MONOCLONAL ANTIBODIES TO HERPES SIMPLEX TYPE 2 ANTIGENS

CHARACTERIZATION OF MONOCLONAL ANTIBODIES TO HERPES SIMPLEX VIRUS TYPE 2 ANTIGENS

by

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ABSTRACT

A HSV 2 immediate early antigen was prepared and used as an immunogen in an attempt to produce monoclonal antibodies to this set of proteins. The results of three screening assays, ELISA, immunodiffusion and radioimmunoprecipitation with cell extracts and Protein A-Sepharose beads indicated that the hybrid cell lines are nonsecretors of antiHSV 2 antibodies. Other monoclonal cell lines from Dr. Bacchetti's laboratory were characterized by radioimmunoprecipitation with Protein A-Sepharose beads and cell extracts.

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TABLE OF CONTENTS

TABLE OF CONTENTS (continued)

TABLE OF CONTENTS (continued)

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

HSV 1 or HSV I:	Herpes simplex virus type 1; currently renamed Human herpesvirus 1.
HSV 2 or HSV II:	Herpes simplex virus type 2; currently renamed Human herpesvirus 2.
NBCS:	Newborn Calf Serum.
PBS:	Phosphate Buffered Saline

INTRODUCTION

The social concern with the rising incidence of herpes simplex virus type 2 infection in the general population and with its association with cervical cancer (Smith et al., 1972; Strnad and Aurelian, 1976; Rawls et al., 1977; Gilman et al., 1980) has made it the focus of many recent scientific studies.

For the herpes carrier, the pain of recurring infections and the associated social stigma are both difficult to bear. After the initial infection, HSV 2 lies dormant in the ganglion cells of the nervous system (Clements and Hay, 1977; Sekizawa et al., 1980; Galloway et al., 1982). Galloway and coworkers (1982) suggest that the synthesis and function of immediate early proteins of HSV as well as some other viral proteins are required to initiate latency. How latency is broken and how the recurring infection is initiated are two areas yet to be addressed.

Clinically, HSV 2 poses many serious problems. For the newborn child and immunosuppressed individuals, the onset of herpes may have many serious and lasting effects ranging from physical disability to death.

Seroepidemiologic studies have demonstrated an association

between antibodies to HSV 2 antigens and cervical carcinomas (Rawls et al., 1977). Strnad and Aurelian (1976) have isolated such a virally-induced protein, AG-4, in cells from a cervical carcinoma. Other antigens such as VP 143, thymidine kinase and ICP 10 have also been detected in herpes-transformed cells. Although viral antigens are found in transformed cells, a consistent 'tumor antigen' is not evident (Galloway and McDougall, 1983). This inconsistency is also evident in the transforming sequence of the viral DNA. There is no apparent set of sequences which are retained or expressed in transformed cells or carcinoma cells suggesting that viral DNA is not necessary for maintenance of the transformed state (Galloway and McDougall, 1983). What factors involved in the oncogenic potential of the virus are as a result difficult to establish.

For the molecular virologist, the virus itself poses a very complex and interesting problem. The large DNA has the capacity to code for more than 50 different polypeptides. Some of the work that has resulted in the characterization of the HSV 2 proteins in terms of their function, properties and genomic location will be considered subsequently. The co-ordinate synthesis and the temporal regulation of these proteins provides an excellent model of regulatory controls at both the transcriptional and translational levels.

Understanding the basic mechanism involved in replication,

infection, transformation and latency can make a significant contribution to the solution of the clinical problems associated with HSV 2 infection.

(II) Classification of Herpesvirus

The many known herpesviruses belong to the family: Herpesviridae and fall into one of the three subfamilies: Alphaherpesvirinae, Betaherpesvirinae and Gammaherpesvirinae. Alphaherpesvirinae, such as herpes simplex type 1 and 2, pseudorabies, bovine mammillitis virus and equine abortion virus, share the following characteristics: (1) a short replicative cycle; (2) a diverse host range; (3) a strong cytopathic effect on infected cells; (4) the establishment of latency in animal infections; and (5) a DNA genome (approximately 87-105 kilodaltons molecular weight) which is composed of two covalently-linked components designated as L(long) and S(short) (Tooze, 1980). Each component contains a stretch of unique sequences that are bracketed by inverted repeats. The unique sequences of L(U_L) are surrounded by repeats designated as ab and a´b´. The unique sequences of S (U_S) are bounded by the inverted repeats designated ac and c´a´. DNA extracted from virions show that the components invert relative to each other to give four isometric structures found in equimolar quantities in the population.

(i) Morphology

The herpesvirion is composed of four structural components. The innermost component, the core, is surrounded by three concentric layers, the capsid, the tegument and the envelope.

Briefly, the electron opaque core consists of the viral DNA wrapped about protein fibres (Lemaster and Roizman, 1980). The capsid, separated from the core by a translucent region, reaches an approximate diameter of 85-100 nm. Approximately 162 capsomers are arranged in such a manner to form an icosadeltahedron (Roizman in Nayak, 1978). Electrophoretic studies of both HSV 1 and 2 nucleocapsids have shown that the nucleocapsid is composed of 5 major and 6-7 minor protein components (Zweig et al., 1979). Although the electrophoretic profiles for the two serotypes are similar, differences do exist. The major capsid protein of HSV 1, VP 5 (or NC-1), approximate molecular weight 154,000 daltons, has a faster electrophoretic mobility than the HSV 2 counterpart ISCP 9 (155,000-mwt) (Cohen et al., 1980). Zweig and coworkers (1979) have reported that nucleocapsid proteins p40 and p45 are larger in HSV 2 than in HSV 1.

Although they differ electrophoretically, the major capsid proteins of HSV 1 and 2, as well as p40 and p45 are immunologically related (Zweig et al., 1979; Cohen et al.,

1980). In the particular example of p40 and p45, Zweig and coworkers (1979) were able to establish with the use of monoclonal antibodies to p40, type-specific as well as cross-reactive antigenic determinants between the two serotypes.

The tegument, defined as the region between the capsid and the envelope, is a layer of amorphous and fibrous material. The amount of tegument present in the virion varies and seems to be strain dependent (Roizman in Nayak, 1978).

Lemaster and Roizman (1980) have isolated a protein kinase which appears to phosphorylate viral proteins only from the capsid-tegument structure. The viral substrates include VP 23, a capsid protein and several other structural proteins (VP 1-2, VP 4, VP 11-12, VP 13-14, VP 18.7, and VP 18.8) which constitute proteins of the tegument or the inner surface of the viral envelope (Lemaster and Roizman, 1980).

The outermost layer and the final structural component of the virion is the envelope (Tooze, 1980). The nucleocapsid which is assembled in the nucleus, associates with the inner lamella of the nuclear membrane modified by the viral glycoproteins and then buds into the perinuclear space (Spear and Roizman, 1972; Compton and Courtney, 1984). The enveloped virion then travels through the endoplasmic reticulum and the Golgi apparatus at which time the immature

glycoproteins are further processed to the mature form (Compton and Courtney, 1984).

Four glycoproteins have been identified on the viral envelope and the surface of infected cells. The four antigenically distinct glycoproteins of HSV 1 have been designated gB, gC, gD and gE. Similar proteins produced by HSV 2 have been given the corresponding names gB, gC (or gF), gD and gE (Zezulak and Spear, 1983). With the use of the monoclonal antibody H966, Roizman and coworkers (1984) have recently identified a glycoprotein gG that is specific only to the HSV 2 system. The glycoproteins play an essential role in viral attachment, penetration and maturation as well as in virus-induced cell fusion. The glycoproteins also provide targets for neutralizing antibodies and other host immune responses (Balachandran et al., 1982; Compton and Courtney, 1984).

(III) Expression of Viral Sequences in HSV-infected cells.

Expression of herpes viral sequences is regulated primarily at the transcriptional level. Hybridization of early and late transcripts from infected cells with viral DNA show that these messages map throughout the genome (Clements and Hay, 1977). Transcripts from cells infected in the presence of cycloheximide (Clements and Hay, 1977; Easton and Clements, 1980) or with ts mutants (Watson and Clements, 1978),

hybridize with restricted portions of the genome. These transcripts have been mapped extensively both in the HSV 1 and HSV 2 system to unique and repetitive sequences on both the L and S arms of the viral genome (Clements and Hay, 1977; Dixon and Schaffer, 1980; Easton and Clements, 1980). The presence of a functional immediate early product has been shown to be necessary for the transition to early and late RNA synthesis (Watson and Clements, 1980).

Easton and Clements (1980) suggest that in addition to the on/off controls as exhibited by the immediate early product transcription is also regulated by an "abundance control". Differences in the hybridization pattern between the transcripts and the different regions of the genome exist between the three regulatory phases as well as within a class (nuclear RNA versus cytoplasmic RNA).

At the translational level, infection with either HSV 1 or HSV 2, induces the synthesis of over 50 different viral-specific polypeptides. Specificity, in terms of virus versus host origin, is based upon three criteria. Firstly, to a first approximation, the rate of synthesis of virally-induced polypeptides will increase at least for a time during infection whereas synthesis of host-cell polypeptides will generally decrease. The pulse-labelling experiments of Powell and Courtney (1975) show a rapid decrease in host--cell synthesis during HSV 2 infection, as indicated by the

reduction in the total acid-precipitable counts shortly after infection. Three hours later, the overall rate of protein synthesis had reached preinfection levels (Powell and Courtney, 1975).

Reports in the literature also suggest that the HSV shutdown of host-cell polypeptide synthesis during infection is a two-step process. The first step is initiated by a virion-associated component. Read and Frenkel (1983) have isolated six HSV 1 mutants which are defective in host-cell shutdown. These vhs (virion-associated host-shut off) mutants fail to shut off host-cell synthesis in cells infected in the presence of actinomycin D. The vhs function is not essential for virus replication since the mutants could produce plaques at 34^{o}C (Read and Frenkel, 1983).

In the absence of actinomycin D, the second stage of host-cell synthesis shutdown took place despite the mutant vhs function (Read and Frenkel, 1983). The seond stage is mediated by an early or late gene product (Honess and Roizman, 1975; Read and Frenkel, 1983). The exact mechanism of inhibition and which products are responsible for host-cell shutdown is yet to be established. Arsenakis and Roizman (1984) have shown that one host-cell protein (130,000 molecular weight) loses its ability to bind double-stranded DNA with the onset of early synthesis. Further work is required to determine whether this loss of binding ability is a

result of a specific modification in the protein induced by the viral gene product. If this is the case, it would provide a potential model as to how the early or late gene products effect shutdown of host-cell synthesis.

Antisera produced as a consequence of infection of an appropriate host will generally react only against virus specified polypeptides. This property is used as the second criterion in determining the specificity (host versus viral origin) of a protein induced during infection. Honess and Watson (1974), using a general antiHSV I serum were able to precipitate 15 polypeptides in HSV-infected BHK-21 cells. Strnad and Aurelian (1976) distinguished type-common and type-specific polypeptides in infected cells by precipitating cell extracts with either an antiHSV 1 and or an antiHSV 2 serum.

Thirdly, the polypeptides can be shown to be genetically encoded on the viral genome. Much work has gone into the physical mapping of HSV-induced proteins, as a means to further understand the expression and regulation of the viral genome. Marsden et al. (1978) have mapped several polypeptides by comparing polypeptide profiles of 29 HSV 1/ HSV 2 intertypic recombinants to the parental profiles. With the aid of restriction enzymes, the recombinant DNA was analyzed to determine the position of the crossover points. As both parental genomes induce a unique polypeptide

the recombinant polypeptides as recognized by an altered electrophoretic mobility can hence be physically mapped to a position on the genome based on the location of the crossover point. Preston et al., (1978) have further extended the use of intertypic recombinants to physically map the location of the immediate early polypeptides.

