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MONOCLONAL ANTIBODIES TO HERPES SIMPLEX VIRUS TYPE 2

Proefschrift

ter verkrijging van de graad van doctor in de landbouwwetenschappen, op gezag van de rector magnificus, dr. C.C. Oosterlee, hoogleraar in de veeteeltwetenschap in het openbaar te verdedigen op vrijdag 3 september 1982 des namiddags te vier uur in de aula van de landbouwhogeschool te Wageningen.
I would like to thank the following people for their help during the various stages of the work described in this thesis. Without them it would never have been written.
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And all the members of the division of virology, for providing such a pleasant atmosphere to work in.
I also want to thank my husband, Robert, whose help with the processing of this thesis. His continuous support has been invaluable.
As a Ph.D is also the completion of a scientific education, I would like to thank my parents for giving me the opportunity to go to university, and for providing such a good home background.
I. The observation by Stevens and Cook, that anti-Herpes IgG is important for maintaining latent infections is invalidated in the light of Sekizawa et al's findings that in the absence of herpes specific antibodies, virus latency was still maintained.


II. Congenital human cytomegalovirus infections are a major cause of abnormalities in the newborn, but in view of the possible oncogenic potential of the virus, the vaccination of young women with live attenuated virus is unjustified.


III. RNA tumor viruses are not important agents of human cancer, but the study of these viruses has led to the identification of genes and proteins involved in the induction of a wide range of neoplastic diseases.


IV. The development of single-cell protoplasts for the study of plant virus replication in the early 1970's heralded a new era in plant virology. However, the outcome has been disappointing in that the progress made in plant virus replication in the past ten years does not compare with the success in the study of animal virus replication following the introduction of animal tissue culture.


V. The recent proposal by Prusiner et al. for a "prion" as the infectious scrapie agent is highly unorthodox. As Kimberlin points out, a much simpler working hypothesis, involving infectious nucleic acid, which fits all the established facts can be found.

VI. The widespread use of live attenuated poliovirus vaccines has led to the virtual elimination of poliomyelitis, but offers no prospects of eliminating the virus. Countries using the live attenuated vaccine should adopt the policy already used in Sweden and the Netherlands and begin using killed vaccines in order to achieve elimination of the virus.


VII. The use of PMSF as a protease inhibitor during the isolation of plant proteins is of little value.


VIII. The study of temperature-sensitive mutants of animal viruses can yield useful information, but the occurrence of silent, double and suppressor mutations make interpretation of results from the study of single viral mutants hazardous.


IX. The English system of competitive University entrance examination is preferable to the Dutch system of a lottery.

Proefschrift van Cornelia S. McLean-Pieper 3 September 1982
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Discussion
SCOPE OF THE INVESTIGATIONS.

In 1975, a method was developed by Kolher and Milstein for the production of large quantities of monospecific antibodies. They isolated a HAT-sensitive mouse myeloma cell line which, after fusion with spleen cells from an immunised mouse, produced hybrid cells of predefined specificity. Large quantities of monoclonal antibodies against any given antigen can be obtained in this way. The technique has been used successfully for the production of monoclonal antibodies against many different antigens, such as tumor antigens, lymphocyte markers, human leucocyte interferon and many viral antigens. (Koprowski et al., 1978; McMichael et al., 1979; Secher and Burke, 1980; Wiktor and Koprowski, 1978; Gerhard et al., 1977.)

These antibodies have proved useful tools to probe and characterise many biological systems. Their application to clinical diagnosis and therapy, and to large scale purification of certain antigens will lead to a further understanding of the antigenic determinants of the virus, the mechanism by which they infect cells, the importance of neutralising antibodies and the interrelationships of different viral proteins. They will provide a source of specific reagents that can be used to clearly define antigenic variations in a given virus type.

In recent years, there have been many reports on identification and characterisation of Herpes Simplex Virus proteins. (for a review: see Spear and Roizman, 1980). The research in this field has been greatly hampered by the complexity of the virus, the great number of virus-specific proteins and the lack of knowledge regarding their interrelationships. Monoclonal antibodies will be useful tools in providing more information about the unique identity and relationships between the proteins coded for by this virus. They will also be of great value as clinical reagents for serotyping HSV strains.

The aim of the work presented in this thesis was to produce monoclonal antibodies against Herpes Simplex Virus Type 2 (HSV-2). At the time of the start of these investigations, no monoclonal antibodies to Herpes Simplex Virus had been reported. We decided not to select for specific viral antigens, but to try and obtain monoclonal antibodies against a variety of different viral proteins.

In the first chapter a short review is given on the significance of monoclonal antibodies in virus research. In addition this chapter contains a review on the relevant properties of Herpes Simplex Virus Type 2, focussing in particular on the glycoproteins of the virus.

The second chapter describes the production of monoclonal antibodies against HSV-2. In total, nine stable hybridoma lines were obtained, each secreting antibodies of the immunoglobulin G class. Several immunisation schedules were compared for their effectiveness in raising an antibody response against HSV-2 in Balb/c mice. A variety of assays for the detection of mouse
anti-HSV-2-antibodies were tested, and the two most suitable systems were used in the subsequent screening of hybridoma's.

In chapters 3 and 4 the further characterisation of these monoclonal antibodies is described. They were tested for their ability to neutralise infectious virus and their type-specificity. If possible, their target-antigen was identified. They were also tested in immunofluorescence and red-bloodcell rosetting assays. In chapter 3, the characterisation of hybridoma's LP1 and LP4, which secrete antibodies directed against the proteins VP16 and ICSP11/12 (the major DNA-binding protein) is presented. Their possible use in the investigation of these proteins and their use in clinical diagnosis is discussed.

In chapter 4, four monoclonal antibodies directed against different antigenic sites on glycoprotein D are presented. These antibodies give further insight in the antigenic structure of this protein, which is an important constituent of the viral membrane. In the same chapter, two antibodies directed against the glycoproteins gC and gB are also described.

In the fifth chapter the protective effect of several of the monoclonal antibodies on Herpes Simplex Virus infection of Balb/c mice is investigated. It was found that antibody LP2 had a significant effect in reducing both infectious virus titres and ear swelling in HSV-1-infected mice. Non-neutralising antibodies did not have any effect, with the exception of antibody LP3, which gave a slightly enhanced inflammatory reaction. The effect of LP2 on the establishment of latent infection is also discussed.

In chapter 6, conclusions are drawn and a short description of the utilisation of some of the monoclonal antibodies in future investigations is given.
CHAPTER 1: INTRODUCTION.

Herpes Simplex Virus Type 2 (HSV-2) is a large DNA virus belonging to the Herpesvirus family. Together with four other Herpesviruses, Herpes Simplex Virus Type 1 (HSV-1), Varicella-Zoster Virus, Cytomegalovirus, and Epstein-Barr virus, it is a natural pathogen of man. The differential characteristics of the Herpesvirus family are a linear, double-stranded DNA-genome, with a molecular weight ranging from $80 \times 10^6$ to $150 \times 10^6$; an icosahedral capsid consisting of 162 capsomers (Wildy et al, 1960); an ill-defined structural component called the tegument (Roizman and Furlong, 1974); and a trilaminar membrane, the envelope, which is acquired by budding through the nuclear membrane (for a review see Spear and Roizman, 1980). The envelope contains both host- and virus-derived material (Wildy et al, 1960).

HSV-1 and HSV-2 have very similar structural and biological properties and share certain common antigenic determinants. Although HSV-1 is generally associated with infections of the oral mucosa and cornea, while HSV-2 causes infections of the genital regions, the symptoms produced by these two viruses are indistinguishable if inoculated into the same site (Rawls, 1973).

The recent interest in Herpes Simplex Virus can be attributed to various factors. 1. HSV causes recurrent epithelial lesions. The virus remains latent in the sensory cells of the trigeminal nerve ganglion; under conditions of stress, fever or exposure to sunlight the virus becomes activated (Stevens, 1973; Wildy et al, 1982). The resulting lesions usually occur on the mucocutaneous junction of the lip and tend to recur at the same site time after time. Although this condition is by no means serious or life-threatening, it is very widespread and causes discomfort and embarrassment to the patients concerned. 2. A number of Herpesviruses that infect humans have been associated with cancer. In particular, interest has focussed on the oncogenic potential of HSV-2 in cervical tissues (review: Rawls, 1973; Spear and Roizman, 1980). 3. Infection of newborn or immunocompromised individuals with Herpes Simplex Viruses can be life-threatening (Nahmias and Roizman, 1973). 4. HSV-1 is the major cause of adult encephalitis (Olson et al, 1967; Miller and Ross, 1968). 5. HSV-2 has become the major cause of venereal infections.

In the last fifteen years or so, much has become known about the molecular biology of HSV-1 and HSV-2 (see Spear and Roizman, 1980). In this chapter a short review will be given of the proteins of HSV-2, and some emphasis will be placed on the glycoproteins as these are likely to be of particular importance in immunity. First, a short review is given about the significance of monoclonal antibodies in viral research in general.
Significance of monoclonal antibodies in viral research.

Soon after the introduction of hybrid myeloma's secreting monoclonal antibodies of predefined specificity, the potential of this technique in producing antibodies to viruses was realised. Antibodies produced by this method have very definite practical advantages over the conventional sera produced by an immunised animal. The mixture of antibody species as well as the variability between sera obtained from different animals have presented a major drawback for the use of conventional sera as standard biochemical reagents. Monoclonal antibodies are defined molecular species, directed against one antigenic site, and are cheap and easy to produce. In addition, there is no variability from animal to animal as with conventional sera. The fact that only one antigenic site is being recognised allows problems of antigenic relationships between proteins, and questions regarding their precursor-product relationship, to be solved unambiguously. Monoclonal antibodies are therefore likely to replace many conventional sera in clinical as well as non-clinical laboratories.

The first monoclonal antibodies directed against a virus were described by Koprowski et al (1977), who produced monoclonal antibodies against influenza virus. Monoclonal antibodies against rabies virus followed soon (Wiktor and Koprowski, 1978) and since then a snowball-effect in the production of monoclonal antibodies against many different viruses has been observed. Monoclonal antibodies have now been obtained against such widely differing viruses as measles (Giraudon and Wild, 1981); murine leukemia viruses (Costron et al, 1979; Harlow et al, 1981; Nowinski et al, 1979); SV-40 tumour antigens (Deppert et al, 1981); poliovirus (Ferguson et al, 1980); Herpes Simplex Virus (Zweig et al, 1979; Pereira et al, 1980; Killington et al, 1981); reoviruses (Lee et al, 1981); retroviruses (Oroszlan and Nowinski, 1980); Sindbis Virus (Roetrig et al, 1981); Adenovirus (Russell et al, 1981) and hepatitis (Wai-kuo Shih et al, 1980).

An example of the potential of monoclonal antibodies for clinical as well as non-clinical application is the research carried out with monoclonal antibodies against influenza virus. Not only were monoclonal antibodies used successfully to distinguish antigenic variants of the virus (Gerhard and Webster, 1978; Kendall et al, 1981), but the complete antigenic topology of the virus haemagglutinin was determined (Wiley et al, 1981). Variation in the antigenic structure of the haemagglutinin is associated with the recurrence of influenza virus in man. When the three-dimensional structure and primary sequence of the 1968 Hong Kong haemagglutinin became known (Wilson et al, 1981), a panel of monoclonal antibodies directed against the haemagglutinin molecule was used to obtain information about the relationship between observed mutations induced by the presence of these antibodies and changed antigenic properties of the haemagglutinin. The observed mutations clustered into four major areas of the haemagglutinin molecule, representing four antigenic sites. Gerhard et al (1981) also used this technique of obtaining mutants by growing virus in the presence of monoclonal antibodies. They obtained 34 PR-8 mutants, using anti-haemagglutinin antibodies, and with the
aid of 58 anti-haemagglutini monoclonal antibodies they also observed four distinct antigenic sites on this molecule. These experiments provided insight in the mechanism of antigenic "shift" and "drift" and the importance of certain antigenic sites in these processes.

Another example of the use of monoclonal antibodies in diagnosis and in the investigation of the antigenic relatedness of virus strains is the work carried out with monoclonal antibodies against rabies virus. Until the discovery of monoclonal antibodies, the knowledge of the serology of this virus was poor. The only method to distinguish rabies virus from the rabies-related viruses was the use of cross-absorbed antisera, and this was not very satisfactory. Monoclonal antibodies defined the rabies-group unambiguously by providing reagents which either react with both the rabies and rabies-related viruses, or with rabies virus only. Yet a third group of antibodies shows specificity for limited numbers of street viruses, so further sub-dividing the rabies virus group. A panel of monoclonal antibodies was used to study a number of variants of the virus, laboratory-induced ones as well as street-variants. These antibodies showed for the first time the great antigenic variation between virus strains, and demonstrated that rabies virus has a similar potential to influenza to undergo antigenic variation. In vivo experiments showed that there was no cross-protection between variants. The frequency of variants of street-strains of rabies, isolated from fatal human cases, seems to be high (Wiktör et al, 1980; Wiktör and Koprowski, 1980). These findings immediately raised the question of the adequacy of existing rabies vaccines, and a modification of the vaccine potency test by including the regional street virus variant in the challenge was proposed.

A more detailed investigation of the antigenic differences between the nucleocapsid proteins of the rabies variants has also been carried out, resulting in an estimate of the number of antigenic sites on the molecule. (Flamand et al, 1980a). Similar studies were done for the glycoprotein (Flamand et al, 1980b). This kind of studies will provide insight in the role of certain antigenic determinants in neutralisation and antigenic variation of viruses.

**Herpes Simplex Virus Proteins.**

Estimates concerning the number of proteins coded for by HSV-1 and HSV-2 have steadily increased in recent years. The approximate number of distinct polypeptides which can be resolved by single dimension electrophoresis is now usually quoted as around fifty (Balachandran et al, 1981; Powell and Courtney, 1975), but in a recent paper, using two-dimensional gel electrophoresis, as many as 230 separate virus-specific polypeptides could be detected in infected cells. (Haarr and Marsden, 1981). One of the difficulties in interpreting these kind of results is the lack of knowledge regarding precursor-product relationships between the different polypeptides. Many of the polypeptide species detected by Haarr and Marsden were shown to be precursors, but there can be little doubt that the
estimate of fifty polypeptides is an under-estimate of the total number specified by Herpes Simplex Virus.

Whatever the total number, the complexity of the polypeptide pattern observed following electrophoresis of infected cell proteins has led to considerable problems of nomenclature. Different groups of workers have used different polypeptide nomenclatures, and it is frequently difficult to relate different nomenclatures. Roizman and coworkers have used a system of numbering polypeptides according to their order of migration in single dimension polyacrylamide gels, the largest polypeptide being given the number 1. Polypeptides from the purified virion (the structural polypeptides) are given VP-numbers (Virion Polypeptide, e.g. VP5 is the major capsid antigen) while polypeptides in the infected cell are given ICP numbers (Infected Cell Polypeptide, e.g. ICP8 is the major DNA-binding protein), and each VP has an equivalent ICP (Spear and Roizman,1972; Honess and Roizman,1973). This nomenclature has the disadvantage that it is inflexible and cannot easily accommodate the discovery of new polypeptides. Furthermore the order of polypeptide migration may be influenced by the electrophoretic gel system used. Marsden et al (1976) used a nomenclature which relies solely on the molecular weight of a polypeptide, each polypeptide being given a number which reflects its molecular weight in thousands. Their system is flexible but has the disadvantage that different laboratories will obtain different molecular weights for the same polypeptide and will therefore give it a different designation. Powell and Courtney (1975), following the example of Roizman's group, numbered the polypeptides of HSV according to their migration order. Each polypeptide was given an ICSP number (Infected Cell Specific Polypeptide). The confusion resulting from these different systems of nomenclature is compounded by the fact that different workers have used different strains of HSV-1 and HSV-2 and different cell types. The polypeptide pattern presented in the literature by different groups often show only limited similarity. In this thesis I shall use where possible the nomenclature of Powell and Courtney (for HSV-2) since these seem to be the most widely used in the literature.

A relatively small number of HSV-specific proteins have been purified and their properties investigated, but the characteristics of the majority of the polypeptides are known only in very general terms - migration in gels, kinetics of synthesis during infection, whether they are glycosylated or phosphorylated and whether they are present in the mature virion. Many of the polypeptides specified by HSV therefore remain poorly defined. Indeed one of the major contributions of monoclonal antibodies to herpesvirus research will be that these antibodies, since they are specific for single antigenic sites, will dissect this complex mixture of polypeptides and allow unambiguous designation of unidentified polypeptides.

Control of viral protein synthesis.

The synthesis of viral proteins is temporally ordered in a cascade fashion. Thus, three groups of proteins can be
distinguished, namely $\alpha$-, $\beta$- and $\gamma$-proteins. The $\alpha$-proteins are synthesized immediately after infection of the cell. To date, six $\alpha$-polypeptides have been identified. Synthesis of $\beta$-polypeptides depends on the presence of functional $\alpha$-proteins (Honess and Roizman, 1974; 1975). Examples of $\beta$-polypeptides are the thymidine kinase and viral DNA-polymerase (Honess and Roizman, 1974; 1975; Powell and Purifoy, 1977; Garfinkle and McAuslan, 1974). The $\gamma$-proteins can only be synthesized when functional polypeptides belonging to both the $\alpha$- and $\beta$-group are present. Structural proteins form the major components of the $\gamma$-group. This designation in $\alpha$-, $\beta$- and $\gamma$-proteins necessarily remains approximate, and it may be that proteins will be found which do not fall into any of these groups.

Polypeptides of the virion.

Estimates of the number of proteins contained in HSV-1 and HSV-2 virions vary between 15 and 33 (Heine et al., 1974). Several of these proteins are glycosylated. Three groups of virion polypeptides can be identified: 1. The proteins present in the nucleocapsid prior to envelopement. 2. Glycosylated polypeptides, which are present in the viral membrane. 3. The remainder of the virion-polypeptides, which are probably constituents of the tegument (Spear and Roizman, 1980).

Glycoproteins: structure and function.

A great deal of interest has been focussed on the second group of proteins, the virus-specific antigens contained on the surface of the envelope and the infected cell. Convalescent antibody is directed against these surface proteins, and it is responsible for the neutralisation of the virus (Plummer, 1964; Nahmias and Dowdle, 1968). The components of the immune system that react with the virion have been shown to react with viral antigens on the surface of the infected cell (Glorioso et al., 1978; Norrild et al., 1979 and 1980). Clearly, these proteins play a major role in the induction of the immune response to the virus in the infected host.

