V L T

507 512
G R V L T T

508 513
R V L T T P

509 514
V L T T P V

510 515
L T T P V V

511 516
T T P V V H

512 517
T P V V H R

513 518
P V V H R A

514 519
V V H R A L

515 520
V H R A L F

516 521
H R A L F Y

517 522
R A L F Y A

518 523
A L F Y A S

519 524
L F Y A S A

520 525
F Y A S A V

521 526
Y A S A V L

522 527
A S A V L R

523 528
S A V L R Q

524 529
A V L R Q P

525 530
V L R Q P F

526 531
L R Q P F L

527 532
R Q P F L A

528 533
Q P F L A G

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P F L A G V

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F L A G V P

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L A G V P S

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A G V P S A

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G V P S A V

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V P S A V Q

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P S A V Q R

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S A V Q R E

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A V Q R E R

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V Q R E R A

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Q R E R A R

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R E R A R R

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E R A R R S
542 547
R A R R S L
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A R R S L L
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R R S L L I
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R S L L I A
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S L L I A S
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L L I A S A
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L I A S A L
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I A S A L C
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A S A L C T
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S A L C T S
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A L C T S D
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L C T S D V
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C T S D V A
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S D V A A A
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D V A A A T
558 563
V A A A T N
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A A A T N A
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A A T N A D
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T N A D L R
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N A D L R T
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A D L R T A
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D L R T A L
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L R T A L A
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T A L A R A
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A L A R A D
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L A R A D H
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A R A D H Q
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R A D H Q K
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A D H Q K T
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D H Q K T L
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H Q K T L F
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K T L F W L
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T L F W L P
579 584
L F W L P D
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F W L P D H
581 586
W L P D H F
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L P D H F S
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P D H F S P
584 589
D H F S P C
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H F S P C A
586 591
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F D L D E S
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D L D E S B
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T R S E T P
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T C E G S T
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C E G S T R
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E G S T R D
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G S T R D I
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T R D I E S
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R D I E S K
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D I E S K R
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I E S K R L
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R T Q N Q R

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AVFMRY
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VFMRYT
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FMRYTP
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TPAGEV
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EVMSVL
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TEGAPS
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EGAPSV
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GAPSVF
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VIHLLA
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IHLLAF
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790 795
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791 796
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792 797
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794 799
DTQPVA
795 800
TQPVAA
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QPVAAI
797 802
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VAAIAP
799 804
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PGFLAA
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AASALG
808 813
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809 814
SALGVV
810 815
ALGVVM