The temporal regulation of viral polypeptide synthesis is well documented in the literature. Studies by Honess and Roizman (1974) of HSV 1 infections show that the HSV 1 reproductive cycle can be divided into three temporal groups: immediate early, early and late. Analysis of infected-cell extracts, pulse-labelled at various times during the reproductive cycle, show that the polypeptides differ in time and in their overall patterns of synthesis. The immediate early polypeptides are the first to appear and their synthesis rate peaks at 2-4 hours postinfection. The early polypeptides reach maximal synthesis at 5-7 hours post--infection whereas the late polypeptides, consisting of the major structural proteins, reach maximal synthesis at 12 hours postinfection.

When cycloheximide is removed from treated infected cells, the immediate early polypeptides are the first to appear suggesting that prior protein synthesis is not required to initiate immediate early synthesis (Honess and Roizman, 1974).

When cells are infected in the presence of amino acid analogues such as L-canavanine, the immediate early polypeptides continue to be made at peak rates. The presence of early or late polypeptides are not detected in these cells. Based on this observation, Honess and Roizman (1975) suggest that functional immediate early polypeptides are necessary for the transition to the "later" polypeptide synthesis.

Studies with temperature-sensitive mutants of HSV 1 have provided further evidence for the importance of immediate early polypeptides in the transition to late synthesis (Preston, 1981; Dixon and Schaffer, 1980;). The temperature-shift experiments by Dixon and Schaffer (1980) using the HSV 1 mutant, tsB21u, have shown that one immediate early polypeptide, VP 175 (or ICP 4) is essential for the transition from immediate early to early and late synthesis. In addition to this observation, ICP 4 was noted to be a necessary requirement for maintaining early and late synthesis throughout infection and also exhibited an autoregulatory function which acts to inhibit immediate early synthesis (Dixon and Schaffer, 1980).

Honess and Roizman (1975) had suggested earlier that functional early and late polypeptides are required to shut--down immediate early synthesis. This statement was based on the observation that when infected cells were treated with

amino acid analogues at 3 hours post-infection, the three groups, immediate early, early and late were synthesized simultaneously and constantly throughout the experiment.

The late polypeptides, like the early polypeptides, require the presence of an infected cell polypeptide before they are synthesized. When cycloheximide is added to infected cells before 2 hours postinfection, late polypeptides are not immediately present after removing cycloheximide. However, delaying the time when the cells are treated with cycloheximide, results in an increase in the initial rate of late synthesis. When cycloheximide is added from 5-7 hours postinfection, the synthesis rates are comparable to those in untreated infected control cells. This time period coincides with early synthesis which would suggest that early polypeptides are involved in the initiation of late synthesis (Honess and Roizman, 1974).

Likewise, early polypeptides synthesis is also regulated by late polypeptides. In cells treated with cycloheximide between 3 to 5 hours until 7 hours, the initial rates of early synthesis exceeds the rates measured in untreated infected controls. The decline seen in the control cells is due to the presence of late polypeptides which are not present in the treated cells (Honess and Roizman, 1974).

The salient features of the cascade regulation can thus

be summarized as follows: (1) In infected, untreated cells, the immediate early polypeptides are the first to appear and reach maximal synthesis at 2 to 4 hours postinfection. In cycloheximide treated cells, they are the first to appear after removing the drug and hence do not require prior protein synthesis. (2) Early polypeptides reach maximal synthesis at 5-7 hours postinfection. Functional immediate early polypeptides, those not made in the presence of amino acid analogues, are required to initiate early synthesis. Likewise, functional early polypeptides, are necessary for the shut-down of immediate early synthesis. Early polypeptides made in the presence of amino acid analogues are ineffective in shutting down immediate early synthesis. (3) Lastly, late polypeptides reach maximal synthesis at 12 hours postinfection. The presence of functional early polypeptides is required to initiate late synthesis. The onset of late synthesis coincides with the decline of early synthesis in infected cells suggesting that late polypeptides turn-off early synthesis (Honess and Roizman, 1974;1975).

Using pulse-labelling experiments throughout the virus growth cycle, Powell and Courtney (1975) were able to discern the three groups of polypeptides in HSV 2-infected cells. To further differentiate the HSV 2 polypeptides, they performed several experiments treating infected cells with

cycloheximide analogous to those described by Honess and Roizman (1974). Although the immediate early polypeptides are easily distinguished, the early and the late polypeptides are difficult to discern after removing the drug cycloheximide from the treated cells. Lengthy treatments with cycloheximide early in infection results in an overall decrease in protein synthesis of both host-cell proteins and HSV 2 proteins as compared to control cells once the drug is removed. Powell and Courtney (1975) suggest that a labile host-factor may be required to initiate herpesvirus protein synthesis.

(IV) Immediate Early Proteins

A vast amount of research has been directed to investigating the properties of the immediate early proteins in an attempt to understand the regulatory controls of polypeptide synthesis. The early studies of Powell and Courtney (1975) suggested that many of the HSV 2 polypeptides undergo post-translational modifications as detected by changes in electrophoretic mobility following a chase period in pulse-chase experiments. Pereira et al., (1977) found that four immediate early polypeptides Intracellular protein (ICP) 4, 0, 22, and 27 underwent phosphorylation as part of the modification process. ICP 4, a functional immediate early protein, is found in three phosphorylated forms: ICP 4a (188 kd), ICP 4b

(198kd) and ICP 4c (207kd). It was also noted that all the immediate early proteins partitioned with the nuclear fraction which is consistent with the theory that they function in the nucleus as a possible regulatory control (Pereira et al., 1977).

Wilcox et al. (1980) have shown that phosphate cycles on and off proteins during infection. ICP 4a and ICP 4c rapidly cycle between the phosphorylated and nonphosphorylated forms as detected by changes in both electrophoretic mobility and intensity during chase experiments. The phosphorylation of ICP 4b however, is a rather stable one for no changes in either parameter could be detected in very long chase experiments (Wilcox et al. , 1980).

How phosphorylation fits into the overall control mechanism is yet to be established. Wilcox et al. (1980) have shown that phosphorylation of immediate early proteins, in particular ICP 4, alters their DNA-binding affinity. Other work done on ICP 4, has suggested that ICP 4 is essential for the switch to early synthesis as reflected by the ineffectiveness of mutants bearing a temperature sensitive mutation in this protein to carry out this transition at the nonpermissive temperature (Dixon and Schaffer, 1980; Preston, 1981). In light of these two observationns, Freeman and Powell (1982) examined the apparent DNA-binding properties of ICP 4 in greater detail. The results suggested that ICP 4

has no inherent DNA-binding ability but binds via a secondary component found in uninfected cells. Based on this observation, Freeman and Powell (1982) suggest that the regulation of synthesis is at a transcriptional level with ICP 4 interacting with the RNA polymerase.

(V) Monoclonal Antibodies

When challenged with a foreign substance, the body's defense system reacts in one of two ways. The first, the cellular immune response as exemplified by tumor and graft rejection, involves the co-operative effort of a network of cells: T-lymphocytes, B-lymphocytes and macrophages. The second response, the humoral immune response, results in the secretion of circulating antibodies produced by sensitized plasma cells.

The humoral response provides the body with a battery of different antibodies ranging in affinity, specificity and avidity, for each plasma cell produces a unique antibody. The polyclonal response is then a clonal expansion of each of these individual plasma cells.

The hybridoma technique developed by Kohler and Milstein (1975; 1976), has provided a means for selectively isolating a clone secreting antibodies with a given specificity The fusion of lymphocytes from sensitized mice with

mouse myeloma cells results in hybrid cells or "hybridomas" which have the properties of immortality in culture and a unique antibody production. With the use of this technique, several hybridomas can be readily screened in search for a clone with a desired specificity.

The use of the hybridoma technology is widespread in the scientific and medical fields. Monoclonal antibodies to a variety of antigens, such as T-cell markers, human IgG, drugs, hormones and enzymes are commercially available for therapeutic or diagnostic use in the clinical setting. Monoclonal antibodies raised to viral antigens have been used to isolate, identify and characterize viral proteins in infected cells (Zweig et al. , 1979; Flamand et al. , 1980; Killington et al. ,1981; Parsons et al. , 1983; Roizman et al. , 1984).

Secher and Burke (1980) report the use of a monoclonal antibody to human leukocyte interferon as an immunoadsorbent to purify interferon for therapeutic uses. Koprowski et al. (1978) have raised antibodies against a human melanoma in attempt of identifying the tumor antigens.

Purpose of this Study

This project involved an attempt to raise monoclonal antibodies to the immediate early proteins of herpes simplex virus type 2. Since the immediate early proteins play an essential role in the regulation of viral polypeptide synthesis, the inherent specificity of monoclonal antibodies would prove to be an invaluable tool in future characterization of these proteins. In addition to attempting to raise these monoclonal antibodies, several existing antiHSV 2 hybridomas were examined and then characterized by immunoprecipitation studies.

MATERIALS AND METHODS

(I) Cell Growth

Mouse L cells were grown in either suspension culture or monolayers in JOKLIK'S modified minimum essential medium with Earle's salts, 75 units/ml penicillin and 50 mcg/ml streptomycin (GIBCO, Grand Island Biological Co.). Suspension cultures were supplemented with 5% newborn calf serum (NBCS) (GIBCO) whereas the monolayer cultures were supplemented with 10% NBCS. The media for both cultures was supplemented with 0.75% sodium bicarbonate. Both cultures were maintained at 37°C.

African Green Monkey kidney cells (Vero) were grown as monolayer cultures in Minimum Essential Medium, MEM F-13, (GIBCO) supplemented with 10% NBCS, 100 units/ml penicillin, 100 mcg/ml streptomycin (GIBCO) and 0.075% sodium bicarbonate. The monolayer cultures were grown at 37°C in glass Brockway bottles.

(II) Virus Growth

Herpes simplex virus type 2 (HSV 2) strain 333 and HSV 1 strain KOS were obtained from Dr. S. Bacchetti, McMaster University. Vesicular stomatitis virus (VSV), the Indiana serotype was used as the control and marker material in several experiments.

(i) Herpes Virus Stock Preparation

Confluent monlayers of VERO cells were infected with a low multiplicity of infection (0.1-0.5 pfu/cell) of either herpes strain. After one hour adsorption at 37°C, the cultures were replenished with Medium 199 (GIBCO), (5% NBCS, 0.075 % sodium bicarbonate). The infected cultures were maintained at either 34°C or 37°C for 2-3 days or until 75-100% of the cells had rounded up.

The cells were harvested and pelleted by low speed centrifugation (1000 rpm), for 5 minutes in an IEC centrifuge. The cell pellet was then resuspended in a small volume of Med-199 (2-5 X 10^7 cells/ml) and sonicated for 30 seconds. Cellular debris was pelleted by a low speed centrifugation (2000 rpm for 10 minutes) in the IEC centrifuge. The resulting supernatant was aliquoted into 1 ml portions and stored frozen at -60°C until use.