Most of the work concerning HSV-glycoproteins has been done with HSV-1, but each of the main glycoproteins in type-1 virus seems to have its counterpart, be it sometimes with a slightly different molecular weight, in HSV-2 (Cassai et al., 1975). Until recently, four major antigenically and functionally distinct species of glycoproteins were identified, namely $gB$, $gC$, $gD$ and $gE$, A fifth glycoprotein, $gA$, is probably a modified form of $gB$ (Eberle and Courtney, 1980; Haffey and Spear, 1980). A new glycoprotein, $gF$, was recently described in HSV-2 by Balachandran et al., (1981). There are probably more glycoproteins than the ones described above, and monoclonal antibodies may provide the tools for identifying them into antigenically distinct classes.

Much of the research into the structure and function of the glycoproteins of HSV has made use of antisera raised against these proteins. Many of these sera were monospecific, i.e. the protein was isolated from an SDS-polyacrylamid gel and antiserum raised against the so purified polypeptide. Some antisera were raised against precipitin bands (monoprecipitin sera), and sera
against gC were sometimes prepared using absorption of hyperimmune serum with a mutant that lacks gC (see Norrild, 1980). These antisera have greatly contributed to our knowledge of the glycoproteins, both for their identification and their processing and function. They have, however, some obvious disadvantages: there always remains some doubt as to the specificity of the antiserum, because of the possible impurity of the antigen to which it was raised. Furthermore, the SDS present in polyacrylamide gels denatures the protein and may modify or destroy certain antigenic sites. These points are illustrated in the fact that the antisera raised by different groups of workers against gC, for instance, seem to have very different properties (Eberle and Courtney, 1980a; Spear, 1976; Powell et al., 1974; Courtney and Powell 1975; Vestergaard and Norrild, 1979; Cohen et al., 1980a). It is obvious that monoclonal antibodies directed against the different glycoproteins will be very useful tools in their further identification and characterisation: the disadvantages mentioned above do not apply to them.

Little is known about the functions of the glycoproteins specified by HSV. Some (or all of them) play a role in virion infectivity, such as attachment to or penetration of cells.

A temperature-sensitive mutant of HSV-1, tsB5 (isolated by A. Buchan, Birmingham), which fails to synthesize gB, is unable to penetrate cells, even though absorption does take place at the non-permissive temperature (Sarmiento et al., 1979). Manservigi (1977), using a different mutant, found that gB was also important in cell fusion. A recent report by Haffney and Spear (1980) confirmed the role of gB in virus infectivity and cell fusion.

The major, fully glycosylated form of gB of HSV-1 has a molecular weight of 126,000D, and a partially glycosylated precursor of 113,000D (pgB) can be found in pulse-labeling experiments (Spear, 1976). gA might be a precursor of gB, as suggested by Eberle and Courtney (1980).

gC has a molecular weight of 130,000 D. Its partly glycosylated precursor has an apparent molecular weight of 110,000 D (Cohen, 1980). Using tunicamycin, an unglycosylated form of the protein could be detected, with a molecular weight of 85,000 D (Pizer et al., 1980). The function of gC is not known, but many syncytial mutants lack gC, and it may be that gC and gB work antagonistically (Manservigi, 1977).

Nothing is known about the function of glycoprotein D, but some detailed work has been done on the processing of this molecule (Pizer et al., 1980). The fully glycosylated form of gD in HSV-1 has a molecular weight of approximately 59,000 D. Using tunicamycin, an unglycosylated precursor of the protein was discovered with a molecular weight of 50,000D. This precursor is not normally found in infected cells, as one or more oligosaccharide chains are immediately attached to it, thus forming a precursor to gD (pgD) with a molecular weight of 52,000D (Cohen et al., 1980). Analysis on two-dimensional gels has shown that the processing of pgD to gD involve 11 discreet steps (Haarr and Marsden, 1981). This work was done using a monoprecipitin antiserum against gD which, because of its possible impurity, left some doubt as to the relatedness of the polypeptides identified as intermediates between pgD and gD. The use of monoclonal antibodies directed against gD has now proved
unambiguously that all the 12 polypeptides are related and have been derived from one common precursor (Harsden et al, 1981). This work was carried out using an HSV-1 strain. The processing of gD for HSV-2 has not been worked out in such detail. gD in HSV-2 has a slightly lower molecular weight than in HSV-1, namely 56,000D, with a partly glycosylated precursor of 51,000D (Eisenberg et al, 1980).

The only other protein that has been associated with a particular function is gE. The appearance of gE on the surface of the infected cell coincides with the appearance of Fc-binding activity. The detergent-solubilised form of gE has an affinity for the Fc-region of immunoglobulin-molecules, and in fact gE can be isolated from virions using affinity chromatography on sepharose beads to which antigen-antibody complexes have been coupled (Baucke and Spear, 1979). The molecular weight of gE of HSV-1 is approximately 80,000 D, with a precursor of about 65,000 D. gE of HSV-2 has a slightly higher molecular weight, about 90,000 D, with a precursor of 67,000 D. There is a certain variability in these molecular weights depending on the virus strain used (Para et al, 1982a and b).

gE is present on the surface of intact virions, which exhibit Fc-binding activity. The role of these Fc-binding receptors in virus replication and biology remains unclear. It has been suggested that the binding of immunoglobulin to the Fc-receptors can in some way interfere with cytotoxic immune reactions, which would prevent the infected cell from being lysed and perhaps even favour the establishment of latency (Westmoreland and Watkins, 1974 Lehner et al, 1975). It has been demonstrated that aggregated IgG can protect infected cells to some extent from immune cytolysis (Adler et al, 1978), but the use of native immunoglobulin did not prevent neutralisation of the virus by an anti-gE serum. The fact that the virion-membrane, including the Fc-receptors, fuses with the cell-membrane when the virus enters the cell, suggests that the protein might be required for some purpose on the surface of the infected cell, or it may be important in mediating the attachment of virions to cells (Para et al, 1982a and b).

Glycoproteins as targets of the immune system.

The presence of the glycoproteins on the surface of the virion and of the infected cells indicates that these molecules are the prime targets for the immune system of the infected host. Indeed it has been shown that the viral glycoproteins are the targets of neutralising antibodies (Powell et al, 1974 et al, 1973), antibody-dependent cellular cytolysis (Norrild et al, 1979), complement-mediated cytolysis (Glorioso et al, 1978), and T-cell-mediated cytotoxicity (Lawman et al, 1980). The different glycoproteins each carry a unique set of antigenic determinants. Some glycoproteins are mainly type-common, while at least one glycoprotein, gC, seems to have only type-specific determinants. The properties of the HSV-glycoproteins as targets of the immune system and their antigenic specificity, will be discussed below. This review will only include results obtained using conventional antisera; recent discoveries in this field using monoclonal antibodies will be discussed later in this thesis.
Glycoproteins as targets of neutralising antibodies.

The first antigenic component of HSV recognised to be the target of neutralising antibodies was the band-II antigen (Watson and Wildy, 1969). Antiserum raised to this antigen was capable of neutralising both HSV-1 and HSV-2 (Sim and Watson, 1973). It has been shown that this antigen corresponds to glycoprotein D. Several antisera to gD have since been prepared, using purified protein bands from gels, chromatographic procedures or immunoprecipitates from crossed immunoelectrophoretic gels (Powell et al, 1974; Powell and Watson, 1975 Vestergaard and Norrild, 1979; Cohen et al, 1978). All these antisera showed neutralising activity for HSV-1, while all but one showed the same ability to neutralise HSV-2. The failure of the anti-gD serum produced by Powell et al (1974) to neutralise HSV-2 was probably due to the denaturation of gD by sodium dodecyl sulphate, as this antiserum was produced against gD purified from SDS-polyacrylamide gels.

It is apparent from the results described above that gD is an important target for the production of cross-reactive neutralising antibodies, although by cross-absorption reactivity against the heterologous antigen can be absorbed out, leaving a low-potency serum which is type-specific.

Another antigen which seems to play a major role in virus neutralisation is gB. Antisera raised against the gA/gB complex have been reported by Vestergaard and Norrild (1979) and Eberle and Courtney (1980a). These antisera have the ability to neutralise homologous as well as heterologous virus. Cross-absorption experiments, however, showed the existence of some type-specific reactivity against this protein as well. It has not been possible to raise antisera against either gA or gB, which is not surprising in the light of the findings by Eberle et al (1980), who suggest that gA is a precursor of gB. The relative importance of gB as a target for neutralising antibodies corresponds to its possible role in virus infectivity, mentioned earlier in this review.

A third antigen which acts as a target for neutralising antibodies to HSV is gC. Antisera to this protein have been raised by several different methods, and they have different neutralising abilities. Some are non-neutralising (Cohen et al, 1980b), some only neutralise in the presence of complement (Courtney and Powell, 1975), and some do not require complement for neutralisation (Vestergaard and Norrild, 1979). All the antisera, however, are type-specific. It is interesting in this context to note that the genetic map position for gC on HSV-1 and HSV-2 is completely different.

Neutralising antibodies against gE have also been found (Baucke and Spear, 1979), but their neutralising ability is dependent on the presence of complement. The serum neutralises HSV-1 better than HSV-2, indicating that there are type-specific as well as type-common sites on the gE-molecule.

In conclusion, it can be said that the two main targets of neutralising antibodies seem to be the glycoproteins gB and gD, but there is a role for gC and gE as well.

Confirmation of the importance of the glycoproteins as targets for antibodies, neutralising and non-neutralising, in humans infected with HSV, came from a recent study by Eberle and
Courtney (1981) who showed the presence of antibodies directed against gB, gC and gD in a number of human sera from individuals infected with HSV-1, HSV-2 or both.

Role of glycoproteins in antibody-dependent cell-mediated cytotoxicity (ADCC) and complement-mediated cytotoxicity (AbC').

Results obtained by several laboratories have shown that sera obtained against individual glycoproteins cause cytolysis of infected cells in both ADCC and AbC' tests (Norrild et al., 1979; Glorioso et al., 1978). Cells infected with HSV-1 can be lysed as early as three hours after infection in the presence of antibody and complement (Cromeans and Shore, 1981). Antiserum against gB, gC and gD are all capable of lysing HSV-1 infected cells in this test (Norrild et al., 1979; Glorioso et al., 1978). In addition, these cells were also lysed by anti-gB, anti-gC and anti-gD sera in ADCC-tests. These findings imply that each of these glycoproteins individually serve as a target for immune cytolysis. In addition, it was shown that anti-gC sera only lyse cells infected with the homologous virus, while anti-gB and anti-gD were capable of lysing both HSV-1 and HSV-2 infected cells. Furthermore, anti-gC sera do not lyse cells infected with HSV-1(MP), a strain which does not produce gC. These results support the conclusion that gA/gB and gD have type-common as well as type-specific sites, whereas gC seems to be totally type-specific.

Glycoproteins as targets of cytotoxic T-cells.

Virus-specific cytotoxic T-lymphocytes recognise viral antigens on virus infected cells only in association with certain antigens coded for by the major histocompatibility complex (Zinkernagel and Welsh, 1976). Studies in most in vitro systems have revealed that the glycoproteins are the antigens recognised by cytotoxic T-lymphocytes (CTL). Two groups of workers have shown that the glycoproteins are the antigens recognised by HSV-1 specific CTL: Both Lawman et al. (1980) and Carter et al. (1981) used an HSV-mutant, tsA1, which is defective in glycoprotein synthesis at the non-permissive temperature. They also made use of tunicamycin and 2-deoxy-D-glucose to inhibit glycoprotein synthesis in the target cells. The target cells infected with the mutant or treated with the inhibitors showed a very marked reduction in their ability to be lysed by CTL. The conclusion that can be drawn from these experiments is that the expression of glycoproteins on the surface of the infected cell is necessary for the T-cell mediated lysis of HSV-infected cells. In a recent report (1981) Eberle and his coworkers suggest that two subpopulations of cytotoxic T-cells exist: those which recognise type-specific and those recognising type-common antigenic determinants. Effector cells generated by priming and restimulating with the same virus recognised both type-common and type-specific antigenic sites on the target cells, but effector cells primed with HSV-1 and restimulated with HSV-2 recognised only type-common determinants. In addition, it was shown that gC is one of the type-specific determinants recognised, by using the mutant MP. It was proved, however, that there is at least one other type-specific surface antigen.
recognised by anti-HSV CTL, apart from gC. This is not surprising in view of the fact that gA/gB, gD and gE all carry type-specific as well as type-common antigenic determinants.

Cross-reactive antigens in the Herpesviruses.

The Herpesvirus group comprises over seventy members, infecting a great variety of different hosts. Very little is known about the relationships between the various members of the group. The serological data on the group do as yet not give any convincing systematic basis for subgrouping of the herpesviruses (Honess and Watson, 1977; Killington et al, 1977).

One of the criteria for the antigenic subgrouping of the herpesviruses has been neutralisation. It has been shown, for instance, that HSV-1, HSV-2 and Bovine Mammalitis Virus (BMV) cross-neutralise each other, while Equine Abortion Virus (EAV) and Pseudorabies Virus (PRV) were only neutralised by homologous antisera (Killington et al, 1977). Many other serological methods, such as immunodiffusion, immunofluorescence or immune agglutination of virus particles, have been used to identify serological relationships between herpesviruses. Although these tests gave an indication of the range of antigenic differences between these viruses, very little work has been done to relate these data to any structural basis.

One of the difficulties in this type of study lies in the complexity of the viruses involved. Even the comparison between such closely related viruses as HSV-1 and HSV-2 is made difficult by the fact that the comparable proteins in these two viruses have different molecular weights and migration characteristics. These problems only get worse when moving to less closely related viruses. The role of antisera against the viruses to be studied is therefore very important. The results published so far have relied on conventional sera, in most instances raised against the whole virus. In one or two cases, antisera raised against one particular protein were used. A short description of the different antigens which seem to be important in cross-reaction between the herpesviruses is given below.

A few early experiments suggested that the major capsid protein might be a cross-reactive antigen between a number of herpesviruses (Ross et al, 1972; Kirkwood et al, 1972; Honess et al, 1974; Killington et al, 1977). In a recent report (Yeo et al, 1981), the proteins and glycoproteins of five different herpesviruses, namely HSV-1, HSV-2, BMV, EAV and PRV have been compared and their antigenic relationship investigated. From immunoprecipitation data using antisera raised against these five viruses it seems clear that one antigen which is strongly cross-reactive between all these viruses is the major DNA-binding protein (ICSP 11/12). This result was confirmed using an antiserum raised specifically against this protein. The major capsid protein is also cross-reactive, but to a much lesser extent.

The glycoproteins, which are the other major group of proteins implicated in cross-reaction through neutralisation studies and immunoprecipitation data, do not have such a wide range of cross-reactivity as the major DNA-binding protein. Their
cross-reactivity seems to be limited to the subgroup to which
they belong. For instance, there is strong cross-reactivity
between the glycoproteins of HSV-1 and HSV-2, and some cross-
reactivity between these two viruses and BMV, as the high-
molecular weight glycoproteins of BMV are precipitated by anti-
HSV-1 and anti-HSV-2 sera. This is in agreement with the results
by Norrild et al (1978). No cross-reactivity could be detected
between the glycoproteins of EAV and PRV.

The data on the cross-reactivity of the glycoproteins
through immunoprecipitation studies thus agree with the findings
in neutralisation studies, where HSV-1, HSV-2 and BMV form a so-
called neutro-seron, while EAV and PRV are two separate
neutroserons, although they are more related to each other than
to HSV-1, HSV-2 or BMV (Honess and Watson, 1977).

Some cross-reactivity was also observed between several
minor components of the five viruses studied in the report by
Yeo et al (1981). The proteins involved in these reactions have
not yet been identified.

Until more members of the Herpesvirus family have been
examined for the presence of the major DNA-binding protein, it
is impossible to say whether this protein can be used as a
marker for the whole herpesvirus group. In the paper by Yeo et
al, it is suggested that this protein might be useful for the
classification of the Herpesvirus group, while the major capsid
protein may help to identify some cross-relationships between
members of the group, and that the glycoproteins will define the
subgroups of the family. If these proteins are going to be used
as "markers" in this way, it will be necessary to have a more
detailed knowledge of their antigenic properties. Monoclonal
antibodies against these proteins will be very helpful in this
kind of research.

Clinical applications of monoclonal antibodies to Herpes Simplex
Virus.

The clinical diagnosis of an infection with HSV is at
present relatively straightforward. The virus is easy to grow
in many tissue culture systems and can be readily diagnosed
using fluorescence methods. High quality rabbit antisera are
available for this purpose. However, the differential diagnosis
between HSV-1 and HSV-2 is not so straightforward. As the
treatment for HSV-1 and HSV-2 is the same at the moment, the
demand for this type of diagnosis in clinical laboratories is
not very high. It is possible, however, that with the present
rapid development of drug-therapy, drugs will be found which
are effective against HSV-1 or HSV-2 only. Two such drugs have
already been reported, namely bromovinyl-deoxyuridine (BVDU) and
Iodovinyl-deoxyuridine (IVDU). Both are active against HSV-1 but
not against HSV-2 (De Clercq et al, 1980). At present, the
differential diagnosis between HSV-1 and HSV-2 relies on
conventional antisera which have been cross-absorbed in order to
make them type-specific. These sera are generally of low potency
and have a residual activity against the heterologous virus.
Monoclonal antibodies could be of great value in facilitating
the rapid diagnosis of HSV-1 or HSV-2. Because of the high
specificity of monoclonal antibodies, it may be necessary to use
mixtures of type-specific antibodies, in order to avoid the
recognition of certain subgroups only, instead of the whole range of HSV-1 or HSV-2 isolates.
CHAPTER 2: FUSION EXPERIMENTS.

As described in the first chapter, HSV-2 is a complex virus which codes for at least fifty virus-specific proteins. Although some progress has been made in the characterisation of some of these proteins using conventional antisera, it is obvious that monoclonal antibodies against any of the viral proteins will be very useful tools in their further identification and characterisation. At the time of the start of the experiments described in this chapter, no monoclonal antibodies against HSV-1 or HSV-2 had been reported. In our efforts to produce monoclonal antibodies to HSV-2, we had the choice of looking at a limited number of antigens (i.e. purified virus, purified membrane antigens, nuclei of infected cells, etc.) or using a "shot-gun" approach and not selecting for any particular group of antigens. As at this time monoclonal antibodies against any of the viral antigens would have been useful, we decided on the latter approach and used HSV-2 infected cell sonicates to immunise mice before the fusion. This would still involve a certain selection, as the different antigens may vary in immunogenicity. Also, in the infected cell certain antigens are produced in much larger quantities than others.

