PRODUCTION AND CHARACTERIZATION OF MONOCLONAL
ANTIBODIES AGAINST HERPES SIMPLEX VIRUS

By

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A Thesis
Submitted to the Faculty of Graduate Studies
in Partial Fulfillment of the Requirements
for the Degree
Master of Science

McMaster University
July, 1980
Production and Characterization of Monoclonal Antibodies Against Herpes Simplex Virus.
MASTER OF SCIENCE (1980)
(Medical Sciences)

TITLE: Production and Characterization of Monoclonal Antibodies Against Herpes Simplex Virus

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NUMBER OF PAGES: 72
ABSTRACT

Methods for the production of hybrid cell lines (hybridomas) secreting monoclonal antibodies against specific antigenic determinants have recently been developed (Kohler G. and Milstein C., Nature 256, 495, 1975). Monospecific antibodies to Herpes simplex virus (HSV) antigens would greatly facilitate the analysis of HSV type-specific determinants, be of use in molecular studies of virus-cell interactions and could have potential applications in immunization against HSV infections. To this end, spleen cells from HSV2-immunized BALB/c mice have been fused to a BALB/c derived HGPRT⁻ myeloma line (Sp 2/0 Ag-14) and the resulting hybrids selected in HAT medium. From 13 successful fusions, 102 hybrid populations secreting antibodies recognizing antigenic determinants specified on HSV2-infected cells have been identified by FITC-immunofluorescence, an ELISA method or a ¹²⁵I-protein A binding assay. Ten of the positive hybrids have been cloned by limited dilutions to generate 124 monoclonal lines reacting specifically with HSV-infected but not with mock-infected cells. High titres of anti-HSV specific antibodies have also been detected in two ascitic fluids recovered from tumors induced in mice by injection of positive hybridomas. Preliminary data on the characterization of two hybridomas in terms of the subclass of immunoglobulins they secrete and the specificities of antibody they define has also been obtained.
ACKNOWLEDGEMENTS

I wish to express my appreciation to my supervisor, Dr. Silvia Bacchetti, who gave generously of her time and patience in helping me fulfill my studies. I would also like to extend my gratitude and appreciation to Dr. R.A. Killington who was an earnest partner in this venture while on sabbatical leave from the University of Leeds, England. The technical assistance of Carol Lavery is gratefully acknowledged as is the invaluable help of Dr. N. Balachandran.

I would also like to thank the other members of the thesis committee, Dr. W.E. Rawls, and Dr. J. Gauldie for their helpful discussion and direction.

I am indebted to Miss Josie Maljar for typing the manuscript.
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1. Introduction

Herpes simplex virus (HSV), also known as Herpesvirus hominis, establishes diverse clinical manifestations upon infection in humans. The virus usually replicates in tissues of ectodermal origin and causes disease upon primary infection (gingivostomatitis) as well as upon reactivation from a latent state (herpes keratitis and herpes labialis) (Lerner, 1976). HSV can be identified from other members of the Herpesvirus group by antigenic analysis and by definition of biological properties such as host range and type of cytopathological effect. HSV may also be differentiated by enzyme neutralization, polypeptide analysis, DNA base composition, or molecular hybridization (Gentry and Randal, 1973). There are two serologically distinct types of herpes simplex virus (Nahmias and Dowdle, 1968). Herpes simplex virus type-1 (HSV-1) which has been observed to infect predominantly the oropharyngeal pathway is the causative agent of the common cold sore (herpes labialis), and is responsible for herpes encephalitis, follicular conjunctivitis, and eczema herpeticum. Herpes simplex virus type-2 (HSV-2) which infects predominantly the genital tract causes a very common venereal disease (herpes genitalis). Genital herpetic infections of the expecting female can also be transmitted to the newborn during parturition (neonatal herpes). HSV-2 is responsible for 31% of HSV infections and 85% of HSV genital infections (Wolontis and Jeansson, 1977).

HSV-1 is a rather ubiquitous virus in the human population. Primary infection by the virus often occurs during early childhood and infected individuals develop neutralizing antibodies during
convalescence. However, recovery from infection does not provide complete immunity from further disease. Once the initial symptoms subside, the virus becomes latent in local sensory ganglia, remaining present in the body throughout life. The virus can be reactivated from this latent stage by certain nonspecific stimuli to cause a recrudescence of the disease, despite significant levels of circulating antibody. The virus is spread orally by both individuals experiencing primary infection and by asymptomatic individuals. Poor personal hygiene, close personal contact, and crowded living quarters favor the transmission of the virus, so that 80-100 percent of adults living in lower socioeconomic environments have antibodies to HSV-1 compared to 30-50% of adults in higher socioeconomic levels (Hirsch, 1979).

Studies of the HSV-2 induced disease show that the virus is sexually transmitted and causes lesions similar to the type-1 disease confined however, to the genitalia. Except in cases of congenital HSV-2 disease, antibodies to the virus are not detected until adolescence. The appearance of antibodies and the acquisition of the disease are correlated with the age of heterosexual activity and have been shown to plateau after the age of maximal sexual activity. Women of a sexually promiscuous nature, especially prostitutes (Duenas et al., 1972), show a greater occurrence of HSV-2 antibodies when compared with monogamous women. Chaste women of religious orders are virtually free of HSV-2 antibodies (Nahmias et al., 1970).

HSV-2 is transmitted by the exchange of contaminated secretions which occur during sexual intercourse. While subjects with the disease shed virus for a considerable length of time, the major source of virus
spread is through sexual contact with individuals who are asymptomatic, however persistently infected. In women, the cervix is the chief site of infection and in males the disease is usually confined to the exterior portions of the penis. The most important factor influencing the severity of the clinical manifestations is the individual's immune status.

As with HSV-1, following primary infection, the virus becomes latent and remains occult in the local sensory ganglia. The virus may be reactivated by nonspecific stimuli to reinitiate the diseased state. Due to the mode of transmission of HSV-2, the disease also shows an increased incidence in lower socioeconomic environments where 20-30% of adults may be infected. Only 7-9 percent of adults in the upper socioeconomic levels have antibodies to HSV-2, (Rawls et al., 1971).

HSV-2 is also suspect of being associated with squamous cell carcinoma of the cervix (Rawls et al., 1968; Melnick et al., 1970; Aurelian, 1973). Rubin (1974) reports that in the United States the malignancy has an incidence of 44 cases per 100,000 women per year. This is the second most common cause of cancer of American women, accounting for approximately 20 percent of all female cancers and an estimated 10,000 deaths annually.

Kessler (1977) listed in a review on cervical cancer numerous venereally transmitted organisms that have been implicated in the cause of abnormal cervical cytology and cervical cancer. However, in a recent study, Thomas and Rawls (1978) showed that mild dysplasia of the cervix might be associated with prior infections by other venereal organisms, whereas severe dysplasia and carcinoma in situ are more significantly
related to serological evidence of prior HSV-2 infections.

A vast amount of research has accumulated in the recent decade supporting an association between HSV-2 and cervical cancer, however it has not yet been possible to unequivocally implicate the virus as being etiologic in causing the cancer (Rawls, 1979).

1.1 Structure and Properties of HSV

Herpes simplex virus is a rather large, complex virus incorporating DNA, proteins, lipids, and carbohydrates into its structure. The viral DNA is double stranded and has a molecular weight of approximately $10^8$ daltons. Its base composition, given as the relative ratio of purines to pyrimidines, is 68.3% for HSV-1 and 70.4% for HSV-2. Consequently, the density of the viral DNA's are 1.727 g/cc and 1.729 g/cc, respectively (Lerner, 1976).

Electron microscopy using phosphotungstic acid staining reveals that the virion consists of a roughly spheric central core of DNA which measures 75 nm in diameter and a stable capsid which measures 100 nm in diameter. It appears to be of icosohedral geometry with a 5;3;2 axial symmetry consisting of 162 capsomers of which 150 are hexagonal and 12 pentagonal in cross-section. The capsid is surrounded by an envelope derived from host cell membranes, and the virion is 145 to 200 nm in diameter.

Virus particles may appear with or without envelopes; both enveloped and naked particles can infect cells although complete virions are more efficient. Naked viral DNA has also been shown to be infec-
tious (Sheldrick et al., 1973; Graham et al., 1973). It is believed that virions enter the host cell either by phagocytosis (viropoxis) (Morgan et al., 1968), or by fusion of the viral envelope with the host cell's plasma membrane (Miyamoto et al., 1971). After initiation of a productive infection, the virus becomes disaggregated within the cytoplasm perhaps with the aid of cellular lysosomes. The viral coat proteins stay within the cytoplasm and the viral DNA is transported into the nucleus (Hochberg and Becker, 1968). Ultimately, newly-formed viral DNA is packaged into capsids in the nucleus. These acquire an envelope at the nuclear membrane, egress through the cisternae of the endoplasmic reticulum and are released from the cell. In this manner, host materials make up major portions of the virion envelope.

Productive viral infection is accompanied by dramatic cytopathological effects including condensation and margination of chromatin, disaggregation of the nucleolus, cessation of host DNA, RNA, and protein synthesis, alteration in structure and immunological specificity of the plasma membrane and alteration in the social behavior of infected cells (Roizman and Furlong, 1974).