(ii) Virus Titration-Plaque Assay

The 24 well Linbro tissue culture plates (Flow Laboratories) were seeded with VERO cells at 10^4 cells/well so as to reach confluency the following day. At this time, a 0.2 ml aliquot of the virus dilution was added to the monolayer. After an adsorption period of 1 hour at 37°C, approximately 0.8 ml of a methylcellulose overlay was added to each well. The infected cells were then incubated at either 34°C or

37°C for 2-3 days or until plaques were visible with the naked eye. At this time, cold PBS was added to the wells to rinse out the methylcellulose overlay. Once the overlay was carefully removed, a small amount of Carnoy's reagent (3 parts methanol to 1 part glacial acetic acid) was added to fix the cells. The plates were then rinsed with water, dried and stained with methylene blue (0.05%) to detect the plaques more readily.

(III) Radiolabelling of Infected Cell Polypeptides

Vero cells were seeded into the 32 oz. Brockway bottles and grown at 37°C. Upon reaching confluency, the cells were infected with HSV 2 at a moi of 10-20 pfu/cell. The inoculum was removed after a one hour adsorption period at 37°C. The appropriate labelling medium or maintenance medium was then added.

To label with L-(^{35}S)-methionine (1060 Ci/mmol) (Amersham), the cells were incubated at 37°C in a methionine-reduced (to 1/20 normal concentration) MEM F-15 medium supplemented with 2% NBCS and containing 20 uCi/ml of ^{35}S-methionine for the indicated labelling period.

To label with ^{32}P-orthophosphate, cells were incubated for several hours in a phosphate-minus medium supplemented with 2% NBCS and containing 200 uCi/ml of ^{32}P-orthophosphate

(carrier free, Amersham).

At the end of the labelling period, the labelling medium was removed. The infected cells were washed once with cold PBS and then prepared either as extracts for polyacrylamide gel electrophoresis or as an antigen source for immunoprecipitation experiments.

(IV) Polyacrylamide Gel Electrophoresis

(i) Preparation of whole cell extracts

Infected cells were scraped into a volume of PBS, and pelleted by centrifugation at 1000 rpm for 5 minutes in the IEC centrifuge. The cell pellet was then resuspended into a small volume of sample buffer (50 mM Tris-HCl pH 7.0, 5 % SDS, 5% 2-mercaptoethanol, 10% glycerol, 6M Urea and 0.1% bromophenol blue) at a concentration of 1 ml per 32 oz. Brockway. The extract was syringed 3X and then boiled for 2 minutes in a boiling water bath. A sample of the extract was removed to determine the TCA precipitable radioactivity.

(ii) SDS-PAGE

The infected cell extract preparations were analyzed on slab gels according to the method of Laemmli (Laemmli, 1970). The gels used in most of the experiments were 9% acrylamide in 0.038 M Tris, 0.1% SDS, 0.1% ammonium persulfate, and 0.03% N,N,N´,N´-tetramethylethylenediamine (TEMED, Sigma).

This was overlaid with a staking gel composed of 6.7% acrylamide, 0.006M Tris, 0.1% ammonium persulfate, 0.1% SDS and 0.5% TEMED. The amount of DATD crosslinker used in the acrylamide was such that DATD was equivalent to 1/40 wt/wt acrylamide (Marsden et al., 1978).

The gels were run at a constant voltage of 200V until the bromophenol blue marker reached the bottom. The gels were then fixed or impregnated with scintillation fluors before drying. To fix the gel, gels were placed in a methanol:water:acetic acid mixture (100:100:14) for at least 1/2 hour. The fixed gels were impregnated with the scintillation fluor PPO (2,5 Diphenyloazole; New England Nuclear) as follows: fixed gels were dehydrated by two washes in dimethyl sulfoxide (DMSO) for an hour each wash. They were then soaked in a 22.2% wt/v PPO/DMSO for two hours. Before drying, the gels were rehydrated by several washes in distilled water.

After fixing or impregnation with fluors, the gels were dried onto Whatman filter paper by low-pressure desication at 100^{o}C. The dried gels were then autoradiographed on Kodak XRP-1 or XAR-5 film.

(V) Hybridoma Production

(i) Virus Antigen Preparation

L cells were infected with HSV 2 at a moi of 200 pfu/cell in the presence of cycloheximide (100 ug/ml) and 5% NBCS. After 5 hours, the cycloheximide block was removed with five washes of 10 ml of PBS and the cultures were then replenished with medium containing Actinomycin D (2 ug/ml) for a further 4 hours (Matis and Rajcani, 1980).

Cells were then harvested, freeze-thawed twice and then scraped into a small volume of buffer (0.02 M Tris-HCl pH 7.5, 0.05% BSA, 2 mM 2-mercaptoethanol). The cell pellet was then sonicated for 30 seconds. To the sonicated material was added a sufficient amount of a high salt buffer to obtain a final concentration of 1.5 M NaCl and 1.5 mM EDTA. This mixture was allowed to sit for 40 minutes at 4^{o}C before centrifugation at 30,000 X g at 4^{o}C. The supernatant was then dialyzed overnight at 4^{o}C against a 0.02 M Tris-HCl pH 7.2 buffer containing 2 mM 2-mercaptoethanol, 2 mM EDTA, 50 mM NaCl and 10% v/v glycerol. The supernatant was then centrifuged at 100,000 X g for 60 minutes at 4^{o}C. The resulting supernatant was then frozen and stored at -60^{o}C until used in the immunization protocol.

(ii)Mice

Inbred Balb/c white mice were obtained from Jackson Laboratories (Bar Harbour, Maine). The mice were 7 weeks old when first inoculated with the antigen.

(iii) Fusion of cells

The plasmacytoma cell line Sp2/0 Ag14 was kindly donated by Dr. Bacchetti. The procedure used for the cell fusion was essentially the same as that described by Killington and coworkers (1981).

Spleens from immunized mice were aseptically removed, finely chopped and passed through a sterile steel screen (60 mesh). The cells were collected in RPMI (GIBCO) plus 20% NBCS and the larger clumps were allowed to settle. The supernatant was then poured into a 15 ml conical tube and the cells were carefully underlaid with 2 ml of NBCS. The cells were then centrifuged at 800g for 10 minutes. Following centrifugation, the pellet was resuspened in 5 ml of 0.1M ammonium chloride (NH_4Cl) and kept on ice for 10 minutes to lyse the erythrocytes. To this mixture was added 10 ml of cold RPMI plus 20% NBCS and the cells were pelleted once again. To the spleen cells, Sp2/0 cells were added so that there were 10^7 Sp2/0 cells to 10^8 lymphocytes. The mixture was then washed twice with serum-free RPMI in the Corning 50 ml centrifuge tubes. The cell pellet was then mixed with 0.2 ml

of a 30% polyethylene glycol solution (PEG-1000 in serum--free RPMI) for 1 minute at which time the cells were pelleted once again at 800g for 8 minutes (or 1000 rpm for 6 minutes). The PEG was then diluted with 5 ml of RPMI plus 20% NBCS. The final pellet was then resuspended into approximately 30 ml of HY-HAT medium (Dulbecco's modified minimal essential medium supplemented with 15% FCS, 20 units/ml insulin, 100mM oxalacetate, 45mM sodium pyruvate, 0.1mM hypoxanthine, 1uM aminopterin, and 40uM thymidine) and then dispersed into 24 well Linbro tissue culture plates (1 ml/well). Surviving cells were then expanded into 25 cm^2 Corning tissue culture flasks. Upon reaching a certain density, cells and samples of culture fluid were frozen for later use.

(VI) Radioimmunoprecipitation with Protein A-Sepharose Beads

(i) Preparation of labelled antigen

Monolayers of VERO cells were mock infected or infected with HSV 2 at a moi of 10-20 pfu/cell and incubated in either a methionine-reduced medium containing 20 uCi/ml of ^{35}S-methionine or in a phosphate-free medium containing 200 uCi/ml of ^{32}P-orthophosphoric acid for the indicated time periods. After the labelling period, cells were harvested and washed 3X with cold PBS. The cells were solubilized with RIPA buffer (0.5M Tris-HCl pH 7.2, 0.15M NaCl, 0.1% SDS,

1% sodium deoxycholate, 1% Triton X-100, 0.1M PMSF, 10 units/ml Aproteinin) by the occasional shake and maintenance on ice for several minutes. The lysate was then sonicated for 1 minute and centrifuged for 30 minutes at 14K rpm at 5°C. The supernatant was collected as the source of antigen.

(ii) Radioimmunoprecipitation

Approximately 500 ul of labelled antigen was added to 10 ul of antibody (ascites fluid or tissue culture fluid concentrated 10 fold by lyophilization and reconstitution with a small volume of buffer) and 100 ul of Protein A-Sepharose beads (Pharmacia) (15 mg beads in RIPA) in a 1.5 ml Eppendorf microcentrifuge tube. The beads were constantly mixed on a spinning disc for a minimum of 2 hours at 4°C. The beads were then collected by centrifugation and washed (by pelleting and resuspension) three times with RIPA buffer. To solubilize the bound protein, 50ul of denaturation buffer (50 mM Tris-HCl pH 7.0, 2% SDS, 5% 2-mercaptoethanol, 10% glycerol and 0.1% bromophenol blue) was added to each tube. The tubes were boiled for 3 minutes and the beads were pelleted by centrifugation. The supernatant was collected and analyzed on 9% acrylamide-DATD gels by electrophoresis for 3-4 hours at 200V.

(iii) Preparation of a HSV soluble protein extract for radioimmunoprecipitations.

Purifoy and Powell (1976) suggest that many of the HSV-2 radiolabelled proteins of infected cells are found in an insoluble form. A high salt extraction protocol has hence been developed which solubilizes these proteins.

Vero cells were infected with HSV 2 at a moi of 10 pfu/cell. After one hour adsorption period, cells were incubated in a methionine-reduced medium containing 20 uCi/ml of ^{35}S-methionine. After 3 hours of labelling, cells were collected, washed with PBS and resuspended in buffer containing 20 mM Tris-HCl pH 7.5, 2 mM 2-mercaptoethanol and 500 ug/ml BSA, to a concentration of 10^7 cells/ml.

After briefly sonicating the cell suspension, an equal volume of a high salt buffer was added to give a final concentration of 1.7 M NaCl and 5 mM EDTA. The mixture was then centrifuged at 30,000 X g for 20 minutes to remove the DNA and protein precipitates.

The extract was then dialyzed overnight against a buffer containing 20 mM Tris-HCl pH 7.5, 50 mM NaCl, 1 mM EDTA, 2 mM 2-mercaptoethanol and 10% glycerol. The light coloured precipitate, containing all the proteins insoluble in a low molarity salt solution, was removed by centrifugation at 100,000 X g at 4^{o}C for one hour. The high spin

supernatant was then used a the antigen for the immunoprecipitation experiments with ascites as the source of the antiHSV antibody.

(VII) Enzyme-linked immunoadsorbent assay (ELISA)

Vero cells, both mock-infected and infected (at a moi of 20 pfu/cell) were harvested after 4 or 18 hours post-infection, sonicated, cleared of cellular debris (1,000 rpm for 5 minutes), and then resuspended into a small volume of a 0.05M sodium carbonate-bicarbonate buffer pH 9.6 (8 ml of 0.2M anhydrous Na_2CO_3 and 17 ml of 0.2M $NaHCO_3$). Fifty microlitres of an optimal dilution of antigen was added to each well of an 96 well Linbro plate and incubated for a minimum of 3 hours at 37°C. The plates were then washed three times using a 0.08% saline solution containing 0.05% Tween 20. Approximately 50 ul of ascites or tissue culture fluid was added to each well and incubated for 30 minutes at 37°C, followed by a wash with the saline-Tween 20 solution. Optimal dilutions of the rabbit antimouse IgG (Cappell Laboratories) (50 ul) and the horse-radish peroxidase conjugated goat antirabbit IgG (Cappell Lab) (50 ul) were incubated in subsequent steps for 30 minutes at 37°C. Following the HRP-goat antirabbit IgG incubation, the plates were washed with saline and a substrate solution (50 ul) was added to determine the amount of peroxidase retained in the wells. The substrate solution consisted of 9 ml of 5-Amino salycylic

acid pH 6 (stock is 0.08% 5-AS, pH with NaOH) and 1 ml of 0.05% H_2O_2. After an incubation time of one hour at room temperature, the reaction was stopped with 100 ul of 1 N NaOH. The wells were diluted with 1 ml of H_2O, and the optical densities were measured at 450 nm. The reactivity was determined by comparing the mock-infected controls to the infected controls.