In the first part of this chapter I describe a number of preliminary experiments which established the most satisfactory immunisation routine. In this section the development of a suitable assay-system for the detection of antibodies against HSV-2 is also described. It was important to have a fast and reliable assay-system, as it was necessary to be able to decide quickly which hybridoma cultures were secreting anti-HSV-2 antibodies and were therefore worth keeping. In earlier reported fusion experiments involving viruses such as influenza and rabies, haemagglutination assays and fluorescence were used. We developed a number of different radioimmunoassays, and present results which enabled us to decide which assay was the most reliable and sensitive.

The second part of this chapter deals with the fusion experiments and the selection of stable, antibody-secreting clones from these fusions.

MATERIALS AND METHODS.

Virus.
The virus strain used was type 2 strain 25766 (obtained from professor K.R. Dumbell, Wright Fleming Institute, London). Virus stocks were prepared by infecting BHK-21 cells with 0.1 pfu per cell for 48 hours at 33°C. Infected cells were harvested, sonicated and the number of plaque forming units measured using BHK-21 cells as described by Russell (1962). Virus stocks to be
used for immunisation of mice for fusion-experiments were grown in 3T3-cells, using the same protocol. All virus stocks were kept frozen at -70°C.

Cells.

BHK-21 cells were grown in Glasgow's modified Eagles medium (GMM), supplemented with 10% newborn calf serum and 10% tryptose phosphate broth (ETC). 3T3-cells were passed in Dulbecco's modified Eagles medium (DMM) with 10% foetal calf serum (FCS). The myeloma cell line P3.NSL/1Ag.3.4.1. (kind gift of Dr. C. Milstein, M.R.C. Cambridge) was passed in GMM supplemented with 5% FCS (GMM-5%FCS). After a fusion, hybridoma cells were kept in GMM-20%FCS, and later in HAT-medium (GMM-20%FCS, supplemented with 0.1M hypoxanthine, 1mM aminopterine and 40 mM thymidine) or HT-medium (as HAT-medium, but without aminopterine). Cells were frozen in liquid nitrogen in 60% FCS-30% GMM-10% glycerol, at a concentration of 2.10^6 cells per ml.

Mice.

Female Balb/c mice aged 5-6 weeks were obtained from Bantin and Kingman Ltd. (Aldbrough, Hull) and used when 6-9 weeks old. In some cases male Balb/c mice, over 20 weeks old, were used for the production of ascites fluid following an injection with hybridoma cells.

Reverse Passive Haemagglutination.

Trypsinised Sheep red blood cells (Tr.Shrbc) were obtained from Professor R.A.A. Coombs (Division of Immunology, Department of Pathology, Cambridge). Cells were extensively washed in saline before use, and made to a 80% suspension in saline. The CrCl₃ stock solution used for the coupling reaction was prepared as described by Bradburne et al (1979) and was a kind gift of Professor R.A.A. Coombs. The solution was stored at 4°C.

In order to couple antibodies to the cells, IgG (purified by DEAE-cellulose chromatography) was extensively dialysed against saline and diluted to a concentration of 2 mg/ml; 50 μl of this preparation was mixed with 50 μl Shrbc-suspension, and 100 μl of a 1/50 diluted CrCl₃-stock solution added with simultaneous mixing. The mixture was left at room temperature for 1 hour, washed 4 times in Hepes-buffered GMM and made to 4 ml with Hepes-buffered GMM, to give a 1% suspension. At this stage the cells could be stored for up to a week at 4°C.

The reverse passive haemagglutination (RPH) test was carried out in Nuncion 96-well round bottom trays. A two-fold dilution series of the antigen (HSV-2) was made in 25 μl saline per well. A 1/3 dilution of the coupled Shrbc in Hepes-buffered GMM (supplemented with 5% FCS), was prepared. One drop of this solution (25 μl) was added to each well, and the results observed after two hours.

Coupling of antibody to plastic surfaces using carbodiimide (CDI).

This was essentially carried out as described by Nash (1976). Plastic of tissue-culture grade was used in all cases. In a 0.2 ml well, 25 μl of a 2 mg/ml solution of purified Rabbit anti-HSV-2-IgG in saline was mixed with 25 μl of a 1 mg/ml solution of carbodiimide in saline. The mixture was left for one hour at room temperature and subsequently washed with jets of
saline. The wells could be filled with saline at this stage and stored at 4°C.

**Labelling of protein with ¹²⁵I.**

The protein to be labelled was dissolved in PBS to a concentration of 1 mg/ml. 25 μl of this solution was mixed with 10 μl ¹²⁵I (1mCi, carrier-free, Amersham Corp.) and 30 μl 10mM Chloramine T. After 3 min. incubation at room temperature the mixture was put on ice and 25 μl 10mM sodium metabisulphite and 10 μl KI added to stop the reaction. The labelled protein was separated from the unbound ¹²⁵I by chromatography on a Sephadex G25 column. The specific activity of the labelled protein was between 5-10 μCi/μg.

**Radioimmunoassay.**

**RIA-1: Target = infected BHK-21 cell monolayers.**

Confluent BHK-21 cell monolayers in 0.28cm wells (Linbro 48-well flat-bottom trays, Flow laboratories) were infected with 10 pfu per cell and incubated for 8 hours at 37°C. The monolayers were then washed in PBS, fixed with 0.2% glutaraldehyde for five minutes at room temperature, and washed extensively in PBS. They were dried at room temperature and stored at -20°C. To prevent non-specific binding, each well was treated with one drop of heat-inactivated FCS (IFCS) before the assay was carried out. The wells were reacted with 50 μl of antibody-solution for 1 hour at room temperature. After washing in PBS, bound antibody was detected using ¹²⁵I-labelled anti-mouse-Fab (kind gift of Dr D. Secher, M.R.C., Laboratory of Molecular Biology, Cambridge). Individual wells were cut out and the isotope was measured in a Philips gamma-counter. Normal mouse serum was used as a negative control.

**RIA-2: Target = Infected cell antigens coupled to plastic.**

This assay was carried out in exactly the same way as RIA-1, with the difference that instead of BHK-cell monolayers HSV-2 antigens were coupled to the wells: 25 μl of an HSV-2 infected BHK-cell sonicate (2.10⁷ pfu/ml), which had been extensively dialysed against saline, was mixed with 25 μl of a 1 mg/ml solution of carbodiimide. After one hour at room temperature the plates were washed, and used for the assay.

**RIA-3: Target = HSV-2 specific antigens selected with hyperimmune antibody.**

Purified Rabbit anti-HSV-2-IgG was coupled to 0.4 ml Linbro wells using CDI. The wells were subsequently treated with a solution of HSV-2 infected BHK-cell sonicate in PBS at 10⁴ pfu per well for 30 min. at room temperature. Unbound protein was washed off using PBS. These targets were then used as described for RIA-1.

**Immunisation Schedule.**

Female Balb/c mice were infected with 10⁴ pfu 3T3-grown virus into the ear pinna. Four weeks later a second injection of 5x10⁴ pfu was given i.p., and two weeks later another injection of 10⁵ pfu via the same route. The final injection of 10⁶ pfu
(inactivated virus using 0.02% formaldehyde) was given intravenously three days before the fusion.

**Fusion.** The fusion was essentially carried out as described by Galfre et al (1977). Immunised mice were sacrificed three days after the final i.v. injection. The spleen was removed aseptically, and gently homogenised to obtain a cell suspension. The spleen cells were washed several times in cold GMM-2%FCS. The myeloma cells (NS1) were harvested whilst in the log-phase of growth and washed in GMM-2%FCS. 2x10^8 spleen cells were mixed with 2x10^7 myeloma cells and centrifuged. The pellet, after having been broken up gently, was placed at 37°C and 1 ml 50% PEG-1500 was added under constant stirring over 1 minute. Increasing amounts of GMM were added over the next ten minutes to a total of 20 ml. The cells were subsequently centrifuged, resuspended in 100 ml GMM-20% FCS and divided among 48 wells (Linbro 24-well trays, 2 ml/well, Flow laboratories). The next day, 1 ml of the medium in each well was replaced with HAT-medium. This procedure was repeated every 48 hours until the medium was beginning to turn yellow. At this stage the supernatants were assayed for the presence of anti-HSV-2 antibodies and the cells from those wells with positive supernatants were passaged for one more week in HAT-medium and 2-3 weeks in HT-medium. After this period supernatants were reassayed and cells from those cultures which continued to secrete antibody were cloned in soft agar. Positive clones were stored in liquid nitrogen and also injected into Balb/c mice, either subcutaneously or intravenously, to produce antiserum or ascites fluid.

**Cloning.** Hybridoma cells were cloned when in the log-phase of growth. Soft agar plates were prepared using Difco-Bacto agar. Each plate contained 10 ml 0.5% agar in GMM-20%FCS, and was allowed to set on the bench for one hour. In the meantime, dilutions of the cells to be cloned were prepared in GMM-20%FCS and a solution of 0.5% agar in GMM-20%FCS was kept ready at 40°C; 1 ml of the cell suspension, containing 10^3 or 10^4 cells, was mixed with 1 ml of the 0.5% agar solution and poured on top of the 0.5% agar plate. The plates were transferred to a gassed incubator at 37°C and colonies which appeared after 3 or 4 days were picked using a microscope. Colonies were transferred to 2 ml Linbro-wells in GMM-20%FCS and culture supernatants assayed as before. Positive clones were gradually transferred to GMM-5%FCS and stored in liquid nitrogen.

**Labeling of secreted immunoglobulins.** In a 2 ml Linbro-well 10^6 hybridoma-cells were incubated for 5 hours in lysine-free minimum essential medium in the presence of 5 µCi ^14^C-lysine. At the end of this period, the supernatant was taken and 20 µl analysed by electrophoresis in SDS-polyacrylamide gels. The procedure for preparing and running the gels is described in chapter 3. Gels were dried down and the isotope detected by autoradiography for 3-4 weeks.
Ouchterlony test for the detection of the immunoglobulin class of the antibodies.

In order to determine the immunoglobulin subclass of the antibodies secreted by the hybridoma cells, culture supernatants were concentrated using a vacuum-filter and tested in an Ouchterlony assay plate (1% agar, 8µl per well). Antisera specific for IgG1, IgG2a, IgG2b and IgG3 were a kind gift of Dr. R. M. I. Parkhouse, Mill Hill, London.

RESULTS.

A. Preliminary Experiments.

Immunisation schedule.

The virus used for the immunisation of mice to be used for a fusion was grown in 3T3-cells. This was done because 3T3-cells are a Balb/c derived strain. It was hoped that the antibody response in the mouse would only be directed against viral antigens, thus preventing the selection of hybridoma's directed against cellular antigens.

The first step in any immunisation schedule was the establishing of latent infection. This was done using intradermal inoculation of the virus into the ear pinna (Hill et al, 1975). Primary infectious doses of $10^3$ and $10^4$ pfu HSV-2 were given to different groups of mice, and secondary immunisations were given either intraperitoneally or intravenously. No Freunds adjuvant was used in any of these instances. At varying times during the immunisation schedule mice were bled and the serum antibody titre measured using neutralisation, reverse passive haemagglutination or RIA-1. The results in table 1 show that injection with higher doses of virus leads to a larger antibody response and that secondary intraperitoneal immunisation gives a slightly lower antibody response as measured by RIA-1 than intravenous immunisation.

Assay systems.

The major criteria for each assay system were speed, reliability and sensitivity. Three different radioimmunoassay systems were compared. In all earlier experiments, anti-mouse-Fab was used as the radiolabelled agent. In later experiments, this was replaced by protein A. In the first type of radioimmunoassay,

RIA-1, (see fig.1a) BHK-cell monolayers in small wells were infected with HSV-2 and fixed in glutaraldehyde. Wells were reacted with serial ten-fold dilutions of a hyperimmune mouse serum, washed and $^{125}$I-anti-mouse-Fab used to detect bound antibody (50,000 cpm/well). Each solution was also tested on uninfected BHK-cells, and normal mouse serum was used as a negative control. The results of the experiment are listed in table 2; the background in all cases is less than 1% of the total input $^{125}$I, and binding ratio's of 6 or 7 are reached at
serum dilutions of 1/100 and 1/1000. Even at a dilution of 1/10,000 a binding ratio of 1.9 is obtained. On uninfected BHK-cells, immune mouse serum and normal mouse serum bind to the same extent, so the reaction with immune serum is specific for HSV-2 infected cells.

RIA-1  RIA-2  RIA-3

Fig 1. Illustration of three different radioimmunoassays to measure antibody. O=viral antigen, Υ=anti-HSV-2-IgG, Y=antibody to be measured, ΥF=I-labelled anti-mouse-Fab.

<table>
<thead>
<tr>
<th>Infection with</th>
<th>1st boost (pfu)</th>
<th>serum titre RPHI</th>
<th>serum titre RIA-1 neutr.</th>
<th>serum titre RIA-1</th>
<th>2nd boost (pfu)</th>
<th>serum titre RIA-1</th>
</tr>
</thead>
<tbody>
<tr>
<td>10³ pfu</td>
<td>10³,i.p.</td>
<td>1/8</td>
<td>1/50</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>10³ pfu</td>
<td>10³,i.v.</td>
<td>1/16</td>
<td>1/100</td>
<td>-</td>
<td>10³,i.v.</td>
<td>1/1000</td>
</tr>
<tr>
<td>10⁴ pfu</td>
<td>10⁴,i.p.</td>
<td>-</td>
<td>1/100</td>
<td>1/500</td>
<td>10⁴,i.p.</td>
<td>1/2000</td>
</tr>
<tr>
<td>10⁴ pfu</td>
<td>10⁴,i.v.</td>
<td>-</td>
<td>1/100</td>
<td>1/500</td>
<td>10⁴,i.v.</td>
<td>1/2000</td>
</tr>
<tr>
<td>10⁵ pfu</td>
<td>10⁵,i.v.</td>
<td>1/16</td>
<td>1/100</td>
<td>1/500</td>
<td>10⁵,i.v.</td>
<td>1/10</td>
</tr>
</tbody>
</table>

Table 1. Comparison of different immunisation schedules. i.v.=intravenous; i.p.=intraperitoneal.

RIA-2. (see fig. 1b). It is possible that some antigens are not resistant to glutaraldehyde fixation. It was therefore thought desirable to have an alternative assay which would present viral antigens in a native form. To this end, different dilutions of an HSV-2-infected BHK-cell sonicate were coupled to 0.4 ml Linbro-wells using carboimide. After washing the wells were blocked with IFCS, and a radioimmunoassay carried out using hyperimmune mouse serum and ¹²⁵I-anti-mouse-Fab at the same concentrations as for RIA-1. As can be seen from table 2, this method also detected viral antigens specifically, but binding ratio's were not as high as were obtained using infected BHK-cell monolayers, except at low serum dilutions, where the results are unreliable. Increasing the amount of viral antigen per well did not raise the number of bound cpm (results not shown).

A third type of radioimmunoassay was tested, RIA-3. (see fig. 1c). In this method the viral antigens were selected by a hyperimmune anti-HSV-2 rabbit serum. To this end, purified anti-HSV-2-IgG was coupled to 0.4 ml Linbro-wells as described in "materials and methods", and subsequently reacted with a dilution of a sonicate of HSV-2-infected BHK-cells. This
target (unlike the target in RIA-1 and 2) is composed of virus-specific antigen and should, in theory, give low backgrounds. Its disadvantage is that it contains only the antigens for which antibodies are present in the serum. A radioimmunoassay comparable to the ones performed using RIA-1 and 2 was carried out. The results are shown in Table 2. A dilution of hyperimmune serum of 1/100 gives the highest binding ratio, but even at a dilution of 1/10,000 antibody can still be detected. The values obtained for normal mouse serum are very low, as expected. The test was repeated using uninfected BHK-cell antigens as a target, and no detectable binding of hyperimmune anti-HSV-2 serum could be found.

<table>
<thead>
<tr>
<th></th>
<th>serum dilution</th>
<th>hyperimmune serum</th>
<th>normal serum</th>
<th>binding ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>RIA-1 infected cells</td>
<td>1/100</td>
<td>3802</td>
<td>600</td>
<td>6.3</td>
</tr>
<tr>
<td>RIA-1 infected cells</td>
<td>1/1000</td>
<td>1296</td>
<td>199</td>
<td>6.5</td>
</tr>
<tr>
<td>RIA-1 infected cells</td>
<td>1/10,000</td>
<td>295</td>
<td>155</td>
<td>1.9</td>
</tr>
<tr>
<td>RIA-1 uninfected cells</td>
<td>1/100</td>
<td>489</td>
<td>522</td>
<td></td>
</tr>
<tr>
<td>RIA-1 uninfected cells</td>
<td>1/1000</td>
<td>219</td>
<td>234</td>
<td></td>
</tr>
<tr>
<td>RIA-1 uninfected cells</td>
<td>1/10,000</td>
<td>181</td>
<td>190</td>
<td></td>
</tr>
<tr>
<td>RIA-2 infected cells</td>
<td>1/100</td>
<td>2130</td>
<td>465</td>
<td>4.6</td>
</tr>
<tr>
<td>RIA-2 infected cells</td>
<td>1/1000</td>
<td>1109</td>
<td>242</td>
<td>4.6</td>
</tr>
<tr>
<td>RIA-2 infected cells</td>
<td>1/10,000</td>
<td>333</td>
<td>122</td>
<td>2.7</td>
</tr>
<tr>
<td>RIA-2 sonicate</td>
<td>1/100</td>
<td>4038</td>
<td>182</td>
<td>22.2</td>
</tr>
<tr>
<td>RIA-2 sonicate</td>
<td>1/1000</td>
<td>1870</td>
<td>142</td>
<td>13.2</td>
</tr>
<tr>
<td>RIA-2 sonicate</td>
<td>1/10,000</td>
<td>417</td>
<td>150</td>
<td>2.8</td>
</tr>
</tbody>
</table>

Table 2. Comparison of three radioimmunoassay systems for the detection of anti-HSV-2 antibody in immune or normal mouse serum. Results are expressed in bound cpm/well, (mean of 2). Binding ratio's are expressed as immune/normal.

DISCUSSION.

Immunisation.