In the case of an abortive infection, the virus either fails to produce progeny and cell destruction does not occur, or the host cell might become transformed.

The process of herpesvirus replication, as reviewed by Roizman and Morse (1978), has been shown to differ from that of other DNA viruses. All viral proteins apparently can be synthesized even if viral DNA synthesis is completely blocked by inhibitors (O'Callaghan et al., 1968), and their synthesis is coordinately regulated and sequentially
ordered (Honess and Roizmann, 1974). The sequential control of mRNA formation is suggested by experiments in which infected cells are treated with an inhibitor of protein synthesis for various lengths of time after infection. When the inhibitor is removed, three different classes of polypeptides are made in a time dependent order, called alpha, beta, and gamma proteins (Honess and Roizman, 1974).

When cells are infected by the virus in the presence of cyclohexamide or puromycin, both cellular and viral protein synthesis is blocked. When the block is released, protein synthesis is allowed to occur. Alpha proteins are operationally defined as the virus specific proteins which do not require prior infected-cell protein synthesis in order to be produced. The alpha group comprises the virus specific proteins synthesized immediately (0.5 to 2 hours) after the withdrawal of inhibitors (Honess and Roizman, 1974).

Although several α proteins have been identified, their function is largely unknown. Recent information suggests that they are involved in the initiation of β protein synthesis, as β polypeptides require the presence in the infected cells of functional α polypeptides in order to be made (Honess and Roizman, 1975). Beta proteins appear within the infected cell between 4 to 8 hours postinfection. Among them are several proteins involved in DNA synthesis, such as viral DNA polymerase and thymidine kinase, as well as proteins involved in the shut-off of host cell metabolism. The synthesis of γ polypeptides require the presence of functional α and β polypeptides; these polypeptides appear late in the infectious cycle with peak synthesis occurring 15 to 18 hours postinfection and comprise largely the structural (virus) proteins.
Synthesis of γ proteins can be at least partially blocked by the drug cytosine arabinoside (Ara C), an inhibitor of DNA polymerase.

The viral proteins, produced in sequence in this manner, appear to be autoregulatory in that α proteins initiate β protein synthesis which feedback to terminate α protein synthesis. β proteins will act to initiate the production of γ proteins which will feedback to shut-off further synthesis of β proteins.

Using high resolution polyacrylamide gel electrophoresis, 51 virus-specific polypeptides are demonstrable in HSV-2 infected cells; 12 are expressed on the surface of infected cells. Nine of them are known to be glycosylated and only 2 are type-specific. Using DNA-DNA hybridization studies, HSV-1 and HSV-2 have been reported to share approximately 50% of their genetic information and are also closely related antigenically (Courtney and Powell, 1975). The bulk of cross-reacting antibodies are to a precipitation band detectable by immunodiffusion and called band II (Watson and Wildy, 1969; Skinner et al., 1974). Antiserum to this band will neutralize both HSV-1 and HSV-2; absorption of this serum with heterologous virus produces a type-specific antibody activity (Naumias et al., 1971).

1.2 Association of HSV-2 with Carcinoma of the Cervix

Infection of the human genital area with HSV-2 is common and has become a significant venereal disease. This is of considerable importance since evidence has been accumulated suggesting that HSV-2 is a candidate oncogenic virus and may be associated with the occurrence
of cervical cancer. The case for this association derives largely from retrospective seroepidemiological studies which show a higher titre of HSV-2 antibodies among women with cervical cancer than among control women without cervical cancer (Rawls et al., 1968; Nahmias et al., 1970; Aurelian et al., 1973; Adam et al., 1974).

The epidemiological data is based largely on measuring neutralizing antibodies as an indication of past infections by the virus, quantitating antibodies to virus-induced antigens in infected cells, corelation of antibody titre with the progression of the cancer and analysis of the class of antiviral immunoglobulin (Rawls, 1979). The variability of the results of seroepidemiological studies has precluded drawing definitive conclusions regarding the etiologic role of the virus in cervical cancer (Rawls, Bacchetti and Graham, 1977). Much of this variability results from the nature of the immune response to antigens of the two virus types. Although HSV-2 has unique biological, biochemical, and epidemiological properties when compared to HSV-1, the two viruses have approximately 50% homology at the DNA level and display major antigenic cross-reactivity. This is of significance since HSV-1 is a much more ubiquitous virus in humans and is commonly encountered before exposure to HSV-2. The difficulty therefore lies in accurately quantitating antibodies to HSV-2 in patients previously infected with HSV-1, by contemporary immunological techniques.

Many assay systems presently available measure antibodies to the cross-reacting antigens as well as the type-specific antigens. The criterion for positivity for HSV-2 antibodies has generally been based on the relative titres of antibodies to HSV-2 compared to HSV-1, or by
quantitating residual type-specific antibodies after the adsorption of sera with cells infected by the heterologous virus (Plummer, 1973).

The production of specific antibody to HSV-2 is known to be much greater among patients without prior exposure to HSV-1 than patients who have had previous infections with HSV-1 (Smith, J.W. et al., 1972). Furthermore, McClung et al. (1977) have shown that following an initial infection with either HSV-1 or HSV-2, about 80% of induced antibody activity is to the cross-reacting antigen, and only 20% is type-specific. Reinfection with either virus type stimulates predominantly those cells producing antibodies reactive to the common antigens. Due to this cross-reactivity of the two viruses, the accuracy with which present antibody assays detect all past infections by HSV-2 cannot be determined (Rawls, 1979).

Antibodies with a variety of specificities are induced during infection with either virus type. Some of these antibodies will be directed against proteins which will be maximally present in a time dependent sequence following an initial infection. Therefore, it is probable that different assay techniques, using cells infected for different periods will detect antibodies to different antigens expressed by the virus (Rawls, 1979). Most seroepidemiological data is based on virus neutralization studies which measure antibody activity directed against glycoproteins expressed on the exterior of the virion envelope (Nahmias and Roizman, 1973). Some studies measure a wide spectrum of antibodies to the virion as well as virus-specified infected-cell proteins, where infected-cell antigen preparations may vary from study to study. Other studies have used specific viral antigens such as Ag-4, an α-protein of 161,000 daltons molecular weight (Aurelian et al., 1976;
Strnad et al., 1978; Arsenakis et al., 1980), or HSV-TAA (herpes simplex virus-tumor associated antigen; 40,000 daltons) (Hollinshead et al., 1976), and VP134 (Anzai et al., 1975) to detect HSV antibodies in sera of cancer patients. Anti-AG-4 antibodies have been demonstrated by complement fixation in about 85% of sera from cervical cancer patients and only minimally in control sera and antibodies to HSV-TAA and to VP134 are also clearly elevated in cases over controls. However, levels of antibodies to these antigens do not correlate with titres of neutralizing antibodies in these individuals (Rawls, 1979). Apart from differences in the immune response to the two virus serotypes and the nature of the assays systems employed, much variation can be accounted for by the inherent nature of transmission and exposure in groups of different socioeconomic settings. Variation in epidemiological case/control studies has also been encountered within different geographical regions. This is likely due to the choice of virus used in antibody assays to quantitate viral antibodies since strain differences may exist and sera may be tested for reactivity to a virus that is not common to a geographical area (Rawls, 1972).

Antibody titres to HSV-2 have been shown to fluctuate with the progression of the cancer. From follow-up studies of patients whom have been treated for cervical carcinoma in situ, antibodies to HSV-2 are shown to decrease during convalescence and to recur with recurrence of the cancer (Catalano et al., 1971; Skinner et al., 1971). This type of pattern of antibody appearance, also demonstrated for antibodies to AG-4 (Aurelian et al., 1973, 1977), suggests very strongly that the viral antigens are associated with the presence of the cancer cells.
Sera from women who had recurrence of the cancer or who died of advanced cervical cancer contained only low levels of antibodies to HSV-2 antigens specified on the surface of infected cells as demonstrated by hemadsorption assays (Christenson and Espmark, 1976) suggesting that perhaps a depressed humoral antibody response allowed the cancer to progress.

Several laboratories have attempted to locate viral determinants expressed on or within cancerous cells. Royston and Aurelian (1970) identified herpesvirus antigens in exfoliated cells from a human cervical cancer using immunofluorescence techniques. Giraudo et al. (1977) could identify and differentiate early and late HSV-2 induced antigens in tumor cells by immunofluorescent techniques. Melnick et al. (1979) detected perinuclear HSV antigens in a cervical cancer cell line utilizing type-specific HSV-2 antisera against nonstructural polypeptides.

Other evidence albeit still preliminary comes from studies which have been undertaken in an attempt to demonstrate viral nucleic acids or viral antigens in tumor cells or transformed cells and tumor induction in animals.