(VIII) Immunodiffusion

To determine whether antibodies were present in the tissue culture fluids and possibly characterize them as either an IgG or IgM, immunodiffusion plates were set up and the majority of the fluids were tested.

A 0.8% agarose in PBS solution was prepared by dissolving the agarose in boiling PBS. The hot agarose solution was then poured into the bottoms of several Petri dish (5 ml for 60 cm^2 dish and 3 ml for 30 mm^2 dish) and allowed to solidify at room temperature for several minutes. Once cooled, the lids were replaced to prevent drying and were stored at $4^{\circ}C$ until use. For immunodiffusion tests, small holes (diameter=3mm) were punched into the agarose with a punch (courtesy of Dr. McCandless, McMaster University). Six equally spaced and equally sized outer wells were punched around a central well. Into the central well was placed 10 ul of either rabbit antimouse IgG or IgM (Cappel

and or Zymed Laboratories). To the outer wells was added 10 ul of tissue culture fluid of the monoclones to be tested. One well, was kept as a control to which was added 5 ul of normal mouse serum. The plates were kept at 4°C for 1-2 days or until precipitin lines could be detected by the naked eye.

The plates were also stained in some instances with Coomassie Blue. To each plate was added 5 ml of a 0.1% Coomassie blue in a methanol/water/acetic acid (100:100:14) solution for 30 minutes. To destain, the plates were washed several times with the methanol/water/acetic acid solution. The stained plates were then stored at 4°C for future reference.

RESULTS

(I) Polypeptides synthesized during lytic infections

As described in the Introduction, infection with Herpes simplex virus type 2 results in the rapid shut-off of host cell proteins and the appearance of viral-related polypeptides. To define the kinetics of protein synthesis under our conditions, monolayers of Vero cells were infected with HSV 2 at a moi of 20 PFU/cell and then labelled with ^{35}S-methionine at four times during the lytic cycle. The proteins were then analyzed one hour after adding the labelling medium. For comparison, an uninfected cell culture was also labelled with ^{35}S-methionine for one hour.

As seen in Fig. 1, the assemblage of labelled proteins in the infected cultures is quite distinct from the mock--infected controls. In particular, comparing the mock--infected with the earliest post-infection labelling period (4-5 hours), it is evident that a number of significant host proteins including the dominant actin band are greatly reduced in label after infection.

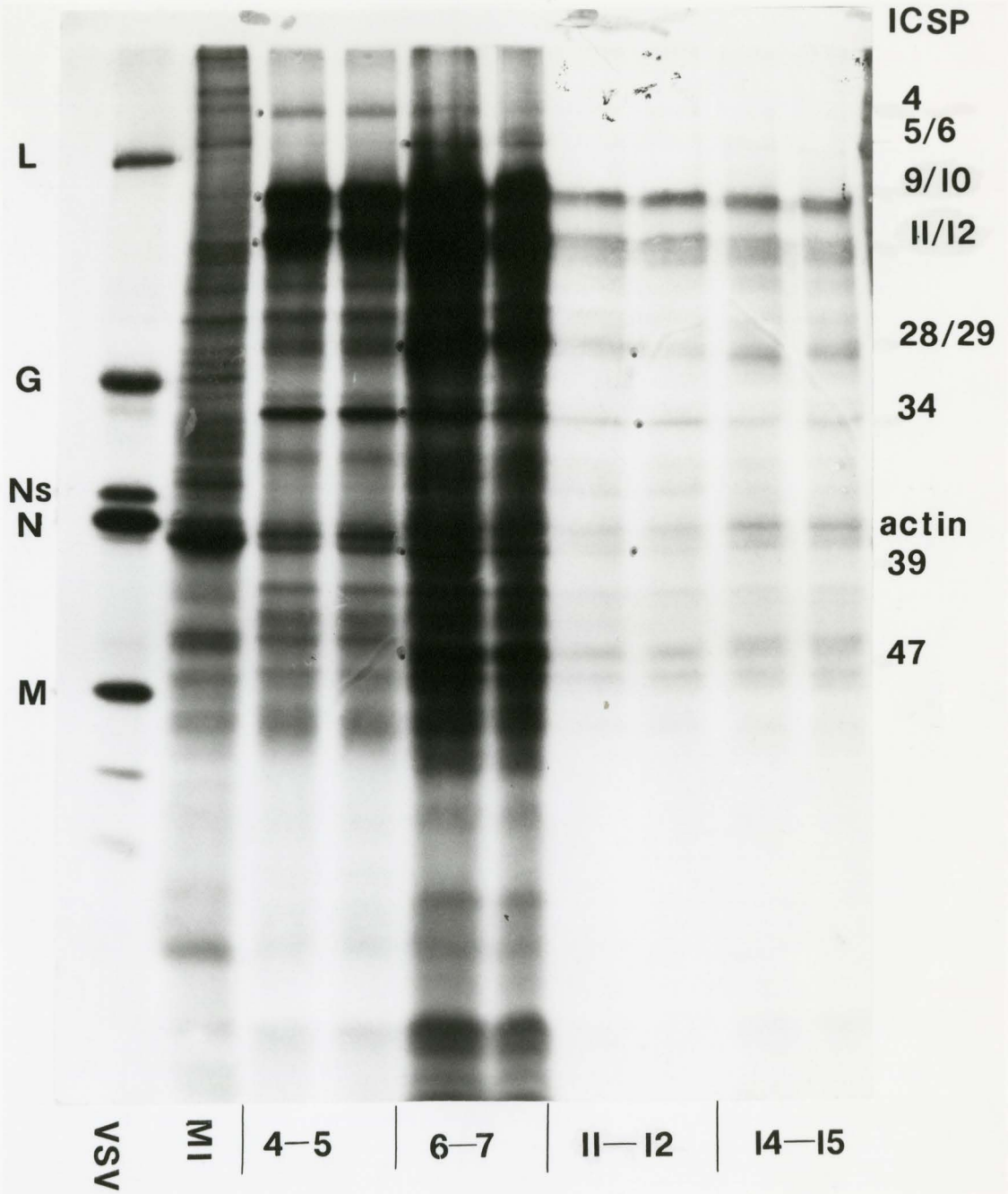
Conversely, there are labelled bands in the 4-5 hour infected culture which do not correspond to any bands in the mock-infected controls. From analogy with reports of previous workers these proteins are identified as "virally-specific"

and are tentatively identified on the basis of electrophoretic mobility using the numerical designation of Powell and Courtney (1975). For example, the protein labelled ICSP 9/10 (mwt=150,000), present in all infected cultures but not in the mock-infected control, is assumed to be a component of the slow migrating doublet identified by Powell and Courtney (1975).

The work done by Powell and Courtney (1975) and Honess and Roizman (1975) showed that the HSV infection is temporally regulated. Comparison of the individual polypeptide profiles represented in Fig. 1, amongst each time point presented, show that certain polypeptides are made at specified times during infection. The polypeptides marked in Fig. 1 are identified according to electrophoretic mobility (molecular weight) and the time and pattern of synthesis. This information was then correlated to the published data found in Powell and Courtney (1975), Strnad and Aurelian (1976) and Bookout and Levy (1980). For example the band marked ISCP 4, shows maximal synthesis, as indicated by the intensity of the labelled band, from 4 to 5 hours postinfection. This band however is not present in the later timepoints of this infection. ICP 4 normally peaks in synthesis at 2-3 hours postinfection however these earlier timepoints were not examined in the experiment described in Fig. 1. Based on the molecular weight and the pattern of

Fig. 1: Polypeptide synthesis induced by HSV 2 infection of VERO cell.

VERO monolayers grown in 30 mm^2 tissue culture plates (10^6 cells) were mock-infected or HSV 2-infected (moi=20 pfu/cell). After one hour adsorption period, the inoculum was removed, the cells were washed once with fresh medium and finally replenished with maintenance medium. Cells were labelled for one hour intervals with ^{35}S-methionine (100 uCi/ml) diluted in a methionine-reduced medium. At the end of the labelling period, the medium was removed. Cells were washed once with cold PBS and then taken up in sample buffer. Duplicates of each sample (50ul) was then analyzed by SDS-PAGE on a 10% acrylamide-0.4% bis-acrylamide gel and run for 4 hours at a constant voltage of 200V. The gel was prepared for autoradiography on Kodak XRP-1 film after impregnation with sodium salicylate.


ICSP
4
5/6
9/10
11/12
28/29
34
actin
39
47
L
G
Ns
N
M
VSV
MI
4—5
6—7
11—12
14—15

synthesis, this polypeptide is assumed to be ICSP 4 as described by Powell and Courtney (1975).

The band marked ICSP 47, approximately 33 kilodaltons molecular weight, is an early polypeptide which reaches peak synthesis from 6 to 7 hours post-infection and then continues to be synthesized throughout infection but at a reduced rate. The polypeptide currently designated ICP 47 in the HSV 2 system is an immediate early protein (personal communication by Dr. Bacchetti). ICSP 39 (47,000 molecular weight) is a second example of a polypeptide reaching maximal synthesis from 6-7 hours post-infection and then continued synthesis at a reduced rate for the remainder of the infection cycle.

The band marked ICSP 34 (58 kilodaltons molecular weight) is strongly labelled during the earlier part of infection, 4 to 7 hours post-infection, and continues to be synthesized, again at a reduced rate during the latter stages of infection.

Fig. 1 also illustrates an uneven labelling between time points despite the fact that equal volumes (equal cell numbers) were added to each well. There are two possible explanations for this observation. Cells infected at high multiplicities of infection tend to round up and detach from the monolayer during the later stages of the infection cycle.

As a result, it is possible that cells were lost when the maintenance medium was replaced with labelling medium.

Secondly, the proteins have been classified into three kinetic groups based on their maximal rates of synthesis. The majority of the viral proteins appear to reach maximal synthesis between 4 to 8 hours post-infection (Powell and Courtney, 1975; Strnad and Aurelian, 1976). In concurrence with the temporal regulation of viral polypeptide synthesis, HSV 2 infection induces an irreversible shutdown in host--cell polypeptide synthesis. The uneven labelling seen in Fig. 1 is then a combination of the viral polypeptides reaching maximal synthesis early in infection and a reduced host-cell polypeptide synthesis throughout infection.

(II) Protein synthesis following a cycloheximide block in the early stages of HSV II infection.

In earlier studies by Honess and Roizman (1974; 1975) on the HSV I infection cycle, treatment of infected cells with the drug cycloheximide was used to delineate the three groups of polypeptides, immediate early, early and late which were involved in the lytic cycle.

As stated in the introduction the immediate early polypeptides are defined by the following criteria: (i) those proteins synthesized upon removal of cycloheximide present at the start of infection; (ii) no prior protein

synthesis is required to initiate immediate early polypeptide synthesis; and (iii) the proteins are normally found 2-4 hours postinfection in untreated cells.