Although intraperitoneal inoculation of viruses or viral antigens has been successful in hybridoma production (Koprowski et al, 1977; Wiktor et Koprowski, 1978; Nowinski et al, 1979), I decided to establish latent infection in Balb/c mice by injection of live virus into the ear pinna. This route was chosen because of the fact that the so produced infection has been well characterised in our laboratory, and it was known that a neutralising antibody response to a titre of 1/40 would develop following infection with 10⁴ pfu of virus (Nash et al, 1980a). The difference between intravenous and intraperitoneal secondary immunisations was small, but i.v. seemed to give a slightly higher antibody response as measured by RPHI and neutralisation. The serum antibody titre was most affected by the dose of virus administered: a higher dose gives rise to a
higher antibody response. The immunisation schedule that was adopted after consideration of these data is outlined in "Materials and Methods" of this chapter. After infection with \(10^4\) pfu in the ear pinna, two increasing doses of live virus are given intraperitoneally, followed by the final intravenous injection three days before the fusion. Secondary immunisations using the intraperitoneal route were chosen instead of intravenous injections because of the fact that the mice frequently died of anaphylactic shock following repeated intravenous challenge with live virus. The antibody titre in mice thus immunised was usually between 1/1000 or 1/10,000, as measured by RIA-1. Although a primary immunisation using a higher dose of infectious virus would give rise to a higher antibody titre (see table 1), a dose of \(10^5\) pfu or more was not practical as a great percentage of the mice thus immunised died following the infection.

In a recent report, it was suggested that infectious virus is inferior to inactivated virus as an immunogen (Killington et al, 1981). This observation was made on the basis of a comparison between the yield of positive hybrids following a fusion with the spleen of mice that were either immunised with live virus or with inactivated virus plus Freund's Adjuvant. A much higher number of positive hybrids was obtained in the latter case. However, in our studies we have found no evidence for a poor immune response to live virus, if administered intradermally. Also, the yield of positive hybrids was higher in our fusions than in the ones described in the above paper. The difference is therefore probably not so much due to the infectivity of the virus as to the route of infection: i.p. or i.v. injection with live virus is inferior to intradermal injection.

Assay methods.

Of the three assay systems compared, RIA-3 was found to be the most sensitive, mainly because of the very low background values. RIA-1 was also satisfactory as far as sensitivity and specificity were concerned. Both the plates and radiolabelled reagents could be stored for reasonable lengths of time. Both assays were limited, however, in the presentation of viral antigens: RIA-1 uses glutaraldehyde to fix the infected cells, and it is not known to what extent this modifies the viral antigens and to what extent cytoplasmic and intranuclear antigens are exposed to antibody. RIA-3 is restricted by the fact that Rabbit anti-HSV-2 immunoglobulin is used to select target antigens, thus selecting for the antigens which give a dominant immune response in the rabbit. Furthermore, the assay involves the use of large amounts of hyperimmune mouse serum. The differences in presentation of the target antigen in the two assays, (RIA-1 and 3), are reflected in the fact that they seem to detect different monoclonal antibodies (see section B).

B: Fusion Experiments.

Three fusion experiments were carried out using the immunisation routine outlined in "materials and methods", which
is based on the results described in section A. After each fusion, the cells were distributed over 48 wells, and cell growth was observed in each well.

The result of the first fusion can be found in fig. 2; supernatants from 48 wells were assayed by RIA-1 and RIA-3, using ¹²⁵I-anti-mouse-Fab. Positive wells were defined as having a binding ratio of 2 or more. The control in both assays was supernatant from NS1 cell cultures. Of the 48 wells, 36 were found to be positive by the assay on infected BHK-cell monolayers (RIA-1) and 31 by the assay using antibody-selected viral antigens (RIA-3). However, many of the wells positive in both assays had a much higher binding ratio in RIA-1 than in RIA-3 or vice versa. This proves that the two assay systems present viral antigens in different amounts and in a different way.

Ten wells with a binding ratio of 4 or higher in at least one of the assay systems were passed for four weeks in HAT-medium and HT-medium. The reason for this was the reported instability of many hybridoma's. It was hoped that passage of the cells for a certain time would select for the hybridoma's stable in the secretion of antibody. At the end of this period, the supernatants were tested again in the two assay systems. Although each of the supernatants was still positive, the binding ratios of six of them had dropped more than 50% compared with four weeks earlier in RIA-1. In RIA-3, all binding ratios were at least halved compared to the first assay. Thus, four hybridoma mixtures were still highly positive in RIA-1 only. These mixtures were cloned in soft agar and positive clones could only be isolated from one of the mixtures. These clones were grown up and recloned in soft agar. The frequency of positive subclones was 100%. The hybridoma was named LP1.

The second fusion was carried out in the same way as the first one, with the exception that only RIA-1 was used in the assay of the 48 wells. A total of 30 out of 48 wells was positive (see fig. 3). The ten most positive wells, with a binding ratio of six or more, were passed for three weeks and the supernatants assayed by RIA-1 and RIA-3. Six wells still had the same or a higher binding ratio as before in RIA-1, and these were also positive in RIA-3. These hybridoma mixtures were cloned, and positive clones could be isolated from four of the mixtures. These were grown and subcloned as for fusion 1. The hybridoma's were called LP3, LP7, LP8 and LP9.
FUSION 1.

<table>
<thead>
<tr>
<th>Supernatant test 10 days after fusion</th>
<th>+ve wells RIA-1</th>
<th>+ve wells RIA-3</th>
<th>shared +ve wells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Selected 10 wells</td>
<td>36/48</td>
<td>31/48</td>
<td>30</td>
</tr>
<tr>
<td></td>
<td>10/10</td>
<td>10</td>
<td></td>
</tr>
</tbody>
</table>

Passed for four weeks in HAT-medium or HT-medium

Number of wells with the same binding ratio as in the first test:

<table>
<thead>
<tr>
<th>RIA-1</th>
<th>RIA-3</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>0</td>
</tr>
</tbody>
</table>

4 hybridoma mixtures cloned under agar.

From 3 no positive clones obtained.

<table>
<thead>
<tr>
<th>LP1</th>
<th>no. of +ve clones</th>
<th>frequency of +ve subclones</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>12/16</td>
<td>100%</td>
</tr>
</tbody>
</table>

Fig. 2. Selection of positive hybridoma's from fusion 1. RIA-1 and RIA-3 as described in chapter 2A. Positive wells were defined as having a binding ratio >2. The binding ratio was defined as: cpm bound from test supernatant/cpm bound from NS1-supernatant.
FUSION 2

<table>
<thead>
<tr>
<th>Supernatants test</th>
<th>+ve wells RIA-1</th>
<th>+ve wells RIA-3</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 days after fusion</td>
<td>30/48</td>
<td>n.d.</td>
</tr>
<tr>
<td>Selected wells</td>
<td></td>
<td>10</td>
</tr>
</tbody>
</table>

Passed for three weeks in HAT-medium and HT-medium.

Number of wells with equal or higher binding ratio than in the first assay:

<table>
<thead>
<tr>
<th>RIA-1</th>
<th>RIA-3</th>
</tr>
</thead>
<tbody>
<tr>
<td>6/10</td>
<td>6</td>
</tr>
</tbody>
</table>

6 cultures cloned in soft agar.

<table>
<thead>
<tr>
<th>nr of +ve clones</th>
<th>frequency of +ve subclones</th>
</tr>
</thead>
<tbody>
<tr>
<td>LP3 2/8</td>
<td>90%</td>
</tr>
<tr>
<td>LP7 7/10</td>
<td>90%</td>
</tr>
<tr>
<td>LP8 1/10</td>
<td>90%</td>
</tr>
<tr>
<td>LP9 2/7</td>
<td>50%</td>
</tr>
</tbody>
</table>

Fig. 3. Selection of positive hybridoma's from fusion 2. RIA-1, RIA-3 and definition of positive wells and binding ratios as for fig. 2.

The assay system for the third fusion differed from that for the first two in that $^{125}$I-protein A was used instead of $^{125}$I-anti-mouse Fab. This excluded the use of RIA-3, as protein A would bind to rabbit-IgG coupled to the plastic. Therefore, all assays were carried out using infected BHK-cell monolayers as targets. Out of 48 wells, 17 had a binding ratio of 2 or more. The cells from the twelve most positive wells were passed for three weeks and reassayed: seven supernatants had an equal or higher binding ratio as before. The cells from these wells were cloned and in soft agar. Positive clones were isolated from four of the seven hybridoma mixtures. These were recloned and the frequency of positive subclones was 100%. The hybridoma's were named LP2, LP4, LP5 and LP6.

The positive supernatants of the hybridoma mixtures of each of the three fusions, that were passed for several weeks after the first assay, were tested for their ability to bind to uninfected BHK-cells. This was done in a radioimmunoassay similar to RIA-1, but using uninfected cells as a target. No detectable binding could be found for any of the hybridoma's.
This experiment was repeated with supernatants of the selected positive clones, and the same result was obtained.

**FUSION 3**

<table>
<thead>
<tr>
<th>Nr of wells +ve in RIA-1</th>
<th>17/48</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nr. of wells selected</td>
<td>12</td>
</tr>
</tbody>
</table>

Passed for three weeks in HAT-medium or HT-medium.

<table>
<thead>
<tr>
<th>Nr. of wells with binding ratio equal or higher as in first assay:</th>
<th>RIA-1</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>7</td>
</tr>
</tbody>
</table>

Cloned in soft agar.

From three cultures no positive clones obtained.

<table>
<thead>
<tr>
<th>+ve clones</th>
<th>frequency of +ve subclones</th>
</tr>
</thead>
<tbody>
<tr>
<td>LP2</td>
<td>12/12</td>
</tr>
<tr>
<td>LP4</td>
<td>12/12</td>
</tr>
<tr>
<td>LP5</td>
<td>3/16</td>
</tr>
<tr>
<td>LP6</td>
<td>4/4</td>
</tr>
</tbody>
</table>

Fig. 4. Selection of positive hybridoma’s from fusion 3. RIA-1 and definition of positive wells and binding ratios as in Fig. 2.

Raising of serum or ascitic fluid.

The hybridoma cells of each line were injected into Balb/c mice subcutaneously or intraperitoneally in order to raise antiserum or ascitic fluid. The antibody titres of these sera or ascitic fluids varied from $1/10^4$ to $1/10^8$, as measured by RIA-1. The variation, however, was between clones: the same clone would give a similar titre every time, varying perhaps by a factor of 2 or 3.

Immunoglobulins secreted by the hybridoma’s.

The immunoglobulins secreted by the stable hybridoma lines isolated from the three fusion experiments described above were characterised. The hybridoma cells were incubated in the presence of $^{14}C$-lysine, and the supernatants analysed on SDS-polyacrylamide gels. The result with a few of the supernatants is shown in fig. 5. All lines produce immunoglobulin of the IgG class. Different clones from the same original well secrete heavy and light chains of the same electrophoretic mobility.
The immunoglobulin-G subclass of each of the hybridoma's was determined using antisera specific for each of the subclasses. The results were as follows: antibodies LP2, LP3, LP4, LP5 and LP6 all belong to the IgG2a subclass, while LP1, LP7, LP8, and LP9 are of the IgG1 subclass. These results can be seen in the table at the end of this thesis.

Fig. 5. Immunoglobulins secreted by some of the hybridoma's, labelled with C-lysine and run on an 10% SDS-polyacrylamide gel. A=LP4; B=LP2; C=LP4; D=LP6; E=LP5; F=LP3. H, L: heavy and light chain of immunoglobulin G.

DISCUSSION.

The number of positive supernatants derived from each of the three fusions described above (77%, 63% and 35% respectively) compares favourably with other reports concerning anti-HSV-1 or anti-HSV-2 monoclonal antibodies (Killington et al, 1981; Zweig et al, 1979). In the case of the third fusion a lower number of positive wells was obtained than in the first two fusions. This can probably be explained by the fact that protein A was used to detect the secreted immunoglobulins, and this method will not detect all immunoglobulin classes or subclasses. It is probable that many secreting hybridoma's were not detected in fusion 3.
The procedure of passing positive hybridoma cultures for several weeks before cloning in order to select for stable hybridoma's worked satisfactory. Admittedly, a large proportion of positive hybridoma's is lost in this way: presumably some cultures contain non-producing cells which overgrow producing cells, while some producing cells themselves may be very unstable. The advantage of this period of passage is that it allows stable hybridoma's to be identified, and it increases the homogeneity of the cultures by selecting for the "fast growers". This reduces the effort involved in cloning: in most instances positive clones were readily obtained from a small number of picked colonies, and in several instances all colonies were positive. One can thus be fairly certain of the stability of the clones. The nine hybridoma's described in this chapter have all been passed for several months without any loss of their ability to secrete antibody.

None of the positive supernatants gave any detectable binding to uninfected BHK-cells. In consequence, the antibody secreted by these hybridoma's is in all cases directed against viral antigens.

The fact that different clones derived from the same original hybridoma culture always secrete immunoglobulins with heavy and light chains of the same electrophoretic mobility suggests that these clones are derived from the same parent. This observation was confirmed in later experiments, when the clones were characterised in more detail. Apparently, the hybridoma cultures are often overgrown very rapidly by one or a few fast growing hybrids. This is in agreement with the findings of Gerhardt et al (1977) in the production of hybridoma's secreting antibodies against influenza virus.

In conclusion, this chapter describes the generation of nine hybridoma's secreting antibody against HSV-2. All antibodies are of the IgG class. A further characterisation of the antibodies is given in the following chapters.
CHAPTER 3: ANTIBODIES LP1 AND LP4.

INTRODUCTION.

In this chapter we describe the further characterisation of two monoclonal antibodies, LP1 and LP4. Their production has already been described in chapter 1. Both antibodies are of the IgG-class. Their target antigens were identified by immunoprecipitation and polyacrylamide gel electrophoresis. Their reactivity with several strains of HSV-1 and HSV-2 was established, and their ability to neutralise infectious virus measured.

MATERIALS AND METHODS.

Cells and virus.

Hybridoma cells were passed in GMM supplemented with 5% FCS. BHK-21 cells were passed in ETC as described in chapter 2. Strains of Herpes Simplex Virus used were as follows: Type 1 strains: strain Cl(101), Dubbs and Kit(1964); strain HPEM, watson et al.(1966); strain F, Ejercito et al.(1968); strain SC16, Hill et al.(1975). Type 2 strains: strain Bry, Thouless and Skinner (1971) strain 25766 (isolated by K.R.Dumbell, Wright Fleming Institute, London); strain 333, (isolated by Rawls, McMaster University, Ontario). Viruses were propagated in BHK-21 cells.

Radioimmunoassay.

The radioimmunoassay used was RIA-1 described in chapter 2, with the difference that $^{125}$I-labelled Staph A protein was used for the detection of antibodies instead of $^{125}$I-anti-mouse Fab.

Fluorescence.

Sub-confluent BHK-cell monolayers were grown on glass microscope slides and infected with HSV-2 at an approximate multiplicity of 3 pfu per cell. Eight hours after infection the cells were fixed in methanol at $-20^\circ$C overnight. Fixed cells were reacted with antibody for 1 hour at room temperature, washed in PBS and then reacted with FITC-labelled, affinity-purified sheep anti-mouse Fab (gift from Dr.A.Muro) for 1 hour at room temperature.

Preparation of isotope-labelled antigen.

Monolayers of BHK-21 cells were infected with HSV-1 or HSV-2 at a multiplicity of 10 pfu per cell and labelled from 3 to 8 hours after infection in methionine-free medium containing 200 $\mu$Ci/ml $^{35}$S-methionine (sp. act.$>600$ Ci/mMole, Amersham Corp.). $^{32}$P-labelled antigens were prepared by incubating cells from 3 to 8 hours post infection in phosphate-free medium containing 22
μCi/ml (32P)-orthophosphate (carrier-free, Amersham Corp.). Cells labelled with either isotope were harvested in PBS at 10^7 cells/ml. Cell suspensions were lysed by the addition of 0.1 volume of lysis buffer (0.9 M NaCl, 0.01M tris-Cl pH 7.4, 7.5 mM L-methionine, 1% Triton and 10% deoxycholate), and incubated 15 minutes at 0°C. Insoluble material was removed by centrifugation at 10,000g for 10 min. The supernatants were made to 0.02mM with respect to phenyl methylsulphonylfluoride (PMSF) and stored at -70°C.

Immunoprecipitation. 20 μl of isotope-labelled antigen was mixed with 5 μl antibody (ascites fluid or serum) and incubated for 30 min. at room temperature. 25 μl of a 50% suspension of protein-A sepharose Cl-4B-beads (Pharmacia Ltd) was added together with 150 μl 0.15M NaCl, 0.01M tris-Cl pH 7.4, 75 mM L-methionine, 1% Triton and 1% sodium deoxycholate. This mixture was kept at room temperature for 2 hours with continuous agitation sufficient to keep the beads in suspension. The beads were collected by centrifugation, washed three times in 0.5M LiCl, 0.1M tris-Cl pH 7.4, 1% 2-mercaptoethanol, and the immune complexes dissolved by addition of 50 μl 0.025M tris-Cl pH 7.0, 2% SDS, 20% glycerol, 1.5% dithiothreitol, 0.02% bromophenol blue. Samples were heated to 100°C for two minutes and electrophoresed for about 4 hours at 12V/cm in 10% or 12.5% acrylamide gels cross-linked with 0.15% bisacrylamide. The buffer system used for electrophoresis was as described by Laemmli (1970). After electrophoresis the gels were stained in 0.1% Coomassie Blue in methanol:acetic acid:water (50:10:40), destained in the same solvent, dried on filter paper and exposed to X-ray film for 2 to 10 days.