A number of studies have suggested that HSV-2 has transforming potential, resulting in the in vitro conversion of normal cells to a potentially malignant state. Duff and Rapp (1971a, b) showed that UV-inactivated HSV-2 could induce transformation in hamster cells. Rat embryo cells (Macnab, 1974) and hamster cells (Kimura et al., 1975) have also been transformed with temperature sensitive (ts) mutants of HSV-2. The transformed cells could grow to form tumors in animals.
Examination of human cervical cancer tissues for evidence of viral DNA has provided, with one exception, limited results. Frenkel et al. (1972) reported detecting a fragment of HSV-2 DNA in a biopsy from cervical cancer tissue. This, however, has not been substantiated by subsequent studies in other laboratories.

It has been shown for other DNA viruses such as Adenovirus (Graham et al., 1974), SV-40 (Aaronson, 1970) and Polyoma (Bourgaux et al., 1965) that the size of viral DNA required for initiating and maintaining the transformed phenotype can be as small as just a few genes (1-3). If this estimate applies also for HSV-2, with a DNA 5 times larger than Ad5, the sensitivity of the available techniques might not be sufficient to identify such a small fragment of the HSV genome in tumor biopsies. Furthermore, the presence of a mixture of normal and transformed cells within a biopsy specimen effectively results in dilution of the amount of viral DNA present.

More recently research has been directed at exploiting the amplification of viral DNA into RNA, by trying to detect viral RNA sequences in tumor cells. Jones et al. (1978) by in situ cytological hybridization of viral DNA to virus-specified messenger RNA have detected viral sequences in cells of 5 of 8 cervical cancer patients tested; no virus material was detected in 3 of 3 non-cancerous controls. Galloway et al. (1979) detected herpes simplex RNA in human sensory ganglia and Puga et al., (1978) demonstrated Herpes simplex virus DNA and mRNA sequences in acutely and chronically infected trigeminal ganglia of mice. Finally, McDougall et al. (1980) have identified RNA comple-
mentary to HSV-2 DNA in frozen sections of tissues undergoing premalignant changes but not in cells of the fully developed squamous cell carcinoma. Although these above examples are few and represent limited samples, they provide further evidence suggesting that HSV-2 may be etiologically associated with human tumors. However, despite the accumulation of molecular and epidemiological data, the association of HSV-2 and cervical cancer remains an open question.

1.3 Contemporary Antibody Assay Techniques

Many of the problems outlined in relation with the seroepidemiological association of HSV-2 and cervical cancer derive from difficulties in accurately detecting HSV-2 antibodies in people previously exposed to the antigenically related HSV-1. Because of the difference in modes of transmission of the two viruses, most patients are infected with HSV-1 prior to becoming infected with HSV-2. It has been shown that reinfection with either virus preferentially amplifies type-common antibody production, and that the relative titres of type-specific antibodies is substantially reduced (McClung et al., 1976). In this respect as already stated, assay methods are required which are sensitive enough to detect low levels of type-specific antibody.

Antibody assays used to date include virus neutralization (Rawls et al., 1968), solid phase radioimmunoassay (Smith, K.O. et al., 1974; Forghani et al., 1975; Kalimo et al., 1977a, b), radioimmunoprecipitation of viral proteins (Anzai et al., 1975) \(^{51}\) Chromium-release (McClung et al., 1976), antibody-dependent cellular cytotoxicity (Shore et al.,
enzyme-linked immunosorbent assay (ELISA) (Gilman and Docherty, 1977; Miranda et al., 1977), indirect hemagglutination (Ohashi and Ozaki, 1979), and fluoroimmunoassay (Smith, J.W., 1979). In each case antibodies to HSV-2 are demonstrable in greater quantities among women with cervical cancer than among controls.

More recently, several laboratories have been trying to isolate type-specific antigens of HSV-2 and utilize them to test sera directly by more sensitive radioimmunoassay techniques. Dreesman et al. (1979) reported the use of a microsolid-phase indirect radioimmunometric assay to detect antibodies against purified HSV type-specific glycoproteins (VP-123 and VP-119) adsorbed to plastic microtitre wells. In this technique, human antibodies reacting with VP-123 (HSV-1 specific) or VP-119 (HSV-2 specific) are detected with radiolabelled rabbit anti-human immunoglobulin. Another approach is given by Vestergaard and Graubelle (1979) who used enzyme-linked immunosorbent assay to identify HSV type-specific antibodies reacting with antigens remaining on the surface of infected cells blocked with type-heterologous sera.

The isolation of a pure HSV-2 type-specific antigen would seemingly be the solution to many of the problems encountered in sero-epidemiological studies, however, the universal use of these techniques would require large amounts of antigen which would be technically difficult to prepare and purify. While the method of Vestergaard does not require purified type-specific antigen, large amounts of standardized type-heterologous blocking serum would be prerequisite to universal application.
A potential solution to these problems may be achieved with the application of the hybridoma technology recently developed by Köhler and Milstein (1975, 1976a) to produce large quantities of monospecific antibodies. This technique has so far been applied in mice and rats. Briefly, the animal is hyperimmunized with a chosen antigen so that he develops a large number of lymphocytes producing antibodies in response to the injected antigen. These lymphocytes are recovered from the animal, usually from the spleen, and fused with myeloma cells in the presence of a chemical fusogen (polyethylene glycol). Myeloma cells used in this technique are mutated at the locus for the enzyme hypoxanthine-guanosine phosphoribosyl transferase (HGPRT) and therefore are sensitive to the drug aminopterin (HAT selection, Littlefield 1964; Fig. 1). In the presence of selective medium, unfused plasmacytoma cells die, while hybrid cells acquire the HGPRT enzyme from the parental lymphocyte which is phenotypically normal at this locus and survive. Unfused lymphocytes do not survive after several passages in tissue cultures. This technique therefore, allows for the perpetuation of the specific secretory capacity of lymphocytes from previously sensitized animals by hybridization with a myeloma cell line adapted to grow in tissue cultures. Lymphocyte-myeloma hybrids (hybridomas) prepared in this manner continue to secrete antibody in tissue culture and cell lines can be clonally selected to provide large sources of antibody with unique specificities. Antibody reagents with such fine specificities have proven to be valuable in many areas of cell biology. Hybridomas have recently been prepared against diverse antigens including plasma membrane antigens (Eisenbarth et al., 1979), differentiation antigens
Figure 1

HAT Selection. Nucleotide precursors required for DNA synthesis are produced by two cellular mechanisms. Nucleotides can be made from de novo biosynthesis (Main pathway) or they can be scavenged from DNA catabolism (Scavenger pathway). The enzyme HGPRT facilitates the recycling of purines from DNA breakdown products back into DNA. Folic acid allows for the biosynthesis of all new nucleotides. Cells which lack the enzyme HGPRT must acquire purine precursors for DNA synthesis via the main synthetic pathway. In the presence of aminopterin, folic acid methylation of purine rings is blocked and therefore cells which are HGPRT− will be killed.
(Bechtol et al., 1979), major histocompatibility antigens (Galfre et al., 1977), B-cells (Gasser et al., 1979), T-cells (Reinhertz, 1979), tumor-specific antigens (Levy, 1979; Herbyn et al., 1979; Yeh et al., 1979; Goodfellow et al., 1979), influenza virus (Koprowski et al., 1977), rabies virus (Wiktor and Koprowski, 1978; Kaplan et al., 1980), vesicular stomatitis virus (Sethi and Brandis, 1980), as well as HSV-1 (Howes et al., 1979) and HSV-2 (Zweig et al., 1979). In these latter cases however, only a limited number of anti HSV antibody secreting hybridomas was obtained (8.8 and 5.1%, respectively). Thus, examples of monoclonal antibodies against HSV-2 would still be of great utility in the study of HSV diseases. In a review article, Rawls et al., (1977) have indicated that herpetic infections are a sufficiently serious medical problem to warrant investigation into the development of effective means of preventing or treating the disease, independent of any possible link with malignancy. A continuous supply of antibody detecting typespecific determinants of HSV-2 would prove useful in directly identifying the exposure of individuals to the virus without consideration of type-common activity, and allow for the universal standardization of immunological assays to do so. Furthermore, antibody preparations of such fine specificity would further research in many areas of Herpes virology. They would be of use in the accurate and rapid clinical diagnosis of HSV infections, the detection and localization of virus-specific antigens on or in cells infected or transformed by HSV, the preparation of affinity-purified viral proteins, and the antigenic analysis of virus strains to mention a few.
Ultimately, monoclonal antibodies will allow for the production of vaccines to counter the many clinical manifestations of HSV infection, by the affinity purification of antigens that would stimulate a strong virus neutralizing antibody response. Also, if HSV-2 is truly associated with human neoplasia and virus determinants are expressed on the surface of cancerous cells, passive transfer, or stimulation of, cytolytic antibodies could be of potential use in tumor therapy. As mentioned, to date there are a few anti HSV monoclonal antibody reagents available. A repertoire of antibodies reactive to all the individual proteins of HSV-2 would greatly facilitate research in this area. To this end, the hybridoma technique has been utilized in this study and a large number of hybridoma cell lines secreting antibodies with specificities for antigenic determinants of HSV-2 have been produced. This work reports the application of this technique and describes the initial characterization of two hybridoma populations.
2. Materials and Methods

2.1 Cell Cultures

African green monkey kidney cells (Vero) were obtained from Flow Laboratories. Mouse L-cells were obtained from Dr. Prevec, McMaster University. Both cell lines were grown as monolayers in plastic bottles (Corning) containing Minimal Essential Medium (MEM-F15) supplemented with 10% v/v heat inactivated fetal calf serum, 100 units/ml penicillin, 100 μg/ml streptomycin, 0.03% w/v 1-glutamine, 0.75 gms/l NaHCO₃, and 0.01 M Hepes buffer. Medium and supplements were purchased from Grand Island Biological Company (GIBCO).