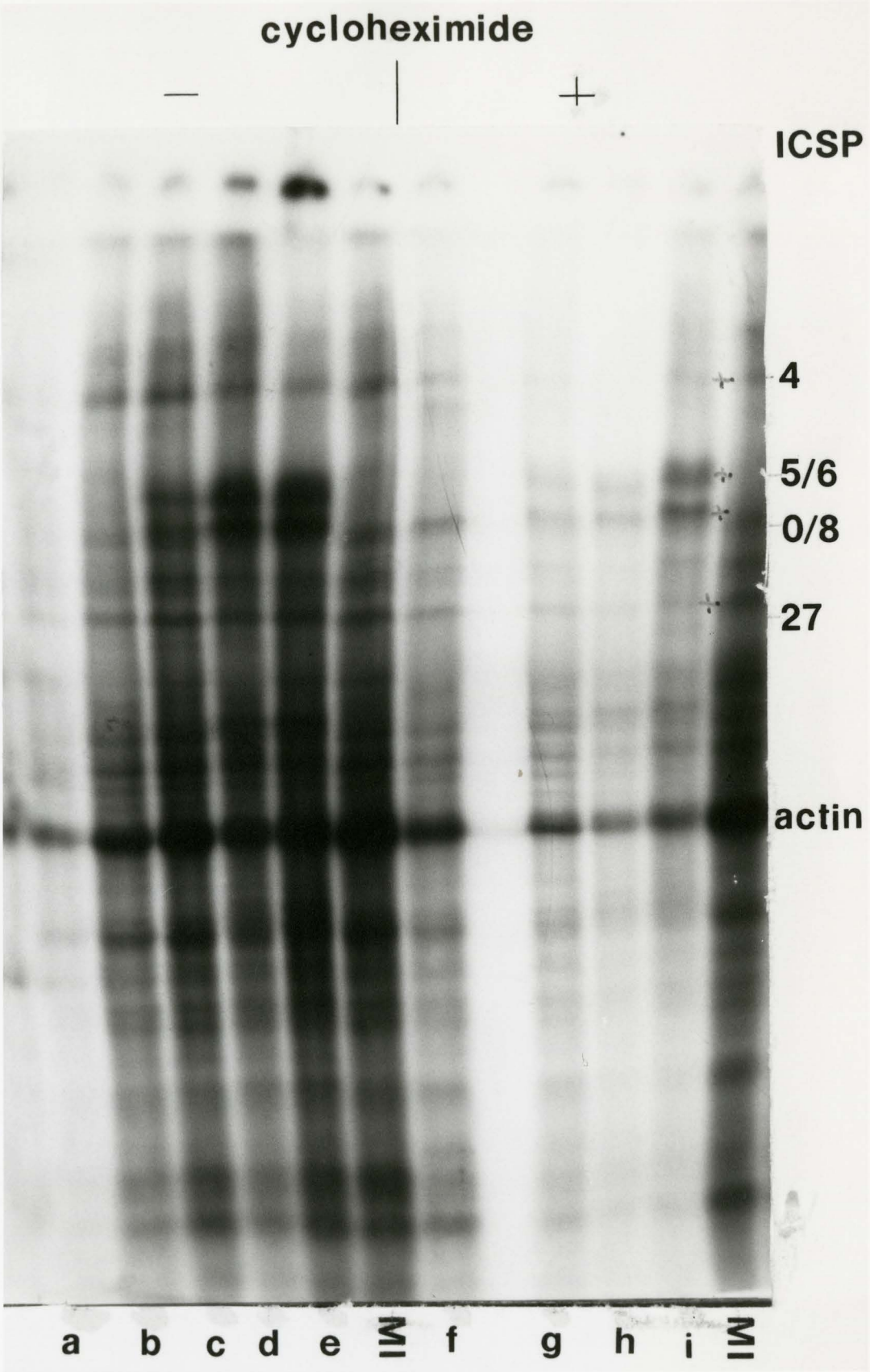
To determine the nature and extent of immediate early protein synthesis in our cell systems, the effect of cycloheximide release was investigated. Briefly, VERO monolayers were mock-infected or infected (moi of 20 pfu/cell) in the presence of cycloheximide (100 ug/ml). After 5 hours, the medium was removed, cells were washed three times with fresh medium and then replenished with either maintenance medium or labelling medium. At designated times thereafter, the cells were labelled for one hour with ^{35}S-methionine (20 uCi/ml) and then harvested and prepared for SDS-PAGE analysis.

As can be seen in Fig. 2, the addition of cycloheximide has greatly reduced the overall level of protein synthesis in the infected cell even as long as three hours after removing the inhibitor. The background level of general cellular proteins appears therefore to have been inhibited and unable to recover. At least two labelled virus-specific proteins can be identified in the proteins synthesized after cycloheximide removal. These proteins, ICSP 5/6 and ICSP 0 (Powell and Courtney, 1975; Pereira et al., 1977), can also be identified in the corresponding non-cycloheximide treated cultures. Currently, ICSP 5/6 has been designated as HSV 2

Fig. 2: Effect of cycloheximide on polypeptide synthesis during early stages of HSV 2 infection.

VERO cells were infected (moi=10 pfu/cell) or mock-infected in the presence of cycloheximide. After 5 hours, the cycloheximide was removed by washing the cells three times, with 5 mls of methionine-reduced medium. At the designated times, cells were labelled for one hour with ^{35}S-methionine (20 uCi/ml). Cells were then harvested and prepared for SDS-PAGE analysis on a 9% acrylamide-DATD gel. The gel was then prepared for autoradiography on Kodak XRP-1

Lanes a-e are infected cells in the absence of cycloheximide. (a) 0-1 hours postinfection; (b) 1-2 hours postinfection; (c) 2-3 hours postinfection; (d) 3-4 hours postinfection; (e) 4-5 hours postinfection. Lanes f-i are infected in the presence of cycloheximide. (f) 5-6 hours postinfection; (g) 6-7 hours postinfection; (h) 7-8 hours postinfection; (i) 8-9 hours postinfection. The last lane is mock-infected, plus cycloheximide, 5-6 hours postinfection.


cycloheximide
—
+
ICSP
4
5/6
0/8
27
actin
a
b
c
d
e
MI
f
g
h
i
MI

10 and ICSP 8 as HSV 2 11/12 (personal communication by Dr. Bacchetti). Since the relative intensities differ signigificantly, it is difficult to determine whether any significant enhancement in the ratio of virus to cellular protein has resulted from the cycloheximide pretreatment.

The largest immediate early polypeptide, ICSP 4, is difficult to see in lanes g to j. It runs above ICSP 6 (ICP 10) at an approximate molecular weight of 175 kilodaltons (Powell and Courtney, 1975). A band of mobility similar to ICSP 4 can also be found in the mock-infected culture. The use of a specific antibody to ICP 4 in a radioimmunoprecipitation experiment would determine if the protein in the mock control is ICP 4 or a host protein.

ICSP 6 (HSV 2 ICP 10), a functionally defined early polypeptide can be detected in lanes h to j. ISCP 6 is defined as an early polypeptide based on two observations. Firstly immediate early polypeptides are defined as those proteins synthesized immediately after removal of cycloheximide from drug-treated infected cells. As can be seen in lane g, only three polypeptides: ICSP 4, 0 and 27 are synthesized after removal of the cycloheximide block. Secondly, in untreated cells, the synthesis of ICSP 6 peaks at 6-7.5 hours post-infection at which time ICSP 4 synthesis has already decreased significantly. As a result, ICSP 6 is better defined as an early polypeptide than an immediate early polypeptide

considering the kinetics of synthesis (Pereira et al., 1977). Recently, it has been shown that HSV 2 ICP 10 is the ribonucleotide reductase induced in mammalian cells by HSV infection (Huszar and Bacchetti, 1981; Bacchetti et al., 1984).

ICP 27, can also be detected in both treated and untreated controls in Fig. 3. It can be clearly distinguished in lanes d and e, but is faintly visible in lanes i and j. The approximate molecular weight of ICP 27, in this gel system used is approximately 80-83 kilodaltons.

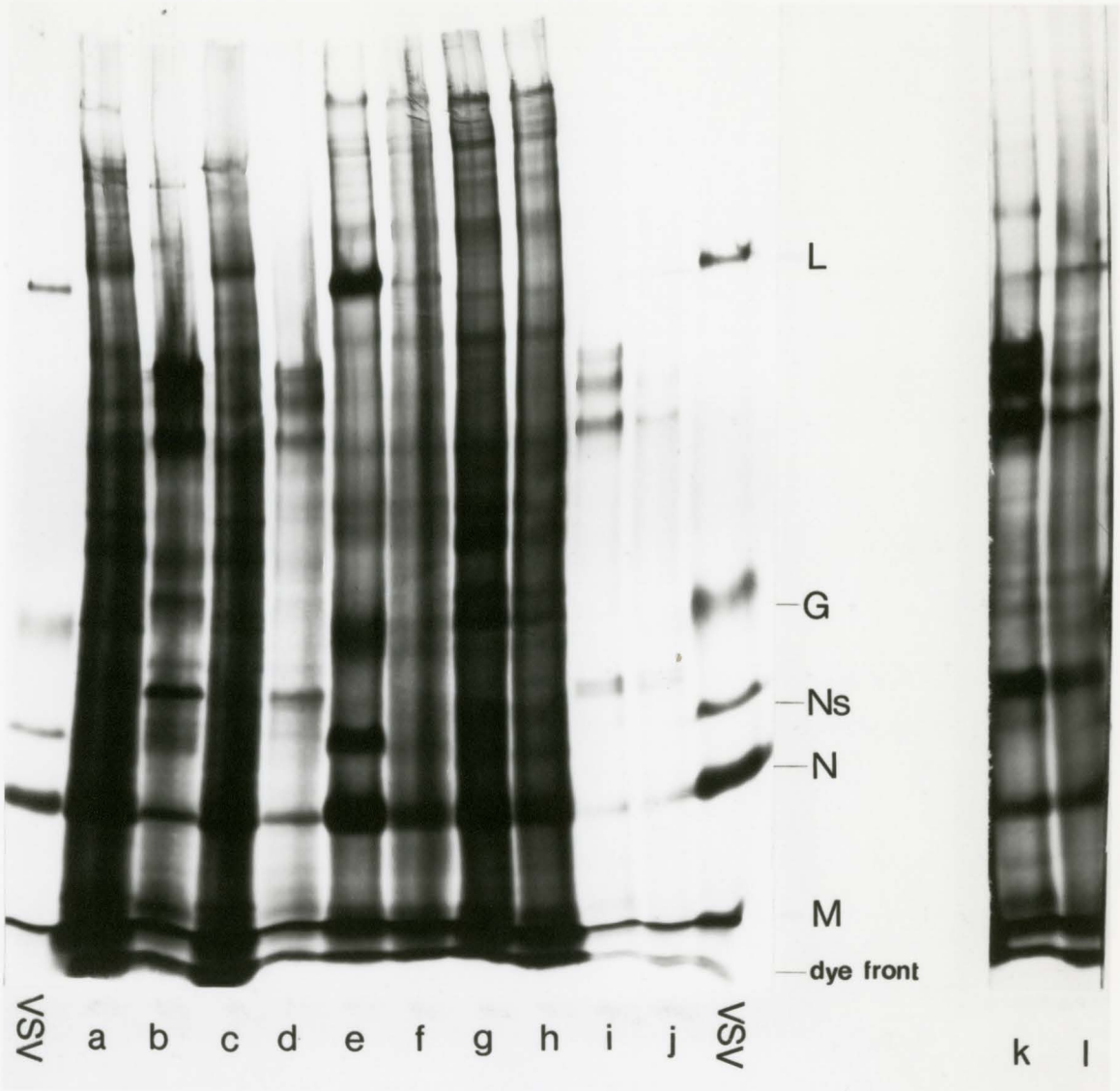
In Fig. 3 is shown a comparison of virus protein synthesis with or without cycloheximide pretreatment in infected and control VERO and mouse L cells. Mouse L cells were subsequently used in the preparation of the immunogen. In order to reduce the stimulation of non-specific (non-viral) antibodies which would arise if a heterologous system were introduced into the recipient Balb/c mice.