Identification of target antigens by treatment of acrylamide gels with antibody. As an alternative to immunoprecipitation followed by electrophoresis in some experiments unlabelled extracts of virus infected cells were subjected to SDS-polyacrylamide electrophoresis and the target antigen identified by treatment of the gel with antibody. 12% polyacrylamide gels were cross-linked with NN'-dial-tartardiamide (Heine et al, 1974) and antigens were disrupted prior to electrophoresis by incubating for 10 min. at 45°C in 2% SDS, 5% 2-mercaptoethanol, 3% sucrose in 0.05 M tris-Cl pH 7.0. After electrophoresis gels were fixed in methanol:acetic acid:water (46:8:46) At -20°C for at least 12 hours and were washed for 24 hours in three samples of PBS. The gels were then shaken for 5 hours in a sealed plastic bag containing sufficient antibody solution to keep the gel surface moist (about 5ml for a 5x11 cm gel slice). Ascites fluids containing monoclonal antibodies were diluted 100-fold in PBS containing 200 μg/ml ovalbumin. After reaction with antibody the gels were washed for 36 hours in PBS and incubated in PBS containing 200 μg/ml ovalbumin and 2 μCi 125I-labelled protein A (specific activity 10 μCi/μg). Gels were again washed for 36 hours to remove unreacted protein A and were stained with Coomassie Blue, destained and dried onto filter paper before autoradiography.
Fig. 1. Electrophoresis of LP1-precipitates. Samples were prepared as described in "Materials and Methods", and electrophoresed on a 12.5% acrylamide gel. A: total $^{32}$P-labelled extract; B: immune precipitate with LP1; D: total $^{35}$S-methionine labelled extract; C: immune precipitate with LP1.

Fig. 2. Detection of LP1-target antigen in unlabelled extracts. Target antigen was identified by reacting the gel with antibody and $^{125}$I-protein A (see methods). 1: uninfected cell extract; 2: HSV-1 infected cell extract; 3: HSV-2 infected cell extract; 4: purified HSV-2 particles; 5: $^{35}$S-methionine labelled HSV-1 infected cell extract. Gel A was reacted with LP1 and gel B with normal rabbit serum.

Fig. 3. Electrophoresis of LP4 immunoprecipitates. A: $^{35}$S-methionine labelled cell extracts. 1: LP4 with HSV-2 infected cell extract; 2: LP4 with uninfected cell extract; 3: normal mouse serum with HSV-2 infected cell extract. 5: total HSV-2 infected cell extract. MDBP is arrowed.

B: purified major DNA-binding protein. Gel was stained with Coomassie Blue. 1: MDBP; 2: MDBP precipitated with LP4; 3: as track 2, but precipitated with LP3; 4: LP4 alone. H and L mark positions of heavy and light chains of IgG.
Neutralisation.

The neutralising activity of monoclonal antibodies was tested by adding 10 μl serum to 10⁴ pfu HSV-2 or HSV-1, in 100 μl of a mixture of 50% GMM-40% PBS-10%FCS. After incubation overnight at 4°C the number of remaining pfu was measured in a suspension assay, as described in chapter 2.

RESULTS.

Identification of target antigens.

Lp1.

In order to identify the target antigen of antibody LP1, HSV-2 infected BHK-21 cells were labelled with ³⁵S-methionine, lysed, and aliquots of the lysate reacted with antiserum obtained from different clones of LP1. The immune precipitates obtained in this way were analysed on polyacrylamide gels. As shown in fig.1a, monoclonal antibody LP1 precipitates a protein with a molecular weight of 65,000 D. Each clone reacts with the same polypeptide (not shown).

In order to determine the nature of the 65K-protein, LP1 antibodies were reacted with a ³²P-labelled HSV-2 infected cell lysate. Fig 1b shows that the 65K protein precipitated by LP1 is phosphorylated. Identical results were obtained with HSV-1 infected cell extracts. Several groups have reported the presence of phosphorylated polypeptides of approximately 65,000 molecular weight in HSV-infected cells. A prominent structural polypeptide of this molecular weight has been designated VP16 in HSV-1 and HSV-2 (Spear and Roizman, 1972; Cassai et al, 1975; Honess and Roizman, 1973), and is phosphorylated (Gibson and Roizman, 1974). This probably corresponds to the polypeptide designated M65 and P65* in HSV-1 infected cells by Maraden et al (1978) and to ICSP 31 in HSV-2 infected cells (Powell and Courtney, 1975). Knopf and Kaemer (1980) identified a 65K phosphoprotein associated with the chromatin in HSV-1 infected cells and RC-37 cells, but could not detect this polypeptide in purified virions. To establish whether the target antigen of LP1 is a virion polypeptide, purified HSV-1 particles were disrupted and subjected to electrophoresis in parallel with extracts of uninfected cells, HSV-1 infected cells and HSV-2 infected cells. The gel was then reacted with antibody LP1 followed by ¹²⁵I-labelled protein A as described in "Materials and Methods". The resulting autoradiograph (fig.2) shows the target antigen to be present in infected cells and in purified particles and to be of similar molecular weight in type 1 and type 2 infected cells. The properties of the LP1 target antigen suggest that it corresponds to polypeptide VP16 in HSV-1 and ICSP 31 in HSV-2. However, this conclusion should be treated with caution in view of the complexities of the published HSV polypeptide patterns in this region of SDS acrylamide gels and the current confusion in HSV polypeptide nomenclature. In any event antibody LP1 should prove useful in helping to achieve a consistent nomenclature among different groups of workers using different virus isolates and different gel electrophoresis systems.
LP4

Electrophoresis of immunoprecipitates prepared from \( ^{35}S \)-methionine labelled cell extracts of HSV-2 infected cells (fig.3a) showed that the target antigen of LP4 was a polypeptide of approximately 130,000 M.W. The electrophoretic behaviour of this polypeptide suggested that it might correspond to the HSV-2 major DNA-binding protein, designated ICSP 11/12 by Purifoy and Powell (1976). This was confirmed by showing that LP4 could react with the purified major DNA-binding protein (Powell et al,1981). This was achieved in two ways. Firstly, purified ICSP 11/12 (a gift from Dr.K.Powell) was precipitated by LP4 and not by a different monoclonal antibody of the same subclass (fig 3b). Secondly, purified ICSP 11/12 blocked the binding of LP4 to HSV-2 infected target cells in a radioimmunoassay, whereas it did not block the binding of a different monoclonal antibody of the same subclass (fig.4). LP4 is therefore directed against the major DNA-binding protein of HSV-2.

Cross-reactivity with other Herpes Simplex Virus strains.

The type-specificity of antibodies LP1 and LP4 was tested by radioimmunoassay. BHK-cell monolayers were infected with three different type 2 strains (25766, Bry and 333) and four different type 1 strains (CI(101), HFEM, F and SC16) at a multiplicity of 10 pfu per cell. The cells were fixed in glutaraldehyde and a 1/1000 dilution of each of the monoclonal antisera was tested on the plates. Controls were normal mouse serum and hyperimmune rabbit serum against HSV-2. Bound antibody was detected using \( ^{125}I \)-protein A. The results in table 1 show that antibody LP1 reacts strongly with all the virus strains tested. Monoclonal antibody LP4, on the contrary binds very efficiently to type 2 infected cells but shows no reactivity with the type 1 strains F, CI(101) and HFEM, and a very faint reactivity with SC16. LP1 and LP4 antisera were also tested on uninfected BHK cells and gave no detectable binding.

Immunofluorescence.

Indirect immunofluorescence with both the antibodies resulted in a predominantly nuclear fluorescence in HSV-2 infected cells, consistent with the target antigen of the antibodies. Antibody LP1 gave a homogeneous nuclear fluorescence while the distribution of antibody LP4 was heterogeneous (fig.5).
Fig. 4. Blocking experiment using RIA-1 and purified MDBP. Wells were treated with increasing amounts of MDBP before reaction with antibody (diluted 1/1000) and 125I-protein A. Controls were antibodies LP2, LP5 and buffer.

Fig. 5. Immunofluorescent staining with LP1 and LP4. Uninfected BHK-cells (c and d) or HSV-2 infected BHK-cells (a and b) were fixed in methanol 8 hours after infection and treated with LP1 (a, c) or LP4 (b, d). Bound antibody was stained using a fluorescent labelled anti-mouse Fab.
<table>
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Table 1. Reactivity of LP1 and LP4 with different virus strains. (serum dilutions 1/1000) using RIA-1. imm=hyperimmune anti HSV-2 serum. nms=normal mouse serum. binding ratio is defined as no. of cpm bound/no. of cpm bound for nms.

DISCUSSION.

In this chapter we described the characterisation of two monoclonal antibodies, LP1 and LP4. Both antibodies were selected against type 2 strain 25766, but show reactivity against two other type 2 strains, 333 and Bry. In addition, LP1 binds to four different type 1 strains tested, but LP4 does not react
with any of these strains. Both antibodies do not neutralise infectious virus (results not shown).

The true monoclonal nature of each of the hybridoma's has been established: clones and subclones of LP1 precipitate the same protein and secrete antibodies with heavy and light chains of the same electrophoretic mobility (see chapter 2B). This is also true for LP4.

The antibody secreted by hybridoma LP1 is directed against the virion polypeptide designated ICSP 31 in HSV-2 and VP16 in HSV-1. This is the first report of an antibody against this polypeptide and LP1 should prove useful in the characterisation of the structure and function of VP16. Antibody LP4 reacts type-specifically with the major DNA-binding protein ICSP 11/12 of HSV-2. This antigen is highly conserved among the members of the herpesvirus group (Killington et al, 1977; Yeo et al, 1981; Littler et al, 1981) and has recently been purified (Powell et al, 1981). Antibodies to the major DNA-binding protein are apparently elevated in patients with cervical carcinoma (Anzai et al, 1975; Melnick et al, 1976) and the protein has been detected by serological techniques in HSV-2 transformed cells (Flannery et al, 1977) and in cervical carcinoma biopsies (Dreesman et al, 1980). This evidence has been interpreted to implicate HSV-2 in cervical carcinoma, but, as Powell et al (1981) point out, given the considerable cross-reactivity of the major DNA-binding protein of different herpesviruses, the use of polyclonal sera to detect this antigen does not define its origin. Since LP4 is type-specific, this antibody should prove a more precise probe for detecting ICSP 11/12 in cervical carcinoma biopsies. Showalter et al (1981) have recently described a monoclonal antibody whose tentative target is the major DNA-binding protein of HSV-1. It is notable that, despite the structural conservation of this antigen among different herpesviruses (Littler et al, 1981) and the broad cross-reactivity of the polyclonal sera raised against it (Powell et al, 1981), both monoclonal antibodies to this antigen are type-specific.
CHAPTER 4: ANTIBODIES AGAINST GLYCOSYLATED PROTEINS.

INTRODUCTION

In the previous chapter data were presented which characterised two monoclonal antibodies against non-glycosylated proteins of HSV-2. In this chapter, six antibodies will be described which are directed against several different glycoproteins of the virus. The glycoproteins of Herpes Simplex Virus are important constituents of the viral membrane. A description of the properties and function of the different glycoproteins was given in the introduction to this thesis. It was shown that each of the glycoproteins can act as a target for neutralising antibodies, cytotoxic T-cells, complement-mediated cytolysis and antibody-mediated immune cytolysis. The antigenic properties of the glycoproteins gB, gC, gD and gE were also discussed, and it was shown that gB, gD and gE have type-common as well as type-specific sites, while gC is completely type-specific.

I present in this chapter the characterisation of one antibody against a protein tentatively designated as gC, one antibody directed against a protein tentatively designated as gE, and four against gD. Two of the anti-gD antibodies (AP7 and AP12) were isolated by Ms. A. Buckmaster (department of Pathology, University of Cambridge), but these are included in this chapter as they are necessary to confirm the existence of three distinct type-common antigenic sites on gD.

A short description of our attempts to identify the target antigen of antibodies LP7, LP8 and LP9 is also given. No target antigen could be identified for these antibodies.

MATERIALS AND METHODS.

Virus strains, cells and medium.
The virus strains used were type 2 strain 25766 and type 1 strain SC16, as described in chapter 3. Propagation of the virus was as described in chapter 2. BHK-21 cells, NS1-cells and hybridoma cells were passed in ETC or GMM-5%FCS as described in chapter 2.

R.I.A.
The assay used was RIA-1 as described in chapter 2, using $^{125}$I-protein A to detect bound antibodies.

Neutralisation.
In order to measure the neutralising activity of antibodies secreted by hybridoma cells into the cell supernatant, 100 µl of supernatant was mixed with $10^4$ pfu HSV-2 or HSV-1, and the mixture incubated overnight at 4°C. A 1/10
dilution was subsequently plated out on BHK-21 cells and the number of plaques scored after two days. The control was supernatant from the cell line NS1. The neutralising activity of monoclonal antiserum or ascitic fluid was tested as described in chapter 3.

**Immunoprecipitations.**

Two methods were used in immunoprecipitations. The first one, A, was as described in chapter 3. The second one, B, was as described by Balachandran et al (1981). Briefly, confluent BHK-cell monolayers were infected with 10 pfu HSV-2 per cell. After two hours adsorption the cells were washed and incubated in methionine-free medium containing 100μCi 35S-methionine per ml, and supplemented with 1% L-glutamine. For the labelling with 14C-glucosamine, the methionine-free medium was replaced by medium containing 20% glucose and 50μCi 14C-glucosamine per ml. After 20 hours incubation at 37°C, the cells were scraped off into PBS, washed three times in PBS and pellets stored at -70°C. The cell pellets were lysed with RIPA-buffer (0.05M tris-Cl pH 7.2; 0.15 M NaCl sodium deoxycholate; 0.1% sodium dodecyl sulphate; 1% Triton X-100) containing 50μg/ml DNAse and 0.1 mM phenylmethylsulfonyl fluoride (PMSP). The lysate was sonicated, centrifuged at 100,000xg for 1 hour, and the supernatant used for immunoprecipitations. To this end, 300μl of supernatant was mixed with 15 μl antibody (serum or ascitic fluid). After 30 min. incubation at room temperature, 100 μl of protein A linked to sepharose beads (Pharmacia Ltd, 15 mg in RIPA-buffer, pre-incubated with an HSV-2 infected Hep-2 cell lysate for 1 hour and washed in RIPA) was added. In some cases, 10μl of Rabbit anti-mouse IgG was added to the mixture before the addition of protein-A sepharose, and incubated for a further 15 minutes. The reaction mixture containing protein-A sepharose was kept for two hours at 4°C under constant mixing, after which time the beads were collected by centrifugation and washed three times in RIPA. The bound proteins were solubilized in 30μl Laemmli's buffer (see chapter 3) and analysed on 10 or 12.5% polyacrylamide gels as described in chapter 3.

**Fluorescence.**

The method used for fixed cells was as described in chapter 3, with the difference that infected cells were fixed for 20 minutes in cold acetone on cardice instead of in methanol. Fluorescence experiments with unfixed cells were carried out using freshly harvested BHK-21 cells. These were washed several times in GMM and pelleted (approx. 2x10⁷ cells per pellet). The pellet was mixed with 20 pfu HSV-2 per cell in a total volume of 200μl. The cells were incubated at 37°C for 1 hour, under frequent mixing. The cells were subsequently washed and incubated in a small petri-dish at 37°C in ETC. After 5 hours the cells were removed from the dish using a pipette, washed in warm PBS and placed on ice. 5x10⁶ BHK-cells per tube were reacted with a few drops of IFCS, washed and incubated with 50μl of a dilution of the antibody on ice for 30 min. The cells were then washed by spinning them through a cussion of FCS, and reacted with 50μl of a preparation of FITC-labelled anti-mouse Fab for a further 30 minutes on ice. After a second wash through IFCS the cells were suspended in mounting buffer (tris-Cl pH9.0:glycerol=1:9) and examined under a microscope.
Rosetting.

Trypsinized Sheep red blood cells were coated with monoclonal IgG-preparations using CrCl₃. The method for the coupling was the same as described in chapter 2. The IgG-fractions were isolated from ascitic fluid using 50% ammonium sulphate followed by extensive dialysis against saline.

BHK-21 cells were infected in suspension as described for "surface fluorescence". Five hours after infection they were washed in PBS, pelleted and resuspended in PBS at 2x10⁸ cells/ml; 35μl of this suspension was added to 35μl of a 1% suspension of coated Shrbc, gently pelleted and left on ice for 1 hour. The pellet was then gently resuspended and the cells examined under the microscope. Rosettes were scored as BHK-cells with 4 or more red-blood cells attached to them.

Coupling of IgG to CNBr-activated sepharose beads.

The IgG-fraction was purified from ascitic fluid or serum using 50% ammonium sulphate. Approximately 10 mg. IgG was coupled to 1 gram of dried gel. The dried gel was washed in 0.001 M HCl, and subsequently mixed with the IgG in coupling buffer (0.1 M NaHCO₃, 0.5 M NaCl, pH 9). After two hours incubation at room temperature under constant rotation, unbound material was washed away using coupling buffer, and any remaining active groups were reacted with 1 M ethanolamine at pH 8.0 for two hours. At the end of this period, the beads were washed three times, each washing cycle consisting of a wash at pH 4.0 (0.1 M acetate buffer; 1 M NaCl) and one at pH 8.0 (coupling buffer).

Purification of IgG using protein A Sepharose.

The IgG fraction of ascitic fluids of monoclonal antibodies which bound well to protein A were purified using protein-A-Sepharose columns. The protein-A was swollen in 0.1 M phosphate buffer (pH 7.8). The serum to be purified was diluted 1/1 with the same buffer and run through the column. After all bound protein was washed off (checked by measuring the O.D.280 nm) the bound IgG was eluted off using 1 M acetic acid. The IgG was immediately dialysed extensively against PBS and stored at -20°C.

RESULTS.

I. Antibodies directed against gD.

The antibodies LP2, LP3, AP12 and AP7 were the result of three different fusion events. Two of these fusions have been described in detail in chapter 2. The third fusion was performed by Ms A. Buckmaster, with the aim of producing neutralising monoclonal antibodies against HSV-2. This fusion differed from the other two in two ways: 1. The mice were infected with 10⁴ pfu HSV-2 in the ear, but no secondary
immunisations were given, except for the final intravenous challenge three days before the fusion; 2. hybridoma supernatants were tested for neutralising activity against HSV-2 as well as by radioimmunoassay. The two monoclonal antibodies described in this chapter which resulted from this fusion are called AP7 and AP12.

The antibodies LP2, LP3, AP7 and AP12 were characterised by neutralisation, immunoprecipitation and fluorescence. Their type-specificity was also determined.

**Immunoprecipitations.**

Immunoprecipitations with antibodies LP2 and LP3 were carried out using the protocol A described in "materials and methods". This procedure had to be modified for the precipitation reaction with LP3, as no precipitated protein could be detected using this antibody and protein-A Sepharose beads. The addition of Rabbit anti-mouse IgG to the reaction mixture solved this problem. Monoclonal antibody AP12 does not bind to protein A at all. In this case the IgG-fraction was purified from ascitic fluids using ammonium sulphate, and this IgG was coupled to CNBr-activated Sepharose beads. These beads were mixed with S-methionine labelled HSV-2 infected cell lysate and the bound antigen washed and eluted in the same way as for protein-A Sepharose beads.