Plasmacytoma cell lines used for fusion with lymphocytes were gratefully received from Dr. Kennett, The Wistar Institute. Cell line P3NS 1/1-Ag4-1 (P3NS 1) is a non-immunoglobulin-secreting variant of the BALB/c mouse cell line P3 (Kohler et al., 1976b) and only expresses intracellular k chains. Cell line Sp 2/0 Ag-14 (Sp 2/0) is itself a hybrid cell line derived from a BALB/c splenic lymphocyte fused to cell line X63-Ag-8 (Shulman et al., 1978) and has lost immunoglobulin heavy and light chain expression. Both these plasmacytoma cell lines lack hypoxanthine-guanosine phosphoribosyl transferase (HGPRT; E.C. 2.4.2.8) and are therefore resistant to the drug 8-azaguanine but are killed by medium containing aminopterin (HAT medium; Littlefield, 1964), (Fig. 1). Both P3NS1 and Sp 2/0 were grown in Dulbecco's Modified Eagles Medium (DMEM) supplemented with 10% v/v heat inactivated fetal calf serum,
antibiotics, buffers, and amino acids as described for MEM-F15 medium.

Hybridomas resulting from fusion events were maintained in HY-HAT medium (Kennett et al., 1978), a modification of DMEM enriched with 0.2 units/ml bovine insulin, 100 mM oxaloacetate, 45 mM sodium pyruvate, 10% v/v NCTC-109 medium, and containing 1% v/v HAT medium (0.1 mM hypoxanthine, 1 μM aminopterin, and 40 μM thymidine; Littlefield, 1964); hybridomas producing HSV-specific antibodies were either maintained in culture for further study, cloned at limiting dilution to produce colonies derived from single cells, or frozen at -186°C in liquid nitrogen in plastic vials (NUNC) containing approximately $10^7$ cells in 1 ml of fetal calf serum containing 5% v/v sterile DMSO. Plasmacytoma, Vero, and mouse L-cells were frozen as above in culture medium containing 5% v/v sterile DMSO.

2.2 Virus Growth and Antigen Preparations

HSV-2 strain 333 and HSV-1 strain KOS (Seth et al., 1974) were used in this study. Stock preparations of 333 were grown in Vero cell monolayers in MEM-F15 medium containing 1% v/v heat inactivated fetal calf serum. Virus was added to the cells at a multiplicity of infection (m.o.i.) of 0.1 plaque forming unit (p.f.u.) /cell, and the infected cells were harvested by scraping with a rubber policeman at a time (usually 24 hours) corresponding to maximal viral cytopathic effect. The cells were pelleted by centrifugation and the virus particles released from the cells into a minimum volume of medium by freeze/thaw treatment followed by sonication to disrupt cellular membranes. Cell debris was removed by centrifugation at 1500 rpm for 10 minutes and the
supernatant containing high titre virus was aliquoted and stored at -70°C. The virus titre was determined by plaque assay on Vero cell monolayers.

Virus antigen used for mouse immunizations was prepared in mouse L-cells. Cells were infected at an m.o.i. of 10 p.f.u./cell and harvested at maximum cytopathic effect. The infected cells were pelleted, washed 3 times in phosphate-buffered saline (PBS) and resuspended at a concentration of 10^8 cells/ml in PBS. The cells were disrupted by 3 freeze/thaw treatments and sonication, and the resulting antigen suspension was clarified of cell debris by centrifugation at 3000 rpm for 5 minutes. This preparation was used as the live virus antigen. Inactivated virus antigen was obtained by treating the above antigen with formalin (0.2 ml of 4% formaline for 5 ml of antigen) overnight at 37°C. Purified virus for mouse inoculations was sedimented from the medium released fraction of L-cells by centrifugation for 2 hours at 14,000 rpm and the resulting virus pellet left at 4°C overnight in PBS. The pellet was resuspended by brief disruption in an ultrasonic bath and centrifuged through a Dextran gradient for 1 hour at 25,000 rpm. The virus, appearing as a refractile band halfway down the gradient, was collected, centrifuged at 20,000 rpm for 1 hour, resuspended in distilled water and stored at -70°C.

Viral antigens for both the Protein-A antibody-binding assay and the ELISA were prepared as described for the immunization antigen except the virus was grown in Vero cells and then treated with trypsin to strip off surface Fc receptors (Bourkas and Menezes, 1979; Dorval et al., 1979). After trypsinization the cells were suspended at 10^8 cells/ml in carbonate-bicarbonate buffer (pH 9.6) for the ELISA antigen or in PBS for protein-A antigen. The details of the antigen preparations for
immunofluorescence screening are described with the assay methodology.

2.3 Immunization/Blastogenesis Studies

Male, 6 week old, BALB/c mice were obtained from Jackson Laboratories (Bar Harbour, Maine) and were used for the production of antisera, blastogenesis studies, and for fusion techniques. Control antisera for assay procedures was raised by injecting mice with live virus antigen (0.05 ml) in the footpads and then serially challenging with inactivated virus either intramuscular or in opposite footpads, and giving final boosts intravenously. Antibody reactivity was confirmed by Protein-A, ELISA, and immunofluorescence.

For blastogenesis studies, mice were immunized with either purified virus, live virus antigen or inactivated virus antigen with Complete Freund's Adjuvant (CFA) or in PBS. Control mice were given only CFA or PBS free of virus antigen. Antigens (0.05 ml) were administered either in the footpads or in the tail vein. Inoculation routes are further detailed in the Results section. The blastogenic response to antigen challenge was monitored by counting total organ lymphocytes and by measuring DNA synthesis by the incorporation of $^3$H-thymidine into nucleic acids. At defined intervals following antigen inoculations, 2 mice were sacrificed from each test group and their spleens and lymph nodes removed. Single cell suspensions were prepared by passing the organs through a stainless steel mesh (size 60), and the cells were collected in RPMI-1640 medium supplemented with 10% v/v heat inactivated fetal calf serum, 100 units/ml penicillin, 100 µg/ml streptomycin 0.03% w/v l-glutamine, 0.75 gms/ml NaHCO$_3$, 0.01 M Hepes buffer, and 3% H$_2$O. Viable cells were enum-
erated by trypan blue exclusion using a haemocytometer and adjusted to $5 \times 10^5$ cells/ml in medium containing $10 \mu$Ci of $^3$H-thymidine (specific activity 20 Ci/mMole, NEN) and incubated in a humid incubator with 5% CO$_2$ at 37°C for 4 hours. Cold PBS containing 0.1% azide was added to stop the reaction, the cells were pelleted and hydrolysed by treatment with 1N NaOH at 56°C and subjected to trichloroacetic acid precipitation (TCA). TCA-precipitable material was collected on glass fibre filters and radioactivity was measured in a liquid scintillation β counter.

2.4 Fusion Technique

The fusion technique used is a modification of the technique described by Kennett et al. (1978), (Fig. 2). Mice were selected for fusion at a time corresponding to maximal mitotic activity as determined in the blastogenesis study. Spleens were aseptically removed and a single cell suspension was prepared by passing the organs through a stainless steel mesh. Red blood cells were removed from the mixture by lysis in the presence of 0.17 M ammonium chloride. The lymphocytes were suspended in DMEM with 15% fetal calf serum and the viable cells were enumerated by trypan blue exclusion with a haemocytometer. $10^8$ spleen cells were then pelleted together with $10^7$ plasmacytoma cells and washed 2x in serum-free medium. Fusion was carried out by suspending the cells in 1 ml of 30% v/v polyethylene glycol-1000 (PEG; BDH Chemicals), (Pontecorvo, 1975; Davidson and Gerald, 1976) in serum-free DMEM for 1 minute followed by centrifugation at 800 x g for 8 minutes. 10-15 ml of serum-free DMEM was added to dilute the PEG and the
Figure 2

Schematic fusion protocol. Spleen cells from hyperimmunized mice are fused with a myeloma cell line to generate large numbers of hybrid populations. These hybrids are then screened by a variety of techniques to detect those secreting specific antibody. Positive cultures can then be clonally selected to produce antibodies of a similar specificity. Monoclonal antibodies can be extracted from culture medium or from fluid draining ascites tumors produced in animals.
$10^8$ spleen cells

PEG fusion

$10^7$ plasmacytoma cells

Fused cells distributed in microtitre plates in HAT medium

Screened for antibody production
1. IPA assay
2. ELISA
3. FITC-IF

Select positive clones

In vitro propagation

Ascites tumor production

Figure 2: Schematic fusion protocol
cells were pelleted again. The fused cells were washed twice in more
tive medium and distributed into two 24-well plastic culture plates
(Linbro) at a volume of 2 mls per well. Medium was changed 2 to 3 days
after fusion to remove any residual antibody that may have been produced
by unfused parental spleen lymphocytes. Hybrid colonies were usually
microscopically visible 8 to 10 days after fusion.