Comparison of the herpes-infected VERO cells without or with cycloheximide (Fig. 3, lanes b and d respectively) confirms the observation that protein synthesis decreases substantially after cycloheximide treatment. Although it is conceivable that the lack of recovery of protein synthesis is due to incomplete removal of cycloheximide, this seems unlikely as the control culture (lanes a and c) has

Fig. 3: HSV 2 infection of VERO and L cells in the presence or absence of cycloheximide.

VERO and L cells were mock-infected or HSC 2-infected (moi=20 PFU/cell) in the presence or absence of cycloheximide (100 ug/ml). After one hour adsorption period, the virus was removed and cells were refed with fresh medium with or without cycloheximide. The cycloheximide block was released 5 hours later by washing 3X with PBS. The cells were then fed with methionine-reduced medium containing 20 uCi/ml of ^{35}S-methionine for 4 hours. After this time, the labelled cells were washed with PBS and prepared for SDS-PAGE analysis. Cell extracts were run on a 9% acrylamide-DATD gel for 4 hours at 200V and then prepared for autoradiography on Kodak XRP-1 film.

Lanes a through d are extracts from VERO cells. (a) Mock-infected in the absence of cycloheximide; (b) HSV-infected in the absence of cycloheximide; (c) mock--infected in the presence of cycloheximide; (d) HSV--infected in the presence of cycloheximide; Lanes e through l are extracts from L cells. (e) VSV-infected in the absence of cycloheximide; (f) VSV-infected in the presence of cycloheximide; (g) mock-infected in the absence of cycloheximide; (h) mock-infected in the presence of cycloheximide; (i) HSV-infected in the absence of cycloheximide; (j) HSV-infected in the presence of cycloheximide; Lanes k and l are a three day exposure of i and j.


L
G
Ns
N
M
dye front
VSV
a
b
c
d
e
f
g
h
i
j
VSV
k
l

recovered essentially all of it's protein synthetic activity after undergoing an identical washing protocol. In this regard, it is interesting to note that protein synthesis in the control L cell culture is reduced by 2 to 5 fold after cycloheximide treatment (compare lane h to g). The VSV proteins in the cycloheximide treated L cells are also very poorly labelled after washing out the protein synthesis inhibitor under the same conditions as the VERO cells.

Viral protein synthesis due to HSV 2 infection is considerably less easy to detect in the L cell system than in the VERO (compare lanes i and j). With a longer exposure of these wells (lanes k and 1), polypeptides synthesized during infection could be detected in both cycloheximide treated and nontreated cell cultures.

(III) Hybridoma Formation

The immunization schedule and antigen source in the two sets of mice used in our attempts to raise monoclonal antibodies to the HSV 2 immediate early (IE) antigen are summarized in Table 1. Mice were injected intraperitoneally with either whole HSV antigen (formalin-inactivated HSV-infected L-cell extracts) or with the immediate early (IE) antigen. prepared from cycloheximide-treated L cell extracts as described in the methods. In the first attempt, the mice were challenged four weeks later with an intraperitoneal

Table 1: Immunization Schedules used in Hybrid Formation

	Day	Antigen[1]	Amount[2]	Adjuvant[3]	Route[4]	No. of fusions[5]	Surviving Hybrids
A	0	whole HSV	0.1 ml	C.F.	i.p.		
	28	IE Ag	0.1 ml	I.C.F.	i.p.		
	60					1	0-contamination
B	0	IE Ag	0.2 ml	C.F.	i.p.		
	28	IE Ag	0.1 ml	I.C.F.	i.p.		
	60					1	0-contamination
C	0	IE Ag	0.1 ml	C.F.	i.p.		
	28	IE Ag	0.1 ml	I.C.F.	i.p.		
	60					1	0-contamination
D	0	whole HSV	0.1 ml	C.F.	i.p.		
	147	IE Ag	0.2 ml	I.C.F.	i.p.		
	161	IE Ag	0.2 ml	I.C.F.	i.p.		
	168	IE Ag	0.2 ml	I.C.F.	i.p.	2	42
E	0	IE Ag	0.1 ml	C.F.	i.p.		
	147	IE Ag	0.2 ml	I.C.F.	i.p.		
	161	IE Ag	0.2 ml	I.C.F.	i.p.		
	167	IE Ag	0.2 ml	I.C.F.	i.p.	2	11

1 Formalin inactivated cell-lysate or IE Ag (2.6 mg/ml).
2 Volume of immunizing material.
3 Complete (C.F.) or incomplete (I.C.F.) Freund's adjuvant.
4 intraperitoneal
5 number of fusions done under immunization schedule.

injection of IE antigen emulsified in incomplete Freund's adjuvant and sacrificed after another four weeks. At this time the spleens were removed for fusion with the myeloma cells.

As a result of fungal contamination of the hybrid cultures from the first fusion attempt there were no surviving cultures to be screened for positive clones.

Since the first set of fusions were not successful, mice primed earlier with whole HSV or IE antigen were given a series of boosters as illustrated in Table 1. Four days after the final booster, the mice were sacrificed and the spleens removed for fusion with the Sp2/0 cells.

The growing mixed clones were expanded from the 24 well plates to the 25 cm^2 Corning tissue culture flasks. About one week later upon reaching high densities, the cells were frozen and kept in liquid nitrogen until needed for further expansion. A total of 42 out of 48 cultures were frozen from group D whereas only 11 out 23 cultures from group E survived and were placed in liquid nitrogen for storage.

(IV) Screening for potential positive anti-HSV II immediate early antigen monoclones.

Three approaches, ELISA (Enzyme-linked immunoadsorbent assay), immunodiffusion and radioimmunoprecipitation were applied to determine whether a hybrid line was a positive

producer or not.

(i) ELISA

The Elisa system was used as an initial screening protocol for detecting positive polyclones. Three antigen sources, a mock-infected, an "immediate early" and a "late" infected antigen were prepared as described in the methods. Microtitre plates were subdivided into three regions and each region was then coated with one of the three antigens as described in the methods.

After coating, samples of tissue culture fluid (TCF) or mouse serum, were added in duplicate to each antigen preparation. The plates were then incubated with rabbit antimouse IgG followed by an incubation with goat antirabbit IgG conjugated with Horseradish peroxidase. The amount of enzyme present was determined with the addition of a substrate solution after washing away any excess conjugate left in the wells. The enzymatic reaction was stopped after one hour and the optical density of each well was then measured at 490nm.

On each assay performed, three control samples were added in addition to the test samples, as a means of monitoring the reliability of the assay results.

The first control, normal mouse serum (NMS), was used

Table 2: A summary of Optical Densities obtained with Various Antisera in the ELISA assay.

Antibody tested	Antigen[1] Mock	Late	Immediate Early
Normal mouse[2] serum			
neat	0.27	0.29	0.25
1/10	0.14	0.18	0.09
1/100	0.02	0.01	0.04
Immune mouse[3] serum			
1/100	0.43	0.51	0.35
1/200	0.30	0.35	0.38
1/400	0.16	0.26	0.31
1/2000	0.13	0.10	0.10
1/5000	0.06	0.04	0.04
13α A3	0.00	0.15	0.00
	0.05	0.13	0.03
	0.01	0.19	0.00
clones 1-6[5]	0.00	0.02	0.02
2-21	0.02	0.06	0.02
1-16	0.03	0.05	0.00
2-19	0.01	0.05	0.00

1 Antigen coated to well of plates as described in the Methods.

2 Normal mouse serum from Cappel Laboratories.

3 Immune mouse serum from mice sensitized with formalin--inactivated virus.

4 O.D. reading at = 490 nm. A reading of 0.10 was taken as a significant positive response.

5 unknown hybrid cell lines.

as a background response. At neat dilutions, NMS produces a significant response on all three antigens, creating a false positive. When serial dilutions of NMS as shown in Table 2, were used in the assay, it was discovered that the readings dropped to background levels rapidly as the dilution of serum increased. To eliminate false positives when screening the hybrids, the samples underwent a similar dilution procedure.

Immune mouse serum (IMS), was used as a positive control. The serum, donated by Dr. Bacchetti, came from mice sensitized to a "late" HSV II antigen. The IMS consistently gave similar readings on the immediate early or on the late antigen as on the Mock-infected antigen. Since the response of IMS was not very consistent between assays and showed high readings on the mock-infected antigen, it was difficult to establish guidelines for determining what represented a positive response.

The third control, tissue culture fluid (TCF) from a known positive monoclone was also used as a positive control and to set the required baselines. TCF or ascites fluid of F13αA3, a positive hybrid cell line to HSV 2 late antigen was a gift from Dr. Bacchetti. In the assay described, F13αA3 gave a strong positive response on the late antigen. The response as indicated in Table 2, was 3-10X that of the reading obtained on the mock-infected antigen. On the IE

antigen, F13αA3 gave a negative response. The minimal three--fold increase between infected and mock-infected antigens was then used as a baseline for determining a positive clone.

In testing the unknown hybrid lines, it was observed that the readings were very low, very close to background levels. Comparing the results from all three antigens, as summarized in Table 2, there exists very little to no difference between the responses of the unknown hybrids on all three antigens. Under these conditions, the assays would suggest that the unknown hybrid cells were non-producers.

(ii) Immunodiffusion

To determine whether the parental clones were producing mouse immunoglobulins, a number of immunodiffusion experiments were performed.

Tissue culture fluid (TCF) of the surviving clones were screened for production of either mouse IgG or IgM. Agarose plates were prepared as described in Materials and Methods. Approximately 10 ul of either rabbit antimouse IgG or IgM was added to the central well of each Ouchterloney plate. To the outer wells, 10 ul of concentrated (100X) TCF or normal mouse serum was added. After an overnight incubation at $4^{o}C$, the plates were checked for precipitin lines. Based upon the

Table 3: Summary of Immunodiffusion results

Fusion Group[1]	Number of Surviving Cultures	Rabbit antimouse IgG[2]			Rabbit antimouse IgM[2]		
		#samples tested	+	-	#samples tested	+	-
Group D # 1	23	22	4	18	23	0	23
Group D # 2	19	19	5	14	22	6	16
Group E # 1	4	3	0	3	4	1	3
Group E # 2	7	6	0	6	6	1	5
TOTAL	53	50	9	41	55	8	47

1 Refer to Table 1--the fusion group is related to the immunization schedule.

2 Antibody placed in central well of immunodiffusion assay arrangement.

Table 4: Positive Mouse IgG/IgM Producing Clones

Group D # 1		Group D # 2		Group E # 1		Group E # 2	
IgG	IgM	IgG	IgM	IgG	IgM	IgG	IgM
1-6		2-8	2-2		3-15		4-3
1-15		2-9	2-3				
1-17		2-11	2-5				
1-23		2-21	2-12				
			2-24				

concept that antibodies can precipitate their respective antigens in a gel system, the formation of a precipitin line would indicate the presence of either mouse IgG or IgM.