The immunoprecipitates were analysed on SDS-polyacrylamide gels. Fig.1 shows that each of the antibodies precipitates two polypeptides with M.W. of 52 K and 57 K. This corresponds to the molecular weights of gD and its precursor. In order to prove that these polypeptides were glycoproteins similar precipitation reactions were done using C-glucosamine labelled extracts, and the same polypeptides were detected on gels (see fig.2; results shown for LP2 and LP3 only). The final proof that these antibodies were directed against glycoprotein D came from an experiment using anti-band II antiserum (gift of Prof.D. Watson, Leeds). The polypeptide bands precipitated by this antiserum co-ran exactly with the LP3-precipitated bands (fig 3).

**Neutralisation.**

The neutralising activity of ascitic fluids obtained from hybridoma's LP2, LP3, AP7 and AP12 was measured. Several dilutions of each antiserum were mixed with $10^4$ pfu HSV-2 and after incubation overnight at 4°C, virus infectivity was measured as described. The results are shown in table 1. This experiment was repeated in the presence of 10μl guinea pig complement, giving a different result for antibody AP7. As shown, antibodies LP2 and AP12 neutralise HSV-2 effectively, while LP3 has no effect on virus infectivity. Antibody AP7 only reduces virus infectivity in the presence of complement. If no complement is present, an increased number of plaques is observed compared to the control value with normal mouse serum.

The neutralising activity of the four antibodies for HSV-1 was also measured: LP2 and AP12 reduce HSV-1 infectivity to the same extent as HSV-2; LP3 is non-neutralising for HSV-1, while AP7 does neutralise HSV-1 in the presence of complement, but fails to give significant enhancement of virus plaques in the absence of complement (results shown for LP2 and LP3 only).
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Table 1. Neutralisation of HSV-2 (10 pfu per vial) by antibodies LP2, LP3, AP7 and AP12. compl. = guinea pig complement, 10μl per vial. Experiments with AP7 and AP12 performed by Ms A. Buckmaster.

Cross-reactivity with HSV-1.

The cross-reactivity of LP2, LP3, AP7 and AP12 with HSV-1 was measured by radioimmunoassay (RIA-1). Several antibody-dilutions were tested on HSV-1 infected BHK-cell monolayers. Antibody AP12 could not be measured in this assay as it does not bind to protein A.

Table 2 shows that LP2, LP3 and AP7 react with HSV-1 as well as with HSV-2 infected cells. This corresponds with the neutralisation data, as AP7 and LP2 neutralise HSV-1 and HSV-2 to the same extent (AP7 in the presence of complement only). The combined results of neutralisation and RIA tests prove that the four antibodies are all cross-reactive with HSV-1.
Fig. 1: Electrophoresis of immunoprecipitates with $^{35}$S-methionine labelled HSV-2 infected cell extracts. A: AP7; B: AP12; C: LP3; D: LP2; E: normal mouse serum; F: total infected cell extract. Molecular weights given are of unlabelled marker proteins.

Fig. 2: Electrophoresis of immunoprecipitates using $^{14}$C-glucosamine labelled HSV-2 infected cell extracts. C: total HSV-2 infected cell extract; HI: precipitation with hyperimmune serum; LP2, LP3: precipitates with antibodies LP2, LP3; 5: total $^{35}$S-methionine labelled infected cell extract.

Fig. 3: Electrophoresis of immune precipitates using $^{35}$S-methionine labelled HSV-2 infected cell extracts. 1, 2: LP3, 2 different subclones; 3, 4: anti band-II serum; 5: combined anti-band II and LP3; 6: normal mouse serum; 7: hyperimmune anti-HSV-2 mouse serum.

Fig. 7, 8: legends on next page.
Fig. 7. Electrophoresis of immunoprecipitates using $^{35}\text{S}$-methionine labelled HSV-2 infected cell extracts. +tunicamycin: 2μg/ml tunicamycin. A, B: total infected cell extract. C, F: precipitates with antibody LP3. D, G: precipitates with antibody LP2.

Fig. 8. Electrophoresis of immunoprecipitates using $^{35}\text{S}$-methionine (A) or $^{14}\text{C}$-glucosamine (B) labelled HSV-2 infected cell extracts. A: 1, 3: monoclonal anti-gC. 4: monoclonal anti-gAB. 5: LP5. 6: LP6. 7: monoclonal anti-gE. 8: monoclonal anti-gF. 9: normal mouse serum. 10: total infected cell extract. B: a: LP5; b: monoclonal anti-gC; c: monoclonal anti-gAB; d: LP6; e: monoclonal anti-gE; f: monoclonal anti-gF; g: total infected cell extract.

Monoclonal anti-gAB, anti-gC, anti-gE and anti-gF were gifts from S. Bacchetti, Canada.

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Table 2. Cross-reactivity of LP2 and LP3 with HSV-1 as measured by RIA-1.

nms = normal mouse serum

**Competition binding.**

The neutralisation results suggest that the four antibodies are directed against at least three different antigenic sites on the gD molecule. In order to obtain more information about the number of antigenic sites to which these antibodies are directed, competition binding experiments were carried out. The IgG fraction of ascites fluids of LP2, LP3 and AP7 was purified using protein A Sepharose columns (see "materials and methods"). The purified IgG was labelled with I-125. Six different dilutions of each of the non-labelled monoclonal antibodies were used to block HSV-2 infected BHK-cell monolayers, before I-IgG was added and the number of counts bound measured. As a control homologous antibody was used to block itself, and as a negative control a monoclonal antibody against gC was used, as well as PBS. The results are shown in fig. 4. Binding of labelled LP2 to the infected cells is blocked effectively by unlabelled LP2 as well as by AP12. Neither LP3 nor AP7 prevent the binding of LP2 to the plates. The binding of labelled LP3 is only prevented by LP3-antiserum, the other
antibodies do not have any effect. The same is true for AP7. In all cases, a slight reduction of binding is observed at low serum-dilutions, even for the control-antibody AP1. This is presumably due to the very high concentrations of IgG in these mixtures. AP7 seems to block slightly more than the other antibodies at a dilution of 1/30, but it does this for LP2 as well as for LP3, so this can not be a specific effect.

The conclusions that can be drawn from this experiment are that LP2 and AP12 are directed against the same or close neighbouring antigenic sites on the gD molecule, while AP7 and LP3 are directed against different antigenic sites.

Fluorescence.

In order to determine the location of the target antigen of the four monoclonal antibodies in the cell, fluorescence experiments were carried out on fixed and unfixed HSV-2 infected BHK-cells with antibodies LP2 and LP3. On fixed cells, fluorescence is observed mainly on the outside of the infected cell, as well as some in the cytoplasm. On unfixed cells, membrane fluorescence could be detected with both LP2 and LP3 (see fig 5.). The controls in each case were uninfected cells and cell treated with normal mouse serum. In the surface fluorescence experiment, an extra control was added in the form of antibody LP1, which is not directed against a membrane protein. The controls were all negative as expected.

Rosetting.

The presence of the target-antigen of LP2, LP3 and AP7 on the surface of infected cells was confirmed by rosetting of infected cells with Sheep red blood cells to which antibody was coupled. (for method, see *materials and methods*). HSV-2 infected cells were mixed with the coupled red blood cells, and the number of rosettes counted. Controls were red blood cells sensitized with antibody LP1 and uninfected BHK-cells. Red blood cells sensitized with polyclonal anti-HSV-2 IgG acted as a positive control. In fig. 5, a table is presented which shows the percentage of rosettes obtained with the different antibodies. LP2, LP3 and AP7 were all positive, but LP3 gave constantly lower values. The negative controls were all less than 2%.
Fig. 4. Competition binding experiments using I-labelled monoclonal IgG.
A: Binding of I-labelled LP3 to fixed HSV-2 infected BHK cell monolayers (prepared as for RIA-1); wells pretreated with increasing amounts of blocking antibody. Assay conditions as for RIA-1.
B: As for A, using I-labelled AP7.
C: As for A, using I-labelled LP2.
Fig. 5. Immunofluorescent staining of fixed (a-e) or unfixed (f,g) HSV-2 infected BHK-cells. Conditions as described in "methods". a: LP2; b: LP3; c: LP5; d: uninfected with LP2; e: LP6; f: LP2; g: LP3.

<table>
<thead>
<tr>
<th>cells coated with</th>
<th>infected</th>
<th>uninfected</th>
</tr>
</thead>
<tbody>
<tr>
<td>LP2</td>
<td>71%</td>
<td>&lt;1%</td>
</tr>
<tr>
<td>LP3</td>
<td>31%</td>
<td>&lt;1%</td>
</tr>
<tr>
<td>LP1</td>
<td>2%</td>
<td>&lt;1%</td>
</tr>
<tr>
<td>imm</td>
<td>70%</td>
<td>&lt;1%</td>
</tr>
<tr>
<td>none</td>
<td>2%</td>
<td>&lt;1%</td>
</tr>
<tr>
<td>AP7</td>
<td>71%</td>
<td>&lt;1%</td>
</tr>
</tbody>
</table>

Fig. 6. Rosetting of HSV-2 infected BHK-cells with Shrbc coated with monoclonal antibody or hyperimmune anti-HSV-2 IgG (imm). Control = uncoated Shrbc (none). a=rosetting with LP2-coated cells, b=LP3-coated cells, c=LP1-coated cells.
Identification of gD-precursor.

The following experiment was designed to detect if the antibodies LP2 and LP3 were directed against the polypeptide or the carbohydrate-part of the gD-molecule. To this end, BHK-21 cells were infected with HSV-2 and labelled with $^{35}$S-methionine as before, but two hours prior to labelling 2μg/ml tunicamycin was added to the cell supernatant. This prevents the attachment of certain carbohydrate residues to newly synthesized proteins (Pizer et al, 1980 Takatsuki et al, 1971). The cells were then labelled and lysed, and immunoprecipitation carried out. The results of this experiment are shown in fig.7. A polypeptide with a molecular weight of 49,000 D was precipitated by both LP2 and LP3. The control of normal mouse serum failed to precipitate any proteins from the lysate.

Antibodies directed against gC and gE.

The antibodies LP5 and LP6 were characterised by fluorescence, cross-reactivity with HSV-1, neutralisation and immunoprecipitation.

Immunoprecipitations.

Immunoprecipitation experiments with LP5 and LP6 were performed according to protocol B (materials and methods), using $^{14}$C-glucosamine and $^{35}$S-methionine labelled infected cell extracts. The results of these experiments are shown in fig.8. Antibody LP6 precipitated two proteins with M.W. of 67K and 80K. These proteins were precipitated both from $^{35}$S-methionine and $^{14}$C-glucosamine labelled cell extracts. As these characteristics correspond to those of glycoprotein E, a monoclonal antibody directed against gE (kind gift of S.Bacchetti, Canada) was used as a reference. A monoclonal antibody against gF, which has similar migration characteristics as gE (Balachandran et al, 1981), was also included. The proteins precipitated by the anti-gE monoclonal antibody and antibody LP6 co-ran exactly on SDS-polyacrylamide gels, which indicates that LP6 is directed against gE.

The two proteins precipitated by antibody LP5 have a M.W. of 110K and 130K. These were detectable on gels using $^{14}$C-glucosamine as well as $^{35}$S-methionine labelled infected cell extracts. These characteristics are those of glycoprotein C. Another monoclonal antibody directed against gC (gift from S.Bacchetti) was used in a parallel precipitation reaction and bound to two proteins with exactly the same electrophoretic mobility as those precipitated by LP5. LP5 was therefore tentatively designated as being directed against gC.

Using labelling in the presence of tunicamycin, it was proved that both LP5 and LP6 were directed against the polypeptide part of gE and gC respectively. (results not shown).
Cross-reactivity of LP5 and LP6 with HSV-1.

The cross-reactivity of LP5 and LP6 with HSV-1 was determined using a radioimmunoassay (RIA-l). The results are shown in table 3. LP5 and LP6 are both specific for HSV-2.

Neutralisation.

Neutralisation experiments were carried out with ascitic fluids derived from LP5 and LP6, as described in "materials and methods". The results are presented in table 4. No specific reduction of virus infectivity was observed using LP5, but neutralisation of HSV-1 and HSV-2 was detected with LP6 at a dilution of $10^{-1}$. At higher dilutions the effect disappeared. The same results were obtained using heat-inactivated or non-inactivated serum, which proves the neutralising activity is not dependent on the presence of complement. This demonstrated that LP6 is cross-reactive with HSV-1 by neutralisation, even though no cross-reactivity was observed using a radioimmunoassay.

Fluorescence.

Fluorescence tests on fixed cells using LP5 and LP6 were carried out as described in "materials and methods". The results are shown in fig. 5. Both antibodies showed fluorescence on the outside of the cell and in the cytoplasm. Fluorescence on unfixed cells showed that both LP5 and LP6 gave rise to membrane-fluorescence (results not shown). Controls of normal mouse serum and uninfected BHK-cells were all negative.

Antibodies LP7, LP8 and LP9.

Antibodies LP7, LP8 and LP9 were all characterised by fluorescence, cross-reactivity with HSV-1, neutralisation and immunoprecipitation. The three antibodies are non-neutralising, even in combination, as can be seen from the results presented in table 4. LP7 and LP8 are cross-reactive with HSV-1, but LP9 is type-specific (see table 3). LP9 gives rise to surface fluorescence, whilst LP7 on fixed cells stained the nucleus and cytoplasm, and LP8 was fluorescent mainly in the cytoplasm (results not shown).

Immunoprecipitation reactions with these antibodies did not result in any detectable bands on a protein gel. Both methods A and B were tried. In each case a Rabbit anti-mouse immunoglobulin serum was added to the reaction mixture, as these antibodies do not bind to protein A. The precipitations were performed with $^{35}$S-methionine, $^{32}$P and $^{14}$C-glucosamine labelled infected cell extracts. The IgG fraction of ascitic fluids of antibody LP8 was subsequently purified, coupled to CNBr-activated Sepharose beads, and these used in a precipitation reaction. The results were again negative.
<table>
<thead>
<tr>
<th>radioimmuno assay</th>
<th>serum dilution</th>
<th>cpm/well (HSV-2)</th>
<th>cpm/well (HSV-1)</th>
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<tbody>
<tr>
<td>RIA-1</td>
<td>LP5 1/1000</td>
<td>2362</td>
<td>114</td>
</tr>
<tr>
<td></td>
<td>imm 1/1000</td>
<td>2668</td>
<td>1950</td>
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<td></td>
<td>nms 1/1000</td>
<td>177</td>
<td>75</td>
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<td></td>
<td>LP6 1/1000</td>
<td>2299</td>
<td>177</td>
</tr>
<tr>
<td></td>
<td>imm 1/1000</td>
<td>2668</td>
<td>3649</td>
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<tr>
<td></td>
<td>nms 1/1000</td>
<td>177</td>
<td>439</td>
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<td>1213</td>
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<td></td>
<td>LP8 1/1000</td>
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<td>nms 1/1000</td>
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<td>587</td>
<td>213</td>
</tr>
<tr>
<td></td>
<td>NS1 *</td>
<td>140</td>
<td>214</td>
</tr>
</tbody>
</table>

Table 3. Cross-reactivity of several antibodies with HSV-1 as measured by RIA-1 or RIA-3. imm=hyperimmune anti-HSV-2 serum; nms=normal mouse serum; *=hybridoma cell supernatant.

<table>
<thead>
<tr>
<th>experiment</th>
<th>antibody</th>
<th>pfu/vial (HSV-2)</th>
<th>pfu/vial (HSV-1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>LP7 1/10</td>
<td>2600</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>LP8 1/10</td>
<td>2550</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>LP9 1/10</td>
<td>2300</td>
<td>-</td>
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<tr>
<td></td>
<td>LP7+LP8+LP9</td>
<td>2460</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>imm 1/10</td>
<td>300</td>
<td>-</td>
</tr>
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<td></td>
<td>nms 1/10</td>
<td>2510</td>
<td>-</td>
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<tr>
<td>2</td>
<td>LP5 1/10</td>
<td>4000</td>
<td>-</td>
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<td></td>
<td>1/100</td>
<td>920</td>
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<td>2400</td>
</tr>
<tr>
<td></td>
<td>1/1000</td>
<td>1620</td>
<td>3770</td>
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</table>

Table 4. Neutralisation of HSV-1 and HSV-2 by different monoclonal antibodies. imm=hyperimmune anti-HSV-2 serum; nms=normal mouse serum.
Antibodies to gD.

The results presented in this chapter show that the monoclonal antibodies LP2, LP3, AP7 and AP12 are directed against gD. The antibodies all recognise type-common determinants on the gD-molecule, and only LP2 and AP12 are directed against the same antigenic site, or at least against sites which are situated so close to each other that the binding of one antibody prevents the binding of the other. This means that there are at least three type-common antigenic sites on gD, one neutralising, one non-neutralising and one for complement-dependent neutralisation. This is in agreement with the findings of Showalter et al (1981), who, using a panel of 10 monoclonal antibodies directed against gD of HSV-1, could distinguish five distinct antigenic sites on this molecule, of which three are type-common. As no competition binding studies were performed within each "group" of antibodies presented by him, this number is necessarily a minimum. Until such studies are carried out, using a large number of monoclonal antibodies against gD, the accurate number of antigenic sites on this molecule cannot be known.

The processing of gD of HSV-1 has been studied in great detail. Cohen et al (1980) and Haarr et al (1981) have shown that pgD(52K) of HSV-1 is processed in 11 discrete steps to gD (59K). Positive identification of several of the observed intermediates as being related to gD came from the use of a monoclonal antibody against gD (Derrick et al, 1981). The processing of gD of HSV-2, however, has not been studied in such detail. Eisenberg et al (1980) showed that gD of HSV-2 has a M.W. of 56K, and a precursor of 51K. But no studies similar to the ones for HSV-1, using tunicamycin in order to identify an unglycosylated precursor, have been reported. In this chapter we present evidence for an unglycosylated precursor of HSV-2, with a molecular weight of 49K. This is similar to the 50K unglycosylated precursor reported for HSV-1 (Pizer et al, 1980), and it seems reasonable to suppose that gD of HSV-2 is processed in a similar way as gD of HSV-1.

The surface fluorescence studies and the rosetting experiments show that the antigenic sites to which LP2, LP3, AP7 and AP12 are directed are present on the surface of the infected cell, and are readily accessible to antibody. The lower percentage of rosettes obtained for LP3-coated red blood cells might mean that this antigenic site is not as accessible as the LP2-AP12 site and the LP7-site, but this result can also be explained by the lower affinity of LP3 for gD.