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812 817
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813 818
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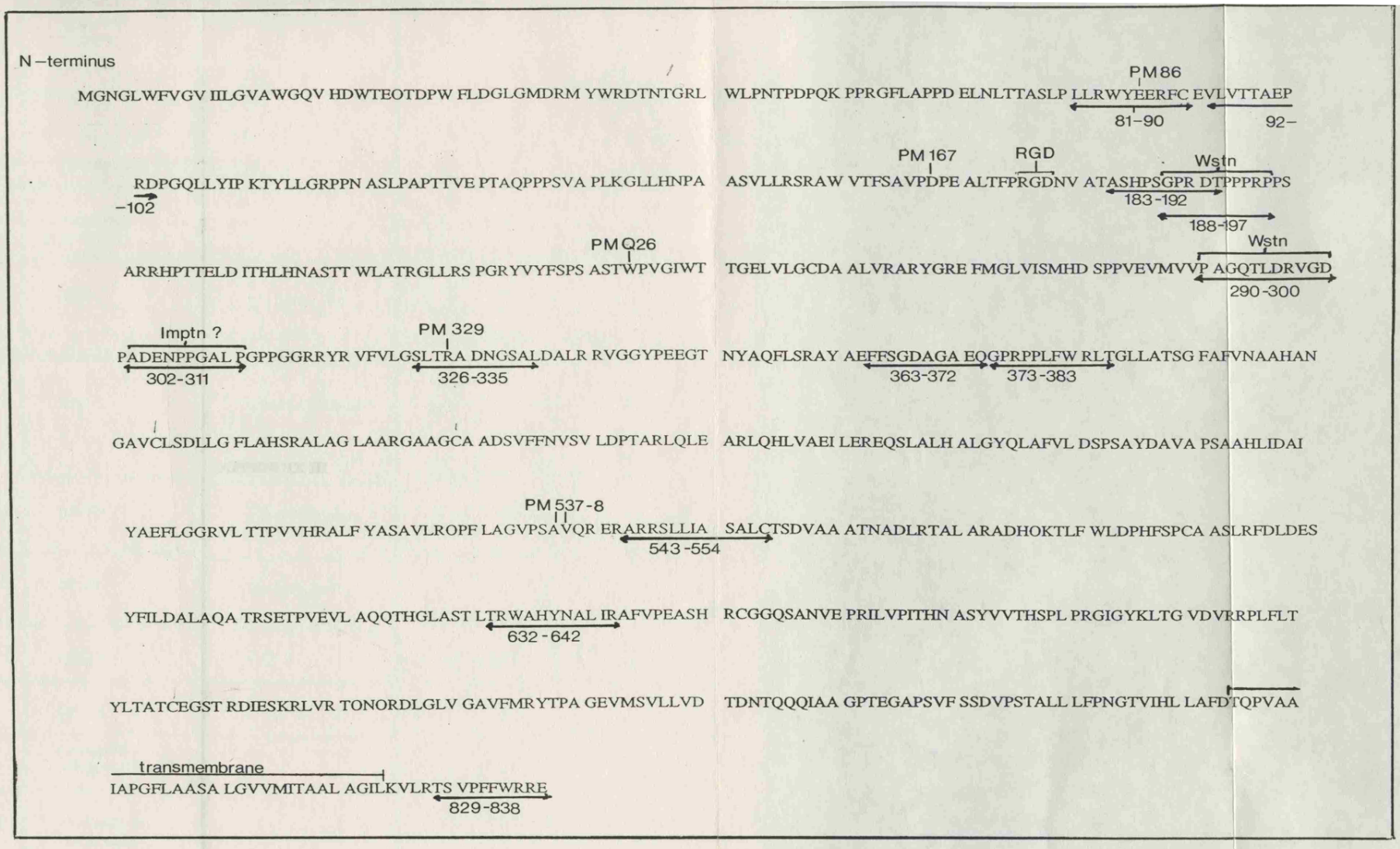
F F W R R E

APPENDIX II



Appendix II Molecular Map.

APPENDIX III

Synthesised Antigenic Peptide	Selection Criteria	Corresponding Hexapeptides	Recognition of Antigenic Peptide on Plate. End Pt. Reciprocal dil. x1000		Antipep Ab. Reactivity with homologous pin Decapeptide.	Antipep Ab. Reactivity with other pin Decapeptide.	Anti-gH Ab Reactivity R3a Plates	R3a Pin	LP-11 Plates	LP-11 Pin	Reactivity with Glycoprotein
81 90 LLRWYEERFC	PM at AA86	-	Yes	(16)	Yes	None	No	No	No	Yes	-
92 102 VLVTTAEFPRD	Proximity to AA86	-	Yes	(256)	Yes	None	No	No	No	Yes	-
183 192 ASHPSGPRDT	R3a	HPSGPR[190]	Yes	(64)	Yes	None	Yes	Yes	No	No (less than the control pep)	-
188 197 GPRDTPPPRP	R3a	GPRDTP[193] PRDTPP RDTPPP DTPPPR	Yes	(64)	Yes	None	Yes	Yes	No	No	gH-1 in Western
290 300 PAGQTLDRVGD	LP-11	GQTLDR[297] RVGDPA[302]	Yes	(64)	No	None	No	No	No	No	gH-1 in Western
302 311 ADENPPGALP	LP-11	DENPPG[308] ENPPGA[309]	Yes	(8)	No	None	No	No	No	No	Immptn (tbc)
326 335 SLTRADNGSA	LP-11	TRADNG[333]	Yes	(2)	No	None	No	No	No	No	-
363 372 FFSGDAGAEQ	LP-11	FSGDAG	Yes	(16)	Yes	None	No	No	No	No	-
373 383 GPRPPLFWRLT	Krchnack ($GPRP2.07x10^4$)	-	Yes	(64)	No	None	No	No	No	No	-
543 554 ARRSLLIASALC	Proximity to PM 536/537	RARRSL[547] ARRSLL[548]	Yes	(2)	Yes	Yes (TSVPFFWRRE)	No	No	No	No	-
632 642 TRWAHYNALIR	Negative Control Peptide	-	Yes	(128)	Yes	3 others	No	No	No	No	-
829 838 TSVPFFWRRE	-	-	Yes	(32)	No	None	No	No	No	No	-

* TBC:To be confirmed.

Appendix III Summary of Experimental Data.

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REFERENCES

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