The results of the Ouchterloney tests are summarized in Tables 3 and 4. A total of 9 samples out of the 50 samples tested showed a positive response for mouse IgG. Likewise, only 8 samples were positive for mouse IgM. These results indicate that there were only 17 clones secreting any sort of mouse immunoglobulin. These assays do not suggest however that these antibodies are directed specifically to the IE antigen of a HSV 2 infection. In an effort to insure the sensitivity of the assay procedure the samples were screened for a third time by means of radioimmunoprecipitation with Protein A-Sepharose beads (Pharmacia) and labelled cell extracts.

(iii) Radioimmunoprecipitation with Protein A-Sepharose beads.

Radioimmunoprecipitation experiments with Protein A-Sepharose beads were used as the final screening step in detecting positive monoclones.

Labelled cell extracts were prepared as described in the methods and used as antigens for the immunoprecipitation experiments. Tissue culture fluid samples were mixed with

mock or infected "early" antigen and with the Protein A beads overnight at 4°C. The beads were collected, washed, and resuspended in extraction buffer to remove the antigen--antibody complexes from the beads. A small volume of this supernatant was taken, precipitated with 10% Trichloroacetic acid and counted in a scintillation counter. A comparison of the TCA precipitable counts of the mock control versus those of the infected runs was used as an initial indication of whether the antibody could precipitate a virally-related protein and produce a positive signal.

The rest of the supernatant was then analyzed by gel electrophoresis on 9% acrylamide-DATD gels.

Prior to any of the immunoprecipitations, samples of the antigen preparations were analyzed on SDS-gels. Fig. 4, is an example of such an analysis. Comparison of the infected antigen with the mock-infected counterpart and with the data from the earlier labelling experiments, showed that the antigens prepared were representative of an early and a late antigen.

Immunoprecipitations with the early antigens and the unknown TCF samples yielded negative results. All the cultures screened showed a negative response on both the mock and infected early antigens. One band of approximately 210 kd molecular weight, is precipitated by all the antibodies

Fig. 4: The antigens prepared for use in the radioimmunoprecipitation assays

VERO monolayers were mock-infected or HSV 2 infected. After one hour of adsorption, the cells were incubated in a methionine-reduced medium containing 20 uCi/ml of ^{35}S-methionine. After the desired labelling time, the cells were harvested, washed and solubilized in RIPA buffer. Approximately 10 ul of the final antigen preparation was analyzed by SDS-PAGE. In this example, the extracts were run on a 9% acrylamide-DATD gel for 4 hours. The autoradiograph is a result of a one day exposure of the dried gel on Kodak XRP-1 film.

Lane (a) Mock antigen; labelled from 1-4 hours postinfection; (b) Mock antigen; labelled from 1-21 hours postinfection; (c) Infected antigen; labelled from 1-4 hours postinfection; (d) Infected antigen; labelled from 1-21 hours postinfection.

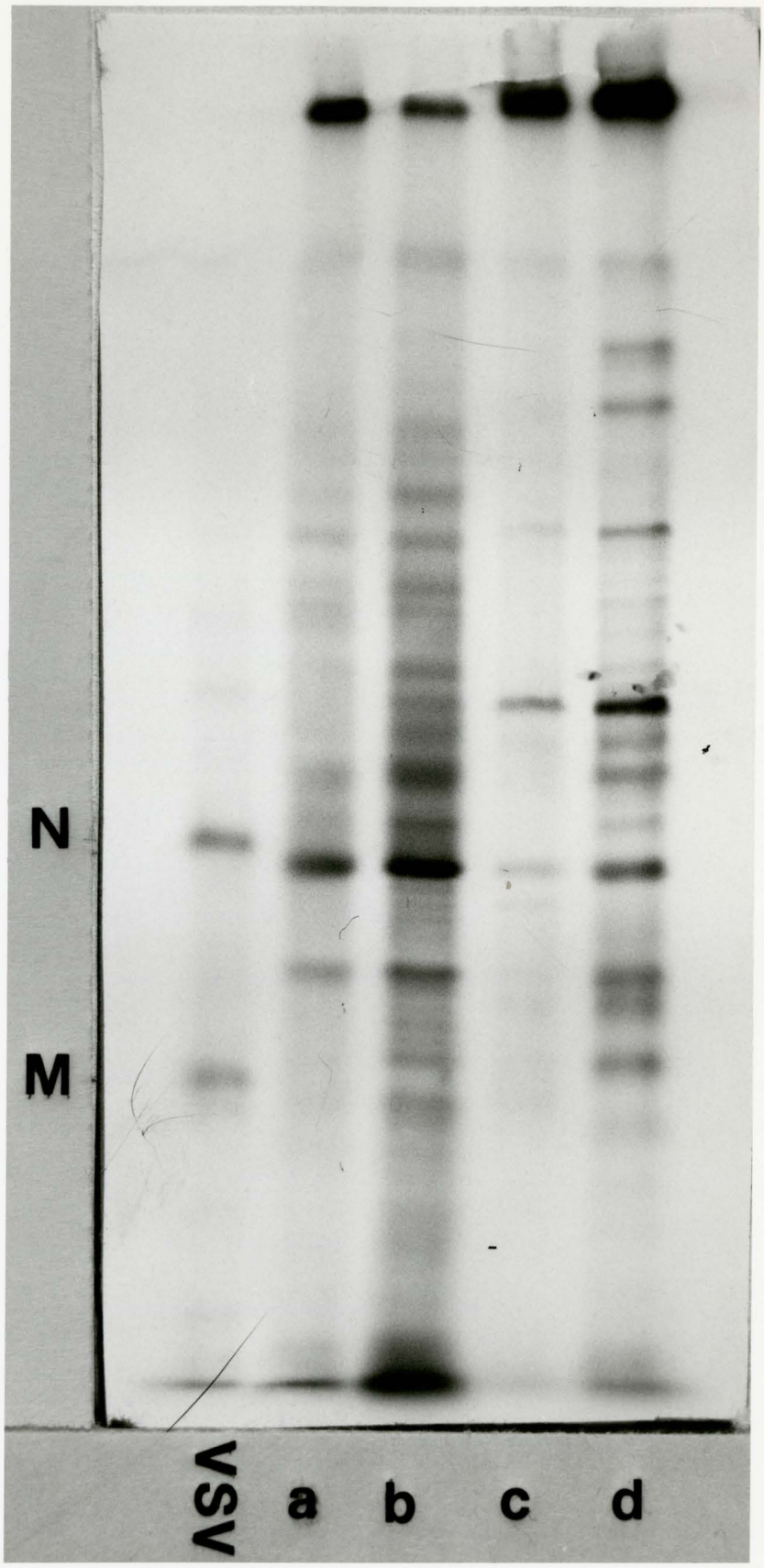

N
M
VSV
a
b
c
d

Fig. 5: Immunoprecipitation of HSV Early Antigens with antibodies of the test monoclonal cells.

Mock or infected early antigens were incubated overnight with Protein A-Sepharose beads and TCF from the test clones. The precipitated antigen was removed from the beads as described in the methods. Samples from each immunoprecipitation experiment were analyzed by SDS-PAGE. Samples in this example were run on a 9% acrylamide-DATD gel for four hours. The dried gel was then exposed to XRP-1 film for 4 days to give this autoradiogram.

The first lane in each pair represents the immunoprecipitation of the antibody with the mock-infected antigen. The second lane is representative of the immunoprecipitation with the infected antigen. Set (a) Normal mouse serum; (b) Immune mouse serum; (c) clone 4-2; (d) clone 1-8; (e) clone 1-10.

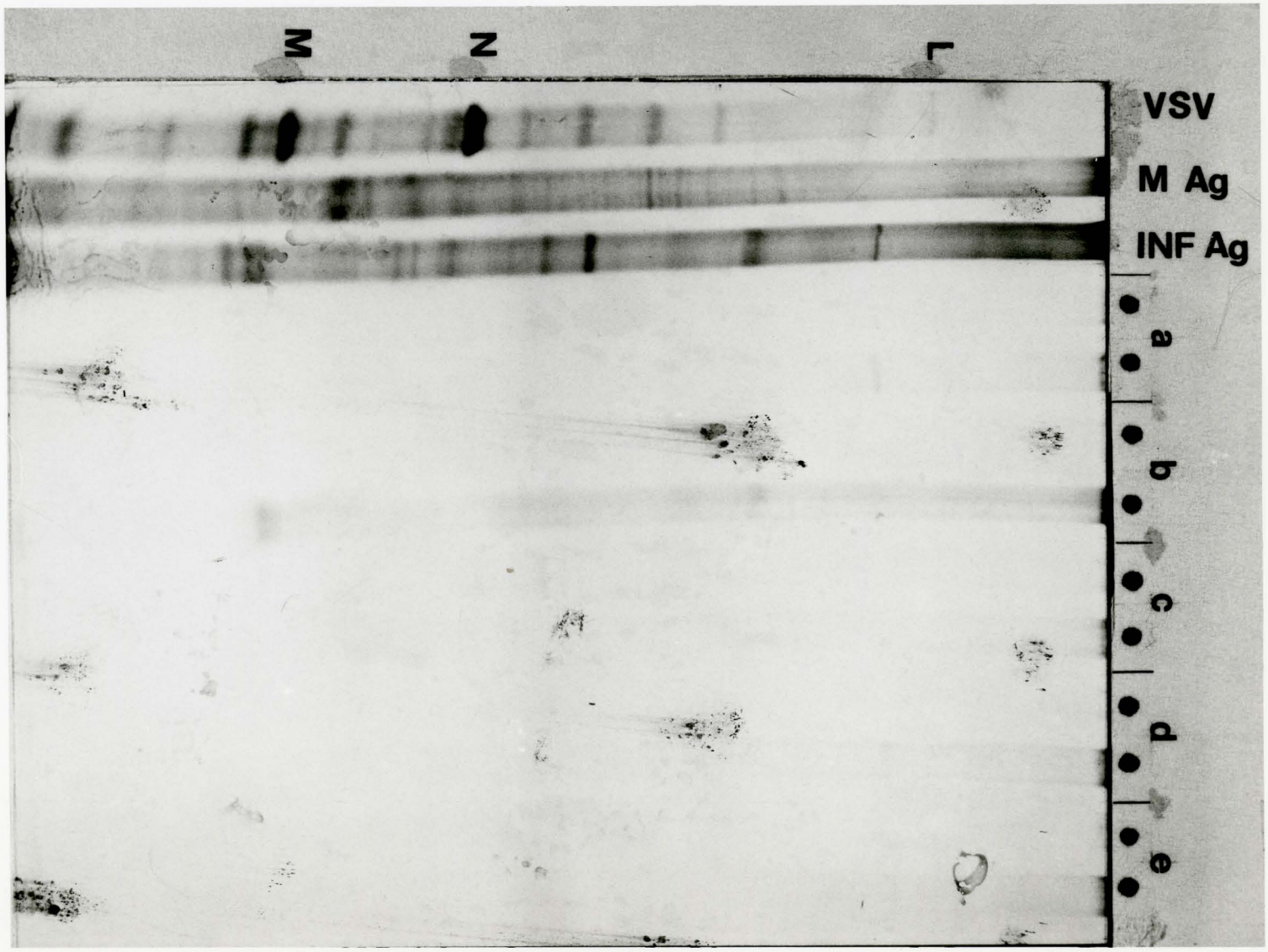
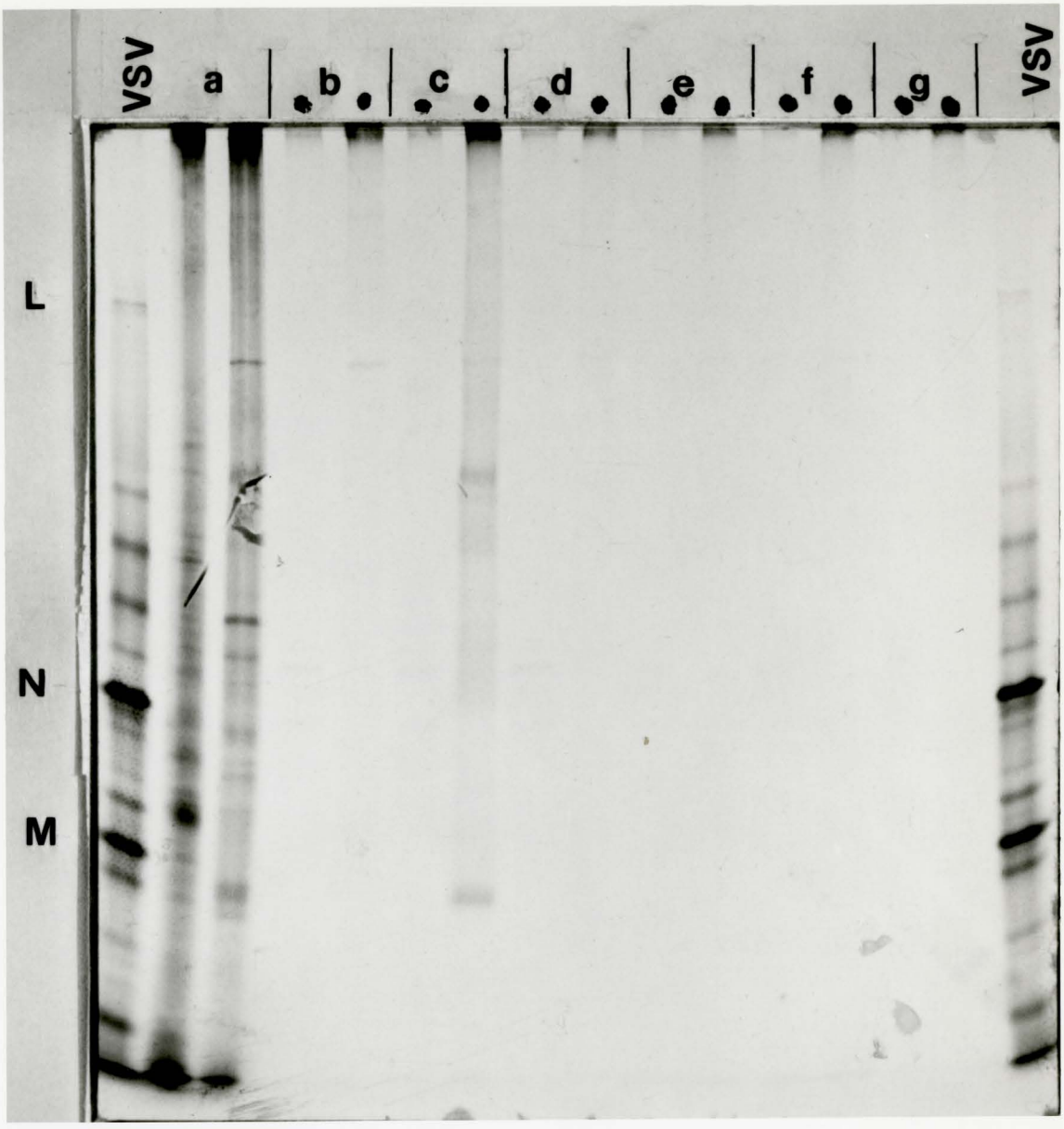