The phenomenon of enhancement of the number of pfu when using HSV-2 and AP7 is at present under investigation. That this is a true effect, and not just an inhibition of the inactivation of the virus in time, has already been demonstrated (Ms.A.Buckmaster, personal communication).

Antibody LP5.

This type-specific, non-neutralising antibody is tentatively identified as an anti-gC antibody. The detection of surface fluorescence using this antibody shows that the
antigenic site to which it is directed is present on the surface of the infected cell.

All the monoclonal antibodies against gC reported so far have been type-specific. Some are neutralising in the presence of complement (Showalter et al, 1981), some are neutralising without complement (Pereira et al, 1980). This corresponds to the results obtained with conventional sera against gC, which were also completely type-specific but differed in their neutralising ability. (Spear, 1976; Powell and Watson, 1975; Powell and Courtney, 1975; Vestergaard and Norrild, 1978; Cohen et al, 1980).

At some stage after the isolation of LP5, seven more monoclonal antibodies against gC were produced by Ms A. Buckmaster. All these antibodies were specific for HSV-2 and neutralised infectious virus in the presence of complement to a varying degree. LP5, as presented in this chapter, does not neutralise infectious virus. However, when competition binding experiments were carried out between the eight monoclonal antibodies against gC, using a radioimmunoassay, it was found that all the antibodies competed with each other. Obviously they were all directed against the same or close neighbouring antigenic sites. This seems to suggest that this particular antigenic site is very dominant in producing an antibody response in the infected host. It may be that this is the only site that is accessible to antibodies. The reason for the inaccessibility of any other antigenic sites might be the fact that gC seems to exist in multimeric forms (Eberle and Courtney, 1982), so that certain parts of the gC-molecule might be shielded by each other.

It is not known if the antibodies described by Showalter et al (1981) or Pereira et al (1980) are all directed against the same antigenic site. Until competition binding experiments with a large number of monoclonal antibodies against gC, resulting from a number of different fusions, are carried out, it is difficult to say anything with certainty about the dominance of one particular antigenic site on this molecule.

Antibody LP6.

Antibody LP6 is tentatively designated as being directed against gE. This was indicated by the immunoprecipitation data. This antigen is present on the surface of the infected cell, as our fluorescence data show. This is in agreement with the results of Para et al (1982a and b), who used a polyclonal anti-gE serum to detect gE on the surface of infected cells and virions. They also described gE for HSV-2 in detail, suggesting a molecular weight of 90,000D for this protein, as opposed to a M.W. of 80,000D for gE of HSV-1. In our gel-system, gE of HSV-2 has a M.W of 80,000D. This difference is probably due to the different virus strain used, and to a different gel system. Varying M.W were found for gE in different strains of HSV-1, and it is likely that a similar variety exists between HSV-2 isolates.

Antibody LP6 has some neutralising ability for HSV-1 as well as for HSV-2, but this could only be detected at very high serum concentrations. The apparent discrepancy between the results of the cross-reactivity test using a radioimmunoassay, where LP6 seems to be type-specific, and the results obtained by neutralisation, can be explained in the following way. It is possible that the RIA demands a higher affinity antibody due to the stringent washing procedures, than a neutralisation assay.
Antibody LP6 might therefore be capable of reacting with HSV-1 in a neutralising assay but fail to react with HSV-1 infected cells in a radioimmunoassay. The finding of a type-common site on gE is, however, in contrast with the results of Para et al, who, using a polyclonal anti-gE serum, suggest that the type-common sites on gE are not the target of neutralising antibodies. The fact that LP6 neutralises only at very high antibody concentrations (a dilution of 1/10, compared to a RIA-titre of 1/100,000) may be the reason for this: it is unlikely that this particular antibody would be present in such high concentrations in a conventional serum.

The neutralising ability of LP6 is not dependent on the presence of complement. The conventional antiserum mentioned above (Para et al, 1982) does only neutralise when complement is present.

Antibodies LP7, LP8 and LP9.

The failure to precipitate any proteins with antibodies LP7, LP8 and LP9 may be due to several reasons. One possibility is that the antigenic site recognised by the antibodies on the fixed radioimmunoassay plates is destroyed by the detergent treatment necessary for the immunoprecipitation reactions. Another reason might be that the antibodies bind to the antigen with a relatively low affinity and binding is not possible under the conditions of the immunoprecipitation procedure. A third possibility is that the target antigen is present in such low concentrations that it is not detectable against the background precipitation. Further experiments using different detergents or very large amounts of labelled infected cell antigen might solve the precipitation problem. The use of affinity columns or western blotting are two other alternative approaches for the detection of the target antigen of these antibodies, which have not yet been tried out for all of them.

In a recent report, the use of monoclonal antibodies against HSV-1 and HSV-2 in serological analysis was demonstrated (Pereira et al, 1982). The antibodies described in this chapter, in particular LP5 and LP6, might be useful in typing clinical isolates. In addition, the different monoclonal antibodies against gC, which compete with each other but do not have exactly the same characteristics, might be useful in answering questions about the occurrence of antibodies with different idiotypes to the same antigenic domain on a particular molecule.
INTRODUCTION

The relative roles of the cellular and humoral immune response in recovery from a primary herpes simplex virus infection are still poorly understood. Initial reports have emphasized the importance of cell-mediated immunity (Roger-Zisman and Allison, 1976; Oakes, 1975) and there seems to be little doubt that indeed cellular mechanisms play an important role in mediating recovery (Nash et al, 1980a and b). However, a number of recent reports have shown that antibody can also play an important role in this respect (Oakes and Lavish, 1981; Worthington, 1980; McKendall et al, 1979; Davis et al, 1979). In normal animals, passively transferred serum hyperimmune to either HSV-1 or HSV-2 can effectively reduce the severity of neurological disease and inhibit viral spread (Oakes, 1978; McKendall et al, 1979; Dix et al, 1981; Luyet et al, 1975). In immunosuppressed animals, the situation is less clear, but it seems that neutralising antibody can have some protective effect on virus infection in some cases (Worthington et al, 1979).

The mechanisms by which antibodies exert their protective ability are known only to a limited extent. Although neutralising antibodies may be important in arresting the spread of virus, neutralisation can not be the only mechanism by which they work, as virus spreads from cell to cell, thus avoiding neutralisation by antibodies (Lodmell et al, 1973). In vitro studies have shown that several other mechanisms, such as antibody-dependent cell-mediated cytotoxicity (ADCC) and complement-dependent cytotoxicity (AbC), may be important in the destruction of virus-infected cells. Antibodies against several glycoproteins, including gD, have been shown to be capable of mediating lysis of infected cells in either ADCC or AbC tests (Glorioso et al, 1978; Norrild et al, 1979).

In a recent study, neutralising monoclonal antibodies directed against gD and gC of HSV-1 were used in passive transfer studies, in order to assess their protective ability against acute virus-induced neurological disease (Dix et al, 1981). Both antisera were effective in reducing viral spread and decreasing the incidence of neurological disease in mice. It was, however, not possible from these studies to determine the extent to which the neutralising ability of the antibodies contributed to the protection. We investigate in this chapter the effect of two monoclonal antibodies against gD, one neutralising and one non-neutralising, on the course of HSV-1 infection in normal Balb/c mice, and demonstrate a role for neutralising antibody in reducing viral infectivity and the inflammatory response. The role of neutralising antibody, as opposed to non-neutralising antibody, in the establishing of latent infection is also investigated.
MATERIALS AND METHODS.

Virus, cells.
The virus strains used were: type 1 strain SC16, and type 2 strain 25766, as described in chapter 3. BHK-21 cells were grown in ETC as described in chapter 2.

Mice.
Inbred female Balb/c mice (obtained from Bantin and Kingman, Aldborough, UK) were used when 5-6 weeks old.

Inoculation of virus and measurement of ear swelling.
10^4 pfu of virus in 20 μl of GMM was inoculated subcutaneously into the pinna of the left ear of anesthetized mice. The thickness of the ear was measured on successive days using a Mitutoyo Engineers screw gauge micrometer. The results were expressed as the difference between infected and uninfected pinnae (Nash et al, 1980a).

Infectivity assays.

a. Ears. The left ear pinnae were removed from mice at different times after infection. Ears were homogenized separately in 1 ml of ETC. The amount of infectious virus was measured on BHK-21 cells (Russell, 1962).

b. Ganglia. Cervical dorsal root ganglia C2, C3 and C4 were removed six weeks after infection and cultured in GMM-1% FCS for 6 days. The ganglia were then homogenized and assayed for virus infectivity on BHK-21 cells.

Sera and ascitic fluids.
Sera and ascitic fluids were obtained as described in chapter 2. LP2, LP3 and LP4 sera had an approximate RIA-titre of 1/10^6. LP2-serum had a neutralisation titre of 1/10^4.

RESULTS.
The course of the infection of Balb/c mice with HSV-1, strain SC16, has been well characterized both in terms of the inflammatory response and virus growth (Nash et al, 1980a and b). In our studies we preferred therefore to work with this type 1 strain where possible. One of the objectives of the experiments described below was the investigation of the relative importance of neutralising versus non-neutralising antibodies in their effect on HSV-infection. To this end, two antibodies against the same protein (gD), one neutralising (LP2) and one non-neutralising (LP3), were compared for their effect on acute infection of mice with HSV-1 (strain SC16). Both antibodies are of the same subclass (2a). The choice of SC16 was possible because both LP2 and LP3 are fully cross-reactive with HSV-1 as measured by radioimmunoassay, neutralisation and immunoprecipitation (see chapter 4).
Fig. 1 Effect of injection of 50 ul antibody on the primary ear thickness response and ear virus titres in mice infected with 10 pfu HSV-1 (SC16). Virus titres expressed as log_{10} pfu.

1a: antibody LP2 and control (no antibody).
1b: antibodies LP3 and LP4, and control.

Antibody injected one day before infection.
Effect of LP2 and LP3 on infection with HSV-1.

In the first experiment, the effect of LP2 and LP3 on the inflammatory response and viral infectivity titres when administered 24 hours before infection was determined. To this end mice were given an intravenous dose of 50 μl antibody (ascitic fluid), and 24 hours later were infected with $10^4$ pfu SC16 in the left ear pinna. Ear thickness was measured each day for 7 days. The growth of virus in the ear was monitored by removing the left ear of groups of mice on days 1, 2, 3, 5 and 7 and measuring the infectivity. The results of this experiment are shown in fig.1a and 1b. The normal course of infection is given by the control group (no antibody), and corresponds with the data published by Nash et al (1980a). The administering of antibody LP2 24 hours before infection resulted in the virtual elimination of virus growth and a marked reduction in ear swelling. Antibody LP3 had no effect on the course of the infection, except for a slightly enhanced inflammatory response in the later stages of the infection. An extra control, namely antibody LP4, which does not react with HSV-1 (see chapter 3), was incorporated in this experiment. This antibody also had no effect on either virus infectivity or ear swelling.

The second experiment was designed to assess the effectiveness of monoclonal antibodies in arresting the growth of virus in the ear when administered after infection was established. In this experiment antibody was administered 24 hours after infection. The results in fig.2 show that LP2 is still effective in reducing viral infectivity in the ear, while the inflammatory response is also diminished. LP3 again had no effect on the course of infection, although a slightly enhanced inflammatory response was observed.

Effect of LP2 and LP4 on infection with HSV-2.

Although the course of infection with type 2 virus in Balb/c mice has not been fully characterised, we tested antibodies LP2 and LP4 in HSV-2 infected mice. LP4 is directed against the major DNA-binding protein, is non-neutralising and specific for HSV-2. The antibody was administered 24 hours before infection. Although the amount of virus infectivity in the ear pinna at these times was low, the reduction in virus titres caused by LP2 was still very marked, as was the reduction in ear swelling. Antibody LP4 had no effect (see fig.3).

In theory it was possible that the effect of LP2 on virus infectivity might be caused by neutralisation of the virus during the assay of the ear, rather than in the animal itself. The neutralising titre of serum in the mouse was still around 1/200 several days after infection. To eliminate this possibility, groups of mice were infected with $10^4$ pfu SC16, and half of the mice treated with 50μl LP2-antiserum 24 hours before infection. Five days after infection the ears of the mice were collected. The ears were assayed in three different groups: in the first group ears of LP2-treated mice were homogenized and assayed separately as before. In the second group the control ears were treated in the same way. In the third group, one ear of
FIG. 2. Effect of antibodies LP2 and LP3 on the primary ear thickness and ear virus titres, when injected 24 hours after infection with 10 pfu SC16.

Fig. 3. Effect of antibodies LP2 and LP4 on the primary ear thickness response and ear virus titres, when injected 24 hours before infection with 10 pfu HSV-2.
an LP2-treated and one ear of a non-treated mouse were homogenized together and assayed. This was done for three mice in each group (six in group 3). The results in table 1 demonstrate clearly that the virus was not neutralised during the homogenisation procedure.

**Effect of LP2 and LP3 on the establishment of latency.**

The effect of antibodies LP2 and LP3 on the establishment of latency was investigated by removing cervical dorsal root ganglia C2, C3 and C4 from HSV-1 infected mice four weeks after infection and assaying them, after a period of culture, for the presence of infectious virus. The mice had been given 50 μl LP2 or LP3 antiserum 24 hours before infection. The results are shown in table 2. Although LP2 did not prevent latency from being established, it did reduce both the number of latently infected mice and the amount of virus recovered from the ganglia following in vitro reactivation. It appeared that neutralising antibody reduced the spread of virus to the ganglia. Antibody LP3 was equivalent to the control.

<table>
<thead>
<tr>
<th></th>
<th>Virus titre in ear (log_{10} pfu)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LP2-treated</td>
<td>0.3, 0, 0.6</td>
</tr>
<tr>
<td>non-treated</td>
<td>3.0, 3.5, 4.2</td>
</tr>
<tr>
<td>LP2-treated</td>
<td></td>
</tr>
<tr>
<td>mixed with</td>
<td></td>
</tr>
<tr>
<td>non-treated</td>
<td>3.8, 3.4, 4.1</td>
</tr>
</tbody>
</table>

Table 1. Virus titre in ears of mice infected with 10^4 pfu SC16, on day 5 after infection. LP2-treated: 50 μl LP2-serum 24 hours before infection. non-treated: no serum received.
Table 2. Proportion of latent ganglia in mice infected with SC16. Mice were given LP2 or LP3 serum 24 hours before infection. Ganglia were removed 4 weeks after infection.

<table>
<thead>
<tr>
<th>virus dose</th>
<th>proportion of +ve latent ganglia</th>
<th>mean titre (pfu) +ve ganglia</th>
</tr>
</thead>
<tbody>
<tr>
<td>LP2-treated</td>
<td>$10^4$ pfu</td>
<td>4/8</td>
</tr>
<tr>
<td></td>
<td>$10^5$ pfu</td>
<td>7/10</td>
</tr>
<tr>
<td>LP3-treated</td>
<td>$10^4$ pfu</td>
<td>5/7</td>
</tr>
<tr>
<td></td>
<td>$10^5$ pfu</td>
<td>10/10</td>
</tr>
</tbody>
</table>

DISCUSSION.

The effect of monoclonal antibody LP2 in reducing the severity of HSV-infection in mice confirms that gD acts as a target for immune responses in vivo, confirming the report by Dix et al (1981). The fact that antibody LP3, which is non-neutralising, did nothing to arrest viral infectivity, while LP2 was effective in reducing virus titres in the ear, argues that neutralisation is one of the mechanisms by which viral spread is reduced. When antibody was administered before infection it was more effective in reducing virus titres than when given after infection.

When considering the case of administering antibody before the infection, these results are in agreement with many other publications (Oakes and Lausch, 1981; Dix et al, 1981; McKendall et al, 1979; Baron et al, 1975). Although in several reports a protective effect could be found even if antibody was administered up to 48 hours after infection, the mechanism by which antibodies work in this case might well be cell-mediated rather than by neutralisation. This was suggested in a report by Oakes and Lausch (1981), who used Fab(2) fragments of Rabbit-anti-HSV-IgG in order to investigate the importance of the Fc-piece of the antibody in conferring protection either before or after infection. They found that the Fab(2)-fragments, which were still capable of neutralising infectious virus in vitro, were effective in promoting recovery of infection if administered before or simultaneous with virus inoculation. However, if transferred 8 hours after infection, no protective effect was observed. These findings argue that, when infection is established, cell-mediated mechanisms, such as ADCC or antibody dependent complement mediated cell lysis, operate rather than antibody-dependent virus neutralisation. In our model, however, non-neutralising antibody had no effect on reducing virus infectivity, neither before or after infection, whereas the neutralising antibody reduced both virus infectivity and ear
swelling dramatically in both instances. Worthington and coworkers (1980) found a protective role for neutralising antibodies in immunosuppressed animals when transferred as late as 6 days after infection. They stressed the importance of the concentration of neutralising antibody in the animal, which must be reasonably high. This might account for some of the discrepancies in published data so far. In our case the antibody-titres remain 1/200 at 14 days after i.v. injection.

Support for the importance of cell-mediated mechanisms in eliminating primary HSV-infection, comes from a series of experiments performed by Dr. A. Kapoor and coworkers (A.K. Kapoor et al., 1982a) in our laboratory. It was found that antibody LP2 had little or no effect on the course of infection in the ear pinna of nu/nu mice, when administered one to three days after infection. However, productive infection in the peripheral and central nervous system is markedly reduced. Although neutralisation by antibody of virus is not effective in eliminating virus from the ear pinna, it is considered that this mechanism may well restrict the spread of virus to sensory nerve endings in the skin and prohibit virus movement to the central nervous system at synaptic junctions. Until more experiments with larger numbers of neutralising as well as non-neutralising monoclonal antibodies are performed, it is not possible to say anything with certainty about the role of neutralising antibodies in protection from virus disease when administered after the infection is established.