2.5 Tumor Induction by Hybridomas

Approximately \(10^7\) hybridoma cells were injected into the peritoneal cavity of mice treated 7 days earlier with 0.5 ml of the immunosuppresive drug, pristane (2,6,10,14-tetramethylpentadecane; Aldrich Chemicals), to produce ascites tumor growth. Peritoneal fluid draining the ascites tumor (ascitic fluid) was collected and used as a source of high titre antibody. The mice were sacrificed and volumes of 5 to 10 ml of ascites fluid were collected from individual mice with a heparinized syringe (100 units heparin/ml ascites fluid). Tumor cells were centrifuged for 10 min at 800 x g from the ascitic fluid and injected into other recipient mice for further ascites production or were put back into tissue culture. Cells handled in this manner have so far retained their ability to form tumors and to secrete specific antibodies. The clarified ascites fluid was aliquoted and stored at -20°C.

2.6 Assay Techniques

Culture medium collected from actively growing hybridoma popula-
tions derived from fusion events was screened for the production of antibodies recognizing HSV-2 antigens expressed by infected cells by one or more of the following methods.

2.6.1 Protein-A Antibody-binding Assay

The protein-A antibody-binding assay (PA) was adopted from the method of Nowinski et al (1979). Briefly, 50 μl of an optimal dilution of 333-infected Vero cell antigen was adsorbed to the wells of plastic microtitre plates (Linbro) in a humid, 5% CO₂ incubator, at 37°C overnight, after which residual unbound antigen was shaken off. To block remaining binding sites in areas of plastic not coated with antigen, 125 μl of 5% BSA w/v in PBS were then added to each well for 2 hours at 37°C. The blocking solution was removed by tipping and shaking the plates and 50 μl of antibody test solution (tissue culture supernatant, ascites fluid, or serum) was added in duplicate wells and incubated for 1 hour at 37°C. Unbound antibodies in the test solutions were washed from the wells with 1% BSA in PBS and 50 μl of a predetermined optimal dilution of Goat or Rabbit anti-Mouse IgG or IgM (Cappel Labs., heavy and light chain specific, IgG fraction only) was added to each of the wells to amplify detection and the plates returned at 37°C. One hour later, after three washes in 1% BSA, 50 μl (10⁵ cpm) iodinated Staphylococcus aureus protein A (Pharmacia) were added to each well for 1 hour at room temperature. Finally the plates were washed thoroughly in PBS, air dried and exposed to KODAK RP X-OMAT film in a cassette with intensifying screens. Wells containing test solutions with antibodies recognizing HSV-2
antigens were detectable as black spots defining the wells after a 1-2 day exposure. This protocol was repeated using uninfected Vero cell antigens or diluted mouse serum as control procedures. Positive hybridomas were selected qualitatively on the basis of the relative intensity of the reactivity on infected cells compared to uninfected cells.

### 2.6.2 ELISA

The methodology of the enzyme-linked immunosorbent assay (ELISA) was essentially similar to the protein-A assay except that an enzymatic reaction replaced the use of isotopic label at the identification step. Vero cells infected with HSV-2 at a m.o.i. of 10 p.f.u./cell and harvested 24 hours later or mock-infected Vero cells were trypsinized, sonicated, clarified of cell debris by centrifugation at 1500 x g, and used as ELISA antigens. 50 µl of an optimal dilution of this antigen in 0.05 M sodium carbonate-bicarbonate buffer, pH 9.6, were added to the wells of plastic microtitre plates (Linbro) and incubated for 3 hours at 37°C. Residual antigen was shaken off and the plates washed with 0.05% Tween-20 in 0.85% saline. Test antibody solutions were added to the wells for 30 minutes at 37°C and again washed thoroughly. The amount of antibody binding to antigen was amplified by the addition of Rabbit anti-Mouse IgG or IgM (Cappel, heavy and light chain specific, IgG fraction) which was then detected by the addition of Horse radish peroxidase (HRP)-conjugated Goat anti-Rabbit IgG (Cappel Lab.). The amount of HRP retained in the wells was determined by adding 50 µl of substrate solution containing H₂O₂ and 5-amino salicylic acid (9 ml of 0.08% 5-AS, pH 6, and 1 ml of 0.05% H₂O₂). After
incubation at room temperature for 1 hour the reaction was stopped by adding 100 µl of 1N NaOH and the contents of the well were diluted with 1 ml H₂O. The optical densities were measured at 450 nm and reactivity against HSV-2 infected cells was determined by comparison with the reactivity on mock-infected control antigen.

Comparison between the protein-A assay and the ELISA was carried out by testing 30 randomly selected hybridoma culture fluids. Greater than 90% agreement in the results by the two assay techniques was observed.

2.6.3 Indirect Immunofluorescent Assay

Detection of antibodies to antigens present in HSV-2 infected cells by immunofluorescence was performed as follows. Vero cells were grown to approximately 50% confluency in MEM-F15 plus 10% FCS and then infected with HSV-2 333 at 0.1 p.f.u./cell in MEM-F15 plus 17 FCS. After an 18 hour incubation period at 37°C, infected cells were washed free of medium with PBS and fixed for 10 minutes with cold acetone; coverslips were then stored at ≤20°C until use. For kinetic studies, infected cells were acetone-fixed at different times post-infection as indicated in the Results section or cells were infected in the presence of inhibitors. Control coverslips containing mock-infected cells were also acetone-fixed and stored at -20°C.

Test and control cell coverslips were wetted first with PBS then with 2 drops of hybridoma culture medium, acites fluid, or serum, and incubated at 37°C for 30 minutes. After a further wash in PBS, 2
drops of fluorescein isothiocyanate (FITC)-conjugated Goat anti-Mouse IgG (Cappel) was added for 30 minutes at 37°C. Residual unbound FITC reagent was washed off with PBS and the coverslips were mounted on glass slides with 9:1 v/v Tris-glycerol solution, pH 9.0, for viewing under the ultra-violet microscope. Positive reacting samples were identified by the relative fluorescent intensity on infected-cells as compared to mock-infected control cells.

2.7 Characterization Studies

Immune precipitation was performed with both concentrated culture medium and ascitic fluid that showed strong reactivity in antibody assays. Cells infected at an m.o.i. of 20 p.f.u./cell and mock-infected cells were labelled for 12 hours with 35S-methionine (30-50 μCi/ml; NEN). The cells were harvested, washed 3 times in cold PBS and solubilized with RIPA buffer (0.05 M Tris-HCl, 0.15 M NaCl, 0.1% SDS, 1% sodium deoxycholate, 1% Triton X-100, 100 units/ml Aprotinin, 0.1 mM PMSF, 1 mM benzimidine) for 30 minutes at 4°C. The lysate was sonicated and centrifuged at 100,000 x g for 60 minutes. One ml of clear supernatant was mixed with 40 μl of protein A-sepharose beads (Pharmacia) (15 mg of beads in RIPA buffer) and kept at 4°C for 2 hours with constant mixing. The beads were collected by centrifugation, washed three times with RIPA buffer and solubilized with 50 μl of sample buffer (10% 2-Mercaptoethanol, 10% SDS). The samples were loaded on a 7.5-15% gradient polyacrylamide gel containing 0.1% SDS and run at constant voltage for 24 hours. The
gels were stained with Coomassie blue, destained, infused with PPO, dried on filter paper and placed in contact with KODAK RP X-OMAT for fluorography. Molecular weight markers and infected cell protein immunoprecipitated with mouse anti-HSV immune serum were electrophoresed along side the test samples.
3. Results

3.1 Blastogenesis Study

Initial attempts to produce hybridomas utilized immunization schedules reported in the literature for other virus systems such as rabies (Wiktor and Koprowski, 1978), and influenza (Koprowski et al., 1977). BALB/c mice were sensitized with an intraperitoneal injection of $2 \times 10^4$ p.f.u. of live HSV-2 and boosted intravenously 6 weeks later with 50 μg of live virus purified on sucrose gradients. Mice were sacrificed 3 days later and spleens were removed for fusion to $P_{\text{NS}}$ myeloma cells. Antiviral antibodies were not detected in sera from the sacrificed mice by microneutralization or solid-phase radioimmunoassay. Spleen cell-myeloma cell hybrids (hybridomas) formed in 119 of 528 wells plated. When these hybrids were screened by a solid-phase radioimmunoassay (Smith, K.O., et al., 1974) no antibody reactivity was detectable on HSV-2 infected cell antigens. One of these 119 cultures (0.8%), however, produced antibodies detectable by the protein-A assay (table I).