M
N
L
VSV
M Ag
INF Ag
a
b
c
d
e

Fig. 6: Immunoprecipitation results between the test clones and an early HSV 2 antigen.

Mock-infected or HSV 2-infected 'early' antigens were incubated with tissue culture fluid samples and Protein A-Sepharose beads overnight at 4°C. The bound antigen-antibody complexes were freed from the beads by treating the beads with elution buffer. Samples of each supernatant were then analyzed by SDS-PAGE. In this example, samples were run for four hours on a 9% acrylamide-DATD gel. The gel was then exposed to Kodak XRP-1 film for 4 days to produce this autoradiogram.

The first lane of each pair is a result of an immunoprecipitation with the mock-infected antigen and the test sample. The second lane represents immunoprecipitation of the HSV 2-infected antigen with the test samples. (a) mock and infected antigen; (b) normal mouse serum; (c) immune mouse serum; (d) clone 2-6; (e) clone 2-17; (f) clone 1-23; (g) clone 2-11.


VSV
a
b
c
d
e
f
g
VSV
L
N
M

tested, including the normal mouse serum. This result, illustrated in Figs. 5 and 6 was considered to be non--specific reaction since the antigen reacted also with normal mouse serum and was thus discounted as a positive result.

In all the immunoprecipitation experiments, immune mouse serum was able to precipitate two bands, a 130 kd and 20 kd. The serum is derived from mice sensitized with formalin-inactivated virus (Killington et al., 1981). Many of the herpes structural proteins are early polypeptides which reach maximal synthesis from 4-8 hours post-infection (Strnad and Aurelian, 1976). Cycloheximide, was not used in the preparation of the immediate early infected antigen. As a result, early synthesis had been initiated when the cells had been harvested as the antigen source allowing some early polypeptides to contaminate the preparation.

(V) Ascites Production of HSV 2 hybridomas

As suggested by the immunodiffusion experiments, a number of the screened clones were producing either a mouse IgG or IgM. Although the specificity of these clones could not be determined by this method, 12 out of the 50 clones were re-injected into mice for ascites production. Ascites fluid, which is an enriched source of antibodies, was then tapped from each mouse two weeks later. Yields of ascites fluid ranged from 20.5ul to 5 ml per mouse. With the use of

ascites fluid (a higher antibody titre) and a different approach to preparing an IE antigen (as described below) it was hoped that the sensitivity of the radioimmunoprecipitation assay would be increased as to pick up any positive monoclones that were overlooked in the earlier assays. The ascites production, as well as the associated radioimmunoprecipitations was done by Mr. Dennis Takeyesu as part of a follow-up study to the earlier screening assays.

(i) Radioimmunoprecipitation of a ^{35}S-methionine labelled extract with test ascites.

Purifoy and Powell (1976) suggest that many of the radiolabelled proteins in an HSV 2-infected cell are found in an insoluble form. As a result, they have developed a high salt extraction procedure which solubilizes these proteins. The same protocol as outlined by Purifoy and Powell (1976) and outlined in the materials and methods was used in preparing the antigen for the following radioimmunoprecipitations.

Approximately 10 ul of ascites fluid or 5 ul of immune mouse serum was incubated with 500 ul of antigen and the Protein A- Sepharose beads. The subsequent precipitates were then analyzed by SDS-PAGE. Figs. 7 and 8 are representative examples of such immunoprecipitation experiments.

Lanes c through k in Fig. 7 are immunoprecipitation

Fig. 7: Immunoprecipitation of an early HSV 2 antigen with ascites fluid.

Approximately 10 ul of ascites fluid or 5 ul of immune mouse serum was incubated with the high speed supernatant antigen as described in the results and with the Protein A-Sepharose beads. The immunoprecipitates were then analyzed on SDS-gels. The dried gel was exposed to Kodak XAR-5 film for 14 days.

Lane (a) the high speed supernatant antigen; (b) immunoprecipitates with immune mouse serum; (c) clone 1-1; (d) clone 1-3; (e) clone 1-6; (f) clone 1-7; (g) clone 1-13; (h) clone 1-16; (i) clone 2-3; (j) clone 2-4; (k) clone 2-9.

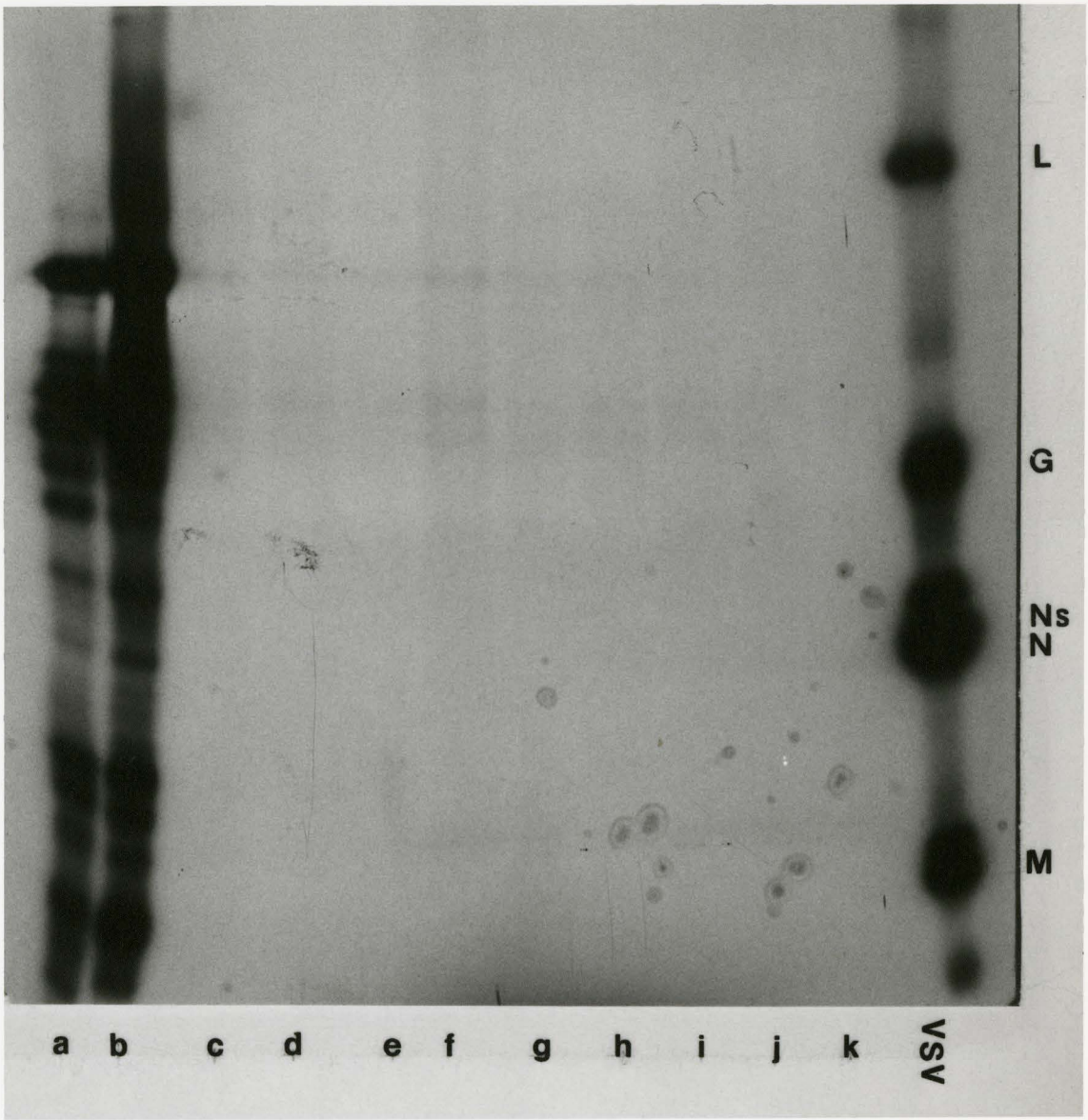