The role of antibodies in preventing the establishing of latent infection has been the subject of much discussion. That latent infection of ganglia will occur even in the presence of hyperimmune serum is indisputable (Klein, 1980; Openshaw, 1979; Price, 1975). Dr. A. Kapoor found that latent infection could be established in nu/nu mice which were given LP2 antibody (unpublished data). However, antibody may restrict the establishment of latent infection to fewer ganglia (McKendall et al., 1979). This is in agreement with our results, where we showed a role for neutralising anti-gD antibody in restricting the number of latently infected sites in the ganglia that are established during the course of infection. This phenomenon was further investigated by Kapoor et al. (1982b), who used B-cell suppressed mice to assess the importance of cellular and humoral immune mechanisms in primary and latent infection. Their findings indicated that, when neutralising antibody was suppressed, there was an increased incidence of latent infection (and primary infection of the ganglia and spinal cord). Together with the observations reported in their paper on the nu/nu mouse system (Kapoor et al., 1982a), these findings argue that neutralising antibody is important in restricting the spread of virus to the nervous system.

The results reported in this chapter are only a start in the direction of elucidating the relative importance of antibody-mediated mechanisms in the recovery of mice from primary infection and the establishment of latency. Monoclonal antibodies will not only provide the opportunity for more detailed studies of the importance of neutralisation versus other antibody-mediated immune mechanisms, but will also provide information about the relative importance of certain antigens in these processes.
DISCUSSION.

In this report the production and characterisation of nine monoclonal antibodies to herpes simplex virus type 2 has been described. The implications of the experiments described in the various chapters have already been discussed. In this section I will therefore concentrate on some current and potential uses of some of the antibodies described.

One example of the use of these antibodies has already been given in chapter 5. The findings of Kapoor et al (1982a) in nu/nu mice suggest that neutralising antibodies are involved in reducing the spread of virus to the central nervous system. In combination with the results in B-cell suppressed mice (A.Kapoor et al,1982b), where an increased incidence of latent infection was observed when neutralising antibody was suppressed, an important role for neutralising antibody in reducing the incidence of latency is indicated. These experiments are currently being extended to include other neutralising and non-neutralising antibodies, and HSV-2 infected nu/nu mice. They will contribute to our understanding of the role of humoral versus cellular immune responses following infection with herpes simplex virus, and may indicate in what instances neutralising antibodies are important.

In a recent report, the use of monoclonal antibodies in serological analysis of HSV-1 and HSV-2 isolates was evaluated (Pereira et al,1982). It was shown by fluorescent techniques that monoclonal antibodies could not only distinguish HSV-1 and HSV-2, but provided the first demonstration of intratypic antigenic variation. In a paper by Cranage et al (to be published) we demonstrate the potential use of antibody LP4, which is specific for HSV-2, in clinical serotyping. This antibody was linked to red blood cells, and used in an haemagglutination assay to distinguish HSV-1 and HSV-2 strains. The usefulness of several type-specific monoclonal antibodies, including LP4, as RPH-reagents for the typing of clinical isolates, is currently being evaluated.

In the above mentioned report by Cranage et al, another application of monoclonal antibodies, coupled to red blood cells, was described. When antibody LP2 or AP7 (both directed against gD) were coupled to sheep red blood cells, they failed to agglutinate in the presence of sonicated or solubilized antigen prepared from either HSV-1 or HSV-2 infected isolates. However, agglutination was achieved upon the subsequent addition of a polyclonal rabbit anti-HSV-2 bridging antiserum. In this system agglutination was only possible if the bridging serum or monoclonal antibody had activity against gD. Although the addition of LP2 to LP2-sensitized cells did not result in agglutination, LP2-linked antigen-sensitized cells could be agglutinated with antibody AP7. The results suggest that the antibody coupled to the red cell selectively binds to monodispersed gD-molecules in the antigen-preparation.

The ability of LP2-linked virus antigen-sensitized cells to measure anti-HSV antibody was used to test a panel of human sera, which had previously been tested for HSV-1 plaque
neutralising activity. A very good correlation was found between results obtained using LP2-linked cells and the plaque reduction assay. The haemagglutination method is obviously much more rapid and simple than the neutralisation assays which are used at present. In addition, it may be possible to use this procedure to measure immune responses to different components of HSV, as apparently only one antigen specificity is captured by the antibody on the red cell. Current procedures such as immunoprecipitation followed by electrophoresis (Eberle and Courtney, 1981) are technically much more demanding.

In an experiment which involved the use of antibody LP4, it was shown that mouse L-cells, transformed with fragments of HSV-2 DNA, expressed the major DNA binding protein of HSV-2 upon superinfection with HSV-1. This was demonstrated using the cell line D21 (Minson et al, 1982), which was infected with HSV-1 and stained with fluorescent labelled antibody LP4. It was found that HSV-2 specific major DNA-binding protein was present in the nuclei of these cells. The use of LP4 represents an easy way of demonstrating the activation of genes coding for the type 2 major DNA-binding protein.

An anti-idiotype serum against antibody LP2 has been prepared in rabbits, which is specific for the LP2-idiotype, has been prepared. This serum did not react with any of the other anti-gD antibodies in our possession. Furthermore, the virus neutralising activity was completely inhibited by the antiserum. Again this was specific for LP2 only. The use of anti-idiotype serum raised against specific monoclonal antibodies could be useful in demonstrating the frequency of certain idiotypes present during natural HSV-infection. In addition, the potential use of anti-idiotypes against monoclonal antibodies could theoretically be useful as putative vaccines or stimulators of protective immune responses as considered by the Jerne Network Theory.

A variety of applications of monoclonal antibodies to HSV in immunological, basic virological and clinical research has been outlined above. At present, the repertoire of monoclonal antibodies to HSV in our laboratory is much larger than the nine antibodies reported in this thesis. It is of course possible to outline more potential applications of these antibodies, such as studies regarding the structure and function of particular antigens, and their potential usefulness in the production of vaccines, but I have concentrated upon the present uses made of the antibodies by workers in our department. It is clear that monoclonal antibodies can be of great value in answering many questions of varying nature. In this context, the exchange of antibodies between different laboratories would be very valuable.
SUMMARY.

In this thesis the production and characterisation of monoclonal antibodies to Herpes Simplex Virus Type 2 is described. The development of a suitable radioimmunoassay for the detection of anti-HSV-2 antibodies, and the selection of an optimal immunisation schedule, is given in chapter 2. Three fusion experiments were performed, resulting in nine stable hybridoma lines. The monoclonal antibodies secreted by these lines were subsequently characterised. A list of these antibodies and their properties, including target antigens, is given at the back of this thesis. Four of the antibodies were directed against glycoproteins of the virus, two reacted with non-glycosylated proteins and for three antibodies no target antigen could be identified. All antibodies were of the IgG class.

Two antibodies, LP2 and LP3, were directed against the same protein, gD. Competition binding experiments involving two additional monoclonal antibodies against this protein showed that there are at least three different type-common antigenic sites on the gD molecule of HSV-2. LP2 and LP3 are directed against different antigenic sites.

Using tunicamycin, an unglycosylated precursor of gD was found with a molecular weight of 49,000D.

The reactivity of antibodies LP1 and LP4 with a number of different HSV-1 and HSV-2 strains was determined. LP4 proved to be specific for HSV-2, while LP1 showed comparable reactivity with HSV-1 and HSV-2 strains.

The ability of antibodies LP2, LP3 and LP4 to protect Balb/c mice from infection with HSV-1 or HSV-2 was assessed. Antibody LP2, which is strongly neutralising, markedly reduced both the inflammatory response and virus titres in the site of infection compared to non-treated mice. Antibody LP3, which is directed against the same protein and is of the same sub-class, had no effect except for a slightly enhanced inflammatory response. Mice treated with antibody LP4 were identical to the control group.

Treatment with antibody LP2 reduced the frequency with which latent infection was established in infected animals, and reduced the virus titres recovered from reactivated ganglia. Again antibody LP3 had no effect.

The implications of the findings described above are discussed at the end of each of the chapters. A review of some of the current and potential applications is given in the Discussion.
In dit proefschrift worden de produktie en karakterisering van monoklonale antilichamen tegen Herpes Simplex Virus Type 2 (HSV-2) beschreven.

HSV-2 heeft een dubbelstrengs DNA-genoom met een moleculair gewicht van ongeveer 96 miljoen, verpakt in een eiwitmantel. Rond de nucleocapside zit een nog ongedefinieerde structuur, die het tegument genoemd wordt, en het geheel is omgeven door een trilaminaire membraan.

In de virusdeeltjes komen ongeveer 33 structurale eiwitten voor. Daarnaast zijn in virus-geïnfecteerde cellen een nog veel groter aantal, in ieder geval meer dan 50, virus-specifieke eiwitten geïdentificeerd. Onze kennis van de structurale en de niet-structurale virus-specifieke eiwitten is nog uiterst beperkt. Het onderzoek hierover wordt bemoeilijkt door de ingewikkelde structuur van het virus en de moeilijkheden bij het identificeren van eiwitten in geïnfecteerde cellen, omdat tussen verschillende eiwitten precursor-produktrelaties mogelijk zijn.

Monoklonale antilichamen tegen virus-specifieke eiwitten kunnen van groot belang zijn bij het onderzoek naar precursor-produktrelaties tussen verschillende eiwitten. Met monoklonale antilichamen kunnen verder ook het aantal antigenen bindingsplaatsen per eiwitmoleculuul worden vastgesteld. Dit kan van groot belang zijn bij het identificeren van HSV-1 en HSV-2 en het onderscheiden van verschillende subgroepen van deze virusen.

Nadat een geschikte methode was ontwikkeld voor de detektie van antilichamen tegen HSV-2 met behulp van een radioimmunoassay, en verschillende immunisatieschema's geëvalueerd waren, werden drie fusie-experimenten uitgevoerd. Deze resulteerden in de isolatie van negen stabiele cellijnen, die antilichamen tegen HSV-2 uitscheidden. Deze antilichamen werden gekarakteriseerd door middel van immunofluorescentie, neutralisatie- en immunoprecipitatie-experimenten. Ook werd nagegaan of de antilichamen reageerden met HSV-1, en werd bepaald tot welke immunoglobulineklasse ze behoren.

Een samenvatting van de eigenschappen van de verschillende monoklonale antilichamen, alsnog een lijst van de eiwitten waartegen ze gericht zijn, wordt gegeven aan het eind van dit proefschrift. Vier antilichamen bleken gericht te zijn tegen geglycosyleerde eiwitten, twee tegen niet-geglycosyleerde eiwitten, terwijl van drie antilichamen het eiwit waartegen ze gericht zijn niet kon worden geïdentificeerd. Twee antilichamen, LP2 en LP3, bleken gericht te zijn tegen hetzelfde eiwit, gD, maar door competitie-experimenten kon worden bewezen dat de antigeen bindingsplaatsen voor deze antilichamen verschillend zijn. Met behulp van nog twee andere monoklonale antilichamen, gericht tegen gD, kon worden aangetoond dat gD minstens drie cross-reactieve bindingsplaatsen bezit.

Met behulp van tunicamycine, dat de glycosylering remt, kon worden aangetoond dat er in geïnfecteerde cellen een niet-geglycosyleerde vorm van gD voorkomt met een moleculair gewicht van 49,000 D.
Voor een aantal verschillende stammen van HSV-1 en HSV-2 werd getest of ze reageerden met de antilichamen LP1 en LP4, met behulp van een radioimmunoassay. LP1 reageerde met alle stammen, maar LP4 reageerde alleen met de stammen behorende tot de HSV-2 groep.

In een serie experimenten werd het vermogen van de antilichamen LP3 en LP2, die beiden gericht zijn tegen gD, om Balb/c muizen te beschermen tegen infektie met HSV-1 of HSV-2, geanalyseerd. Deze proeven toonden aan dat LP2 de ontsteking ten gevolge van de virusinfektie, en ook de virus titers op de plaats van de infektie, sterk reduceerde, terwijl LP3 geen effekt had, behalve een geringe vergroting van de ontsteking. Een derde antilichaam, LP4, dat reageert met het major DNA-binding protein, werd ook getoetst in het Balb/c muizen systeem. De resultaten van deze proeven wijzen erop dat neutraliserende antilichamen een rol spelen zowel bij de reductie van de primaire infektie, als bij de reductie van de verspreiding van het virus. Dit effekt op de verspreiding van het virus lijkt ook een reductie in het aantal dieren, dat door de virusinfektie latent wordt, tot gevolg te hebben. Dit leek waarschijnlijk door de resultaten van een experiment waarbij het effekt van LP3 en LP2 op het voorkomen van latentie werd onderzocht. Het injekteren van LP2 reduceerde zowel het aantal latente muizen als de titers van het virus, dat uit de gereactiveerde ganglionen kon worden verkregen.

In de diskussie aan het eind van dit proefschrift wordt een kort overzicht gegeven van de verschillende manieren waarop deze antilichamen kunnen worden gebruikt in fundamenteel en klinisch-gericht onderzoek van Herpes Simplex Virus. Een aantal van de antilichamen beschreven in dit proefschrift hebben inmiddels hun waarde al bewezen in fundamenteel, immunologisch en klinisch-gericht onderzoek.
<table>
<thead>
<tr>
<th>Antibody</th>
<th>Target antigen</th>
<th>Reactivity with HSV-1</th>
<th>Fluorescence</th>
<th>IgG subclass</th>
<th>Neutralisation</th>
</tr>
</thead>
<tbody>
<tr>
<td>LP1</td>
<td>VP16</td>
<td>+</td>
<td>nucl/cyt</td>
<td>1</td>
<td>-</td>
</tr>
<tr>
<td>LP2</td>
<td>gD</td>
<td>+</td>
<td>surface</td>
<td>2a</td>
<td>$1/10^4$</td>
</tr>
<tr>
<td>LP3</td>
<td>gD</td>
<td>+</td>
<td>surface</td>
<td>2a</td>
<td>-</td>
</tr>
<tr>
<td>LP4</td>
<td>MDBP</td>
<td>-</td>
<td>nuclear</td>
<td>2a</td>
<td>-</td>
</tr>
<tr>
<td>LP5</td>
<td>gC</td>
<td>-</td>
<td>surface</td>
<td>2a</td>
<td>-</td>
</tr>
<tr>
<td>LP6</td>
<td>gE</td>
<td>-</td>
<td>surface</td>
<td>2a</td>
<td>$1/10$</td>
</tr>
<tr>
<td>LP7</td>
<td>?</td>
<td>+</td>
<td>nucl/cyt</td>
<td>1</td>
<td>-</td>
</tr>
<tr>
<td>LP8</td>
<td>?</td>
<td>+</td>
<td>cyt</td>
<td>1</td>
<td>-</td>
</tr>
<tr>
<td>LP9</td>
<td>?</td>
<td>-</td>
<td>surface</td>
<td>1</td>
<td>-</td>
</tr>
</tbody>
</table>

Table with properties of nine monoclonal antibodies. MDBP = Major DNA binding protein. nucl. = nuclear; cyt. = cytoplasmic.
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PARA, M.F., BAUCKE, R.B. and SPEAR, P.G. (1981). Immunoglobulin G (Fc)-binding receptors on virions of herpes simplex virus type 1 and transfer of these receptors to the cell surface by infection. J. Virol. 34: 512.


<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>AbC'</td>
<td>antibody-dependent complement mediated cytotoxicity.</td>
</tr>
<tr>
<td>ADCC</td>
<td>antibody-dependent cell-mediated cytotoxicity.</td>
</tr>
<tr>
<td>BMV</td>
<td>Bovine Mammalitis Virus.</td>
</tr>
<tr>
<td>CDI</td>
<td>carbodiimide.</td>
</tr>
<tr>
<td>Ci</td>
<td>curie</td>
</tr>
<tr>
<td>cpm</td>
<td>counts per minute.</td>
</tr>
<tr>
<td>CTL</td>
<td>cytotoxic T-lymphocyte.</td>
</tr>
<tr>
<td>D</td>
<td>dalton.</td>
</tr>
<tr>
<td>DMM</td>
<td>Dulbecco's modified medium.</td>
</tr>
<tr>
<td>EAV</td>
<td>Equine Abortion Virus.</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylene diaminetetraacetate.</td>
</tr>
<tr>
<td>ETC</td>
<td>Glasgow's modified Eagles medium, supplemented with tryptose phosphate broth and calf serum.</td>
</tr>
<tr>
<td>FCS</td>
<td>foetal calf serum.</td>
</tr>
<tr>
<td>GMM</td>
<td>Glasgow's modified Eagles medium.</td>
</tr>
<tr>
<td>hr</td>
<td>hour</td>
</tr>
<tr>
<td>HSV</td>
<td>Herpes Simplex Virus.</td>
</tr>
<tr>
<td>ICP</td>
<td>infected cell polypeptide.</td>
</tr>
<tr>
<td>ICSP</td>
<td>infected cell specific polypeptide.</td>
</tr>
<tr>
<td>IFCS</td>
<td>heat-inactivated foetal calf serum</td>
</tr>
<tr>
<td>IgG</td>
<td>immunoglobulin G.</td>
</tr>
<tr>
<td>i.p.</td>
<td>intraperitoneal.</td>
</tr>
<tr>
<td>i.v.</td>
<td>intravenous.</td>
</tr>
<tr>
<td>M</td>
<td>molar.</td>
</tr>
<tr>
<td>min</td>
<td>minute.</td>
</tr>
<tr>
<td>ml</td>
<td>milliliter.</td>
</tr>
<tr>
<td>ul.</td>
<td>microliter.</td>
</tr>
<tr>
<td>M.W.</td>
<td>molecular weight.</td>
</tr>
<tr>
<td>nms</td>
<td>normal mouse serum.</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline.</td>
</tr>
<tr>
<td>pfu</td>
<td>plaque forming units.</td>
</tr>
<tr>
<td>PMSF</td>
<td>phenyl methyl sulphonyl fluoride.</td>
</tr>
<tr>
<td>PRV</td>
<td>pseudorabies virus.</td>
</tr>
<tr>
<td>Rb</td>
<td>rabbit.</td>
</tr>
<tr>
<td>RIA</td>
<td>radioimmunoassay.</td>
</tr>
<tr>
<td>RPH</td>
<td>reverse passive haemagglutination.</td>
</tr>
<tr>
<td>RPHI</td>
<td>reverse passive haemagglutination inhibition.</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulphate.</td>
</tr>
<tr>
<td>Shrbc</td>
<td>sheep red blood cells.</td>
</tr>
<tr>
<td>Tr.Shrbc</td>
<td>trypsinized sheep red blood cells.</td>
</tr>
<tr>
<td>VP</td>
<td>virion polypeptide.</td>
</tr>
</tbody>
</table>
CURRICULUM VITAE.


Sinds 1979 is zij als research assistant werkzaam in de Division of Virology, Department of Pathology, Cambridge University in Cambridge, alwaar het in dit proefschrift beschreven onderzoek werd verricht.