Due to the low yield of positive hybridomas, subsequent experiments were aimed at developing different immunization schedules and better assay techniques for screening hybridoma culture fluids. The efficacy of novel immunization schedules was evaluated by monitoring the blastogenic response in spleen cells, the production of antibodies to the virus, and the yields of hybridomas secreting antibodies.
TABLE I. Responses to various immunization schedules. (1)

<table>
<thead>
<tr>
<th>Date</th>
<th>Virus, IF</th>
<th>Virus, LF</th>
<th>IF</th>
<th>LF</th>
</tr>
</thead>
<tbody>
<tr>
<td>5/18</td>
<td>No</td>
<td>No</td>
<td>18</td>
<td>18</td>
</tr>
<tr>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<tr>
<td>3/24</td>
<td>No</td>
<td>Yes</td>
<td>14</td>
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<td>3/9</td>
<td>200</td>
<td>200</td>
<td>50</td>
<td>50</td>
</tr>
</tbody>
</table>

**Notes:**
(1) The table above summarizes responses to various immunization schedules.
(2) The cell lysates and lysates of infected cells were used as antigens.
(3) IF and LF stand for immunofluorescence and light microscopy, respectively.
(4) The number of cells obtained from spleens and the in vivo response was indicated.
(5) The HSV-2 stocks were prepared in mouse embryo fibroblasts.
(6) Additional notes are included at the bottom of the page.
BALB/c mice were inoculated with either formalized or non-treated HSV-2 antigen or with purified HSV-2 or variations of all three via a variety of routes. Examples of two successful immunizations are illustrated in Table I. Mice immunized with formalized antigen plus adjuvant in the footpad were found to have no increase in the number of spleen cells and a 50% reduction in $^3$H-thymidine incorporation in spleen cells 6 days after immunization. An intravenous boost with formalized antigen on day 10 was followed 4 days later by a dramatic increase in spleen cell numbers (137%) and in $^3$H-thymidine incorporation (700%) and 39.6% of the hybrids formed were found to be secreting antibodies to virus-infected cells by the protein-A assay. Sera obtained from these mice had anti-HSV-2 antibodies detectable by protein-A assay at titres greater than 1:1000. Mice sensitized with formalized virus plus adjuvant in the footpad, boosted with the same antigen in the footpad on day 10 and again with formalized virus intravenously on day 20 were found to have an increase of both spleen cell numbers (445%) and $^3$H-thymidine incorporation (300%) 4 days after the last injection. Antiviral antibody was readily detectable by protein-A in the sera of these mice and 37.5% of the hybridomas formed from the spleen cells were secreting antibody to HSV-2 infected cell antigens.

Two additional examples are presented in Table I. Mice sensitized with formalized virus intraperitoneally with adjuvant and boosted either once or twice with formalized virus by the same route were found to respond with only low levels of serum antibody. The yields of hybridomas secreting antibody to infected-cell antigens were 10.4% and 12.5% respectively, for mice receiving one or two boosts.
The inoculation of control antigens (without the presence of virus) show only minimal deflection from the normal blastogenic state of healthy mice (data not shown).

3.2 Hybridoma Production

Mice were subjected to immunizations paralleling blastogenic profiles which induced the greatest mitotic stimulation and in some cases mice received repeated boosts in an "ad hoc" fashion in preparation for further fusions. At the appropriate intervals, usually 3 or 4 days after the last inoculation, spleens were removed from the animals for fusion with plasmacytoma cells. In some experiments lymph nodes were also removed from the animals and fusion of pooled lymph node cells and plasmacytoma cells was carried out. The plasmacytoma cell line P.3 NS1 was substituted with line Sp 2/0 which has been reported as better for hybridoma production (Shulman et al., 1978).

From 13 independent fusions a total of 348 hybrid populations were obtained. Of these, 331 were screened for their ability to secrete antibodies of the IgG or IgM class by the $^{125}$I-Protein A method; some of the hybrids were also screened by FITC-IF. Culture medium from each hybridoma population was initially tested only on infected Vero cells (and not on mock-infected cells) primarily for the sake of rapidity. At this stage all of the hybrids screened were considered polyclonal since they were isolated from wells in which approximately $2 \times 10^6$ cells had been plated and no direct attempt to isolate single cell progeny had been made.
### Table 2

**Reactivity of Hybridomas**

<table>
<thead>
<tr>
<th>Number of positive lines</th>
<th>$^{125}$I-Protein A</th>
<th>FITC-IF</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IgG</td>
<td>IgM</td>
</tr>
<tr>
<td>49</td>
<td>38</td>
<td>20</td>
</tr>
</tbody>
</table>

**Table 2.** Reactivity of polyclonal hybridomas. Medium from hybridoma cultures was tested for antibody against HSV-2 infected Vero cells by the Protein A methods or by indirect immunofluorescence as described.
Of the 331 hybrids screened, 102 (30.8%) showed reactivity on HSV-2 infected cells. Inoculation schedules are shown for 83 of these hybrids in Table 1, the rest resulting from the "ad hoc" continuation of these schedules. Of the 102 positive hybrids, 26 were positive for IgG production as detected by the Protein-A method and 18 as detected by FITC-IF. Thirty-eight hybridomas were found positive for IgM production and a further 20 secreted both IgG and IgM as detected by the Protein-A assay (Table 2). Several hybridomas were detected as positive by both Protein-A and FITC methods; these are included in the total only under one category.

Following the preliminary screening, 12 hybrid cultures showing good reactivity in antibody assays were cloned at limiting dilutions in 96-well plastic microtitre plates (Linbro) to generate monoclonal cell populations. Of the resulting 457 clones, 249 were screened for antibody production on both infected and mock-infected control cells. A total of 124 (49.8%) of these monoclones were found positive for anti-HSV-2 antibodies by ELISA (91) or FITC-IF (33). Of these, 31 were detected by both methods (Table 3).

Several polyclonal and monoclonal cell populations were also injected into mice to induce tumors and the resulting ascites fluids were found to contain high titres of antibodies of the same class defined by the cells of origin. To this date, antibody production appears a stable property of the positive hybridomas as no loss of reactivity has been observed after long term in culture, freezing of the cells, or repeated passage in mice.
Table 3

**Reactivity of Monoclonal Hybridomas**

<table>
<thead>
<tr>
<th></th>
<th>ELISA (IgG)</th>
<th>FITC-1' (IgG)</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>number of positive clones</td>
<td>91</td>
<td>33</td>
<td>124</td>
</tr>
</tbody>
</table>

Table 3. Reactivity of monoclonal hybridomas. Medium from cultures of monoclonal hybridomas was tested against HSV-2 infected and mock-infected Vero cells by ELISA or indirect immunofluorescence as described. The number of positive clones refers to clones reacting only with infected cells and not with control cells.
3.3 Characterization Studies

In order to obtain greater concentrations of antibody for characterization studies, ascites tumors were produced by injecting hybridoma cells into the peritoneal cavity of BALB/c mice \(10^7\) cells/mouse, sensitized with 0.5 ml pristane 1 week earlier. Ascites tumor production was initially attempted with the polyclonal population designated FL30A3 and FL30A5. Tumors were also produced in mice with a monoclonal obtained from FL30A5, designated FL30A5-2-B7. Several studies were undertaken to characterize the specific reactivity pattern of the antibodies secreted by these cell lines. Immunofluorescent studies were performed to detect the cellular localization of the virus-specific determinants in infected cells. The kinetics of appearance of these viral antigens was similarly determined by immunofluorescence. The serotype specificity of these antibodies was also tested by IF on both HSV-2 (333) infected cells and HSV-1 (KOS) infected cells. Finally, the viral products that could be immunoprecipitated by these antibodies were analyzed by polyacrylamide gel electrophoresis.

3.3.1 Antigen Localization

Acetone-fixed HSV-2-infected Vero cells (18 hours after infection) and mock-infected (control) cells were exposed to fluids from ascites derived from hybridomas FL30A3 and FL30A5. Mouse antibodies binding to determinants expressed on the infected cells were then reacted with FITC-conjugated Goat anti-Mouse IgG and the coverslips were mounted on microscope slides for viewing under an UV-microscope.
Antibodies present in the ascitic fluid of an FL3xA3 tumor reacted with viral antigens expressed within the nucleus of infected cells (Fig. 3.1). This reactivity was detectable up to an endpoint dilution of 1/10000. FL3xA5 ascites fluids contain antibodies that react with viral determinants expressed diffusely within the cytoplasm of infected cells (Fig. 3.2) and was detectable at an endpoint dilution of 1/10,000. Neither ascitic fluid gives any reactivity detectable on mock-infected control cells. Sera from mice immunized with HSV-2 stain both the nucleus and cytoplasm of HSV-2 and HSV-1 infected cells but do not stain mock-infected cells (Figs. 3.3, 3.4 and 3.6 respectively).

3.3.2 Kinetics of Appearance of Viral Antigens

The kinetics of appearance of viral antigens detected by hybridoma antibodies was determined by reacting ascites fluids from FL3xA3 and FL3xA5 with infected cells fixed at different times after infection, and assaying for immunofluorescent activity (Table 4). Both antibody populations detect antigens appearing at 2 to 4 hours post-infection; maximum reactivity was reached at 8 hours, and persisted till at least 18 hours post-infection. To establish the class of viral protein detectable by these hybridoma antibodies, experiments were carried out with the use of inhibitor drugs. Cells were infected and incubated in the presence of cycloheximide for 4 hours after which the drug was removed and protein synthesis allowed to continue for 1 hour. These cells were then acetone-fixed and exposed to the ascites fluids of FL3xA3 and FL3xA5. As shown in Table 4, no reactivity was detectable by IF in either case suggesting that viral \( \alpha \) proteins are not involved. When
Figure 3

Fluorescent reactivity of hybridoma antibodies. Coverslips of Vero cells, infected with either HSV-2 or HSV-1 or mock-infected and acetone-fixed 18 hours later were incubated with medium from hybridoma cultures; (1) hybridoma F13αA3 ascites fluid with HSV-2-infected Vero cells, (2) hybridoma F13αA5 ascitic fluid with HSV-2-infected Vero cells, (3) mouse anti-HSV-2 serum with HSV-2-infected Vero cells, (4) mouse anti-HSV-2 serum with HSV-1-infected Vero cells, (5) hybridoma F13αA5 ascites fluid with HSV-1-infected Vero cells, (6) mouse anti-HSV-2 serum with mock-infected Vero cells. (400 x magnification; 60 sec exposures).
<table>
<thead>
<tr>
<th>Virus</th>
<th>Time p.i.</th>
<th>Drug</th>
<th>FL3αA3*</th>
<th>FL3αA5**</th>
</tr>
</thead>
<tbody>
<tr>
<td>HSV-2</td>
<td>2</td>
<td>-</td>
<td>Neg.</td>
<td>1+</td>
</tr>
<tr>
<td>HSV-2</td>
<td>4</td>
<td>-</td>
<td>1-2+</td>
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<tr>
<td>HSV-2</td>
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<td>-</td>
<td>2+</td>
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</tr>
<tr>
<td>HSV-2</td>
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<td>Neg.</td>
<td>Neg.</td>
</tr>
<tr>
<td>HSV-2</td>
<td>6</td>
<td>Ara C</td>
<td>1-2+</td>
<td>2+</td>
</tr>
<tr>
<td>HSV-1</td>
<td>8</td>
<td>-</td>
<td>Neg.</td>
<td>3+</td>
</tr>
<tr>
<td>Control</td>
<td>-</td>
<td>-</td>
<td>Neg.</td>
<td>Neg.</td>
</tr>
</tbody>
</table>

* Nuclear fluorescence  
**Cytoplasmic fluorescence

Table 4. Kinetics of appearance of viral antigens as detected by hybridoma ascites fluid antibodies. Vero cells grown on coverslips were infected with HSV-2 or HSV-1 in the presence or absence of inhibitors and harvested at different times after infection. Infected cells were fixed in acetone and reacted with FL3αA3 or FL3αA5 antibodies. Viral antigens detected by these antibodies were identified upon the onset of their appearance in infected cells.
cells were infected for 6 hours in the presence of cytosine arabinoside (Ara C) which inhibits DNA synthesis, thus blocking also the synthesis of most viral γ proteins, fluorescence was detectable with both hybridoma fluids at an intensity comparable to that observed at a similar time in the absence of drug.

3.3.3 Serotype Specificity

This study was performed to determine whether Fl3αA3 and Fl3αA5 ascites fluid antibodies reacted with viral proteins specified solely upon HSV-2 infection or also upon HSV-1 infection. As shown in Table 4, antibodies produced by hybridoma Fl3αA5 reacted with an antigen specified upon infection of Vero cells with both serotypes of HSV. The localization of the KOS-specified antigen is also cytoplasmic (Fig. 3.5). On the contrary, antibodies produced by hybridoma Fl3αA3 did not react with KOS-infected cells (Table 4). Again, neither antibody reacted with mock-infected (control) cells.

3.3.4 Radioimmune Precipitation Studies

Antibodies recovered in the ascites fluids produced by hybridomas Fl3αA3 and Fl3αA5 were used to immunoprecipitate HSV-2 specified proteins which were then subjected to analysis by SDS polyacrylamide gel electrophoresis (RIP-PAGE). Antibodies present in the ascitic fluid of both Fl3αA3 and Fl3αA5 reacted with HSV-2 infected cells to give multiple bands (both major and minor) (Fig. 4, channel 3 and 5 respectively). Fl3αA3 antibodies precipitated virus determinants which
Figure 4

Radioimmune precipitation of viral antigens by hybridoma antibodies. Vero cells were infected with HSV-2 or mock-infected in the presence of 20 μCi/ml of 35S-methionine for 1-12 hours as described in Materials and Methods. Products immunoprecipitated by hybridoma antibodies in ascites fluids or immune sera were analyzed by SDS polyacrylamide gel electrophoresis. Channel 1, immune mouse sera with infected cells; Channel 2, immune mouse sera with mock-infected cells; Channel 3, Fl3αA5 with infected cells; Channel 4, Fl3αA5 with mock-infected cells; Channel 5, Fl3αA3 with infected cells; Channel 6, Fl3αA3 with mock-infected cells.
migrated in the regions of 78K, and 38K (major bands) of molecular weight. Two minor bands were present in the regions corresponding to molecular weights of 83K and 58K. Antibodies produced by hybridoma culture F13αA5 also precipitated viral antigens of HSV-2 infected cells, the major species migrating at 94K with minor bands present at 105K, 97K, 82K, 77K, 69K, 64K, and 40K. Neither F13αA3 or F13αA5 antibodies precipitated antigens present in mock-infected (control) Vero cells (Fig. 4, channels 4 and 6 respectively).

The identity of all the products precipitated by these hybridoma antibodies has not yet been established. However, to discount the possibility that the complex immunoprecipitation pattern was caused by the polyclonal nature of the line, a monoclonal of F13αA5 was similarly analyzed by RIP-PAGE. No change in the immunoprecipitation pattern was observed when infected-cell antigens were reacted with antibodies of F13αA5-2-B7 monoclonal (data not shown).
4. **Discussion**

The serological response to herpes simplex viruses involves the formation of numerous antibodies of different isotypes and different antigenic specificity. Many of these antibodies are known to cross-react with virus proteins specified by both HSV-1 and HSV-2. Current serological assays attempting to measure antibodies to HSV-2 are therefore hampered by the presence of antibodies to HSV-1 cross-reacting with HSV-2. The seroepidemiological association of HSV-2 with cervical cancer has been complicated by this problem, and hence a more definitive proof is required to unequivocally substantiate the association between this virus and the cancer. The recent success of several laboratories at producing somatic cell hybrids which secrete monospecific antibodies to given antigens suggested a similar approach to produce antibody reagents with specificity for unique proteins of HSV-2. The capacity to produce monospecific antibodies would be invaluable in serological typing, in correlating neutralizing titres with specific antibody production, in the identification and purification of specific virus antigens, in defining more clearly potential tumor-associated antigens, and in the identification of antigens of importance in protection against herpes simplex virus infections.

The hybridoma technique allows for the continuous production of large amounts of monospecific antisera without the arduous task of extracting antibodies from animal sera. Hybridoma cell lines of desired specificity can be grown in culture and frozen for long term
storage, thereby avoiding the expense of maintaining a sensitized animal colony. As of yet, hybridoma reagents have only been prepared in small rodents.

This work describes the initial results of a study to produce monospecific antibody reagents of potential use in furthering the study of herpes simplex viruses. A prime consideration in this effort was to ensure that the virus produces an efficient antibody response, thereby indicating the presence of a large number of antibody-forming cells in the mouse. It is these antibody-forming cells which are partners in fusions with a continuous cell line. The success of fusion and the frequency of hybrid formation appears to be dependent to some extent on the numbers of antibody-forming cells and their mitotic status at the time of fusion (Stähli et al., 1980).

The importance of a proper immunization schedule can be brought to light with a description of the immune response to an antigen and the process of antibody formation. The first introduction of antigen, the primary stimulus, invokes what is called the primary response. Immediately after introduction of the antigen, the inductive or latent period ensues where little or no antibody is detected in serum. It is during this period that the antigen is recognized as foreign and processed, and an unknown signal is transferred to the appropriate cells destined to make antibody. This period is characterized by cellular proliferation and differentiation, followed by active biosynthesis of specific antibody. Antibodies produced during the primary response are usually detectable 4 to 6 days after the primary stimulus, the lag period varying with the dose, the route of injection, the particulate
or soluble nature of the antigen, the type of adjuvant used, the animal species, and the sensitivity of the assay used to detect newly formed antibodies. On a cellular basis, the number of differentiated plasma cells increases soon after immunization, while peak cellular synthesis of antibody precedes the peak serum antibody response by several days. The presence of antibody in serum is short-lived and declines rapidly after peak concentrations are reached. The response to most antigens is characterized by the initial predominance of IgM antibody; the IgG class of antibody appears somewhat later in the primary response. Numerous antibodies are formed with specificities for the diverse antigenic determinants present on an antigen. In this respect, the humoral immune response is heterogeneous, producing a population of antibodies of different immunoglobulin class, affinity, and specificity.

Upon a second exposure to the same antigen there is a markedly enhanced response that is characterized by the accelerated appearance of antibody-forming cells and the expression of antibody. The secondary response is further characterized by a shorter latent period, a more rapid rate of antibody synthesis, and a higher peak titre of antibody persisting for a longer period of time than primary response antibodies. This secondary, or anamnestic response, is the basis for the rationale for giving booster doses of antigen. The enhanced secondary response is related to the number of antigen-sensitive cells, called memory cells, produced during the primary response. Following the primary stimulus, a precursor cell divides and differentiates into a number of specific antibody-forming cells (lymphoblast formation, or blastogenesis); during this primary proliferation event, a small number
of memory cells are produced. Upon secondary challenge with antigen the response is more dramatic because the pool of sensitized memory cells undergo blastogenesis. This enhanced blastogenesis response which results in large numbers of antigen-specific lymphoblasts, is therefore favourable for the production of hybridomas with greater efficiency (Stähli et al., 1980). IgG antibodies are characteristic of the secondary antibody response.

The low frequency (0.8%) of hybridomas obtained in our initial experiments and the inability to detect serum antibodies to HSV-2 infected cell antigens could have been related to an inefficient stimulation of blastogenesis. Those results suggested to us the need for a more rigorous study of antigen preparations and immunization routes to increase the yield of hybridomas. The blastogenesis experiments reported were therefore performed with the sole purpose of identifying potentially useful immunization regimes and as such do not allow for a indepth analysis of the immune response to HSV-2. By identifying optimum blastogenic activity in response to a particular boost series, more suitable mice could be selected for fusion experiments.

Several interesting observations became evident from our study. When mice were challenged with live virus (2 x 10^4 p.f.u., i.p.) approximately half of the animals died from viremia. Surviving mice used for fusions did not allow for the production of hybridomas secreting anti HSV-2 antibodies. One possible interpretation of these results is that injection of live virus overwhelms the animals' immune system so that its response is not sufficient to contain virus spread. The inability to recover positive hybridomas along with the apparent lack of serum
anti HSV-2 antibodies suggests that live virus was possibly inducing a state of immunosuppression which might result in low yields of anti-HSV-2 lymphoblasts. Instances of HSV-2 induced immunosuppression are well documented in the literature (Cappel, 1976); the mechanism by which HSV induces a suppression in lymphocyte blastogenesis is however not well understood. Since HSV is known to be infectious in lymphocytes (Kirchner et al., 1976; Nahmias et al., 1976; Henle and Henle, 1975) it is possible that the disruption of cellular metabolism brought about by virus replication renders the cells non-functional. HSV infection of cells also induces numerous changes in the cell plasma membrane. Besides the induction of HSV-specific membrane antigens the virus causes the induction of immunoglobulin Fc receptors (Dorval et al., 1979). These receptors could be visualized to act with a "mopping-up" effect on circulating antibodies thereby further compromising the humoral immune system. The interference with cellular metabolism upon infection of lymphocytic cells appears however, to be more significant in affecting the immune response to the virus.

Inactivated (formalin-treated) virus used as an antigen allowed mice to be serially boosted without threat of immunosuppression or mortality from viremia. As illustrated in Table 1, secondary and tertiary boosts with inactivated virus antigen given in the footpads were sufficient to induce and sustain blastogenic activity (127 to 445% increase in spleen cell numbers and a 167 to 700% increase in $^3$H-thymidine incorporation). Peak responses were recorded 4 days after the last inoculation at which time mice were used for fusions. Final boosts in preparation for fusion experiments were administered in the
tail vein to localize blastogenic activity within the spleen, an immunocompetent organ which by filtering the blood and coming in contact with circulating antigens is a convenient source of a large number of sensitized lymphoblasts readily accessible for fusion procedures. As shown, animals that were prepared for fusions in this manner allowed for the successful production of positive hybridomas in appreciable frequency (40%). On the other hand, mice that were prepared for fusions by serial intraperitoneal inoculations of purified virus gave rise to positive hybridomas at a much lower frequency (10%). This is perhaps because only low amounts of antigen are absorbed into the circulation from the peritoneal cavity resulting in an insufficient boost effect as opposed with antigen injected into the tail vein. This observation is also supported in the literature by other accounts of HSV hybridoma production. Zweig et al. (1979), following a serial intraperitoneal inoculation schedule produced hybridomas secreting antibodies against virus nucleocapsids at a frequency of 5.1% (positive/hybrids tested). These results were slightly improved by Howes et al. (1979); using intraperitoneal injections followed by a final boost in the tail vein they recovered positive hybridomas at a frequency of 8.8%. Our technique therefore allowed for the more efficient production of hybridomas; however, as our numbers represent hybrids screened only on infected cells, the final yield may be slightly overestimated.

The 124 positive monoclonal populations recovered represent 50% of the clones derived from 12 parental polyclones. This high frequency of positives could indicate that HSV-2 specific antibody-forming cells were prominent within at least some of the "polyclonal" cultures. As
several of these polyclones were passaged in tissue culture for approximately 2 months before being monocloned, it is possible that antibody-forming cells might have survived as the dominant clone. In this respect, monoclones could have been selected for by retaining the cells in culture for a long period of time.

This possibility becomes relevant when one analyzes the immunoprecipitation patterns of antibodies in the ascites fluids of the polyclonal cultures F13αA3 and F13αA5. Antibodies present in the ascites fluid of F13αA3 are of the IgG class and identify antigens present only in the nucleus of infected cells as detected by immunofluorescence. F13αA3 antibodies do not react with HSV-1 (KOS) infected cells and therefore appear to be specific for HSV-2. When viral proteins are immunoprecipitated by F13αA3 antibodies, several bands can be detected by SDS polyacrylamide gel electrophoresis. The major bands identified are of 76K, and 38K molecular weight with minor bands present at 83K and 46K.

Antibodies detected in the ascites fluid produced by hybridoma F13αA5 are also of the IgG class and appear to react with a viral β protein (as is the case for F13αA3). These antibodies differ from F13αA3 in that they identify a viral antigen in the cytoplasm of HSV-2 infected cells and also cross-react with an antigen expressed in the cytoplasm of KOS-infected cells. F13αA5 antibodies, when reacted with HSV-2 infected cells immunoprecipitate multiple bands. The pattern differs from that of F13αA3 in that the major species identified for F13αA5 antibodies migrates at 94K with a series of minor bands which also differs from those of F13αA3. It is unknown at present whether
these bands represent a polyclonal antibody response by these cultures, whether they are degradation products of a single precipitated species, or whether they are viral products non-specifically absorbed to, or trapped within, the staphylococcal protein-A-antibody-antigen complex during the radioimmunoprecipitation process. The identity of the bands and therefore the clonal nature of the cell lines from an immunological standpoint remains to be determined.

Recent data with a monoclonal of FL3xA5, designated FL3xA5-2-B7 (data not shown), provides an interesting observation. When FL3xA5-2-B7 monoclonal antibodies are used in the radioimmunoprecipitation process, viral determinants form the same pattern as FL3xA5 when analyzed by SDS polyacrylamide gel electrophoresis. The fact that a monoclonal produces a similar immunoprecipitation pattern as the parental "polyclone" strongly suggests that the parent is itself a cellular monoclonal.

There have been other reports of production of hybridomas secreting antibody to herpes simplex viruses since this work was begun. Koprowski (1978) mentioned the production of hybridomas to HSV-1 in a recent symposium but detailed results have not been presented. Howes et al. (1979) reported the production of 5 hybridoma cell lines secreting HSV-1 specific antibodies. Two of these cell lines produced IgM antibodies, while three made IgG antibodies. None of these antibodies were found to have neutralizing ability in a plaque-forming assay either individually or together. These authors could not identify the products precipitated by the antibodies and also reported the presence of multiple bands of unknown origin although results were neither presented or discussed. Zweig et al. (1979) most recently reported
the production of cell lines making antibodies against nucleocapsids of HSV-1 and HSV-2. These antibodies were able to identify type-homologous regions of the major nucleocapsid protein (p40) present in both HSV-1 and HSV-2. Furthermore, these antibodies were shown to cross-react with another minor nucleocapsid protein (p45) which was thought to possess similar antigenic determinants. The detection of similar antigenic determinants on different proteins of the virus could possibly account for the presence of multiple bands in the electrophoretic gel patterns of proteins precipitated by F13A3 and F13A5.

Our work has reported the production of 124 monoclonal hybridoma cell lines with antibody reactivity to antigens of HSV-2 derived from 12 independent polyclones and of a further 40 uncloned hybridoma cultures which could potentially yield large numbers of monoclonal cell lines. In terms of the number of viral proteins that can now be detected it is possible that several clones would be producing antibodies with specificities for the same antigenic determinant. So far, we have seen that F13A3 and F13A5 identify different viral proteins and that several monoclones of F13A5 react with the same determinants as the parental line (data discussed only for F13A5-2-B7).

Further efforts are needed in order to characterize the remaining cell lines in terms of the viral proteins identified by their antibodies. A repertoire of monoclonal antibodies reactive to all or most of the proteins of HSV would be an invaluable resource in furthering research in this field.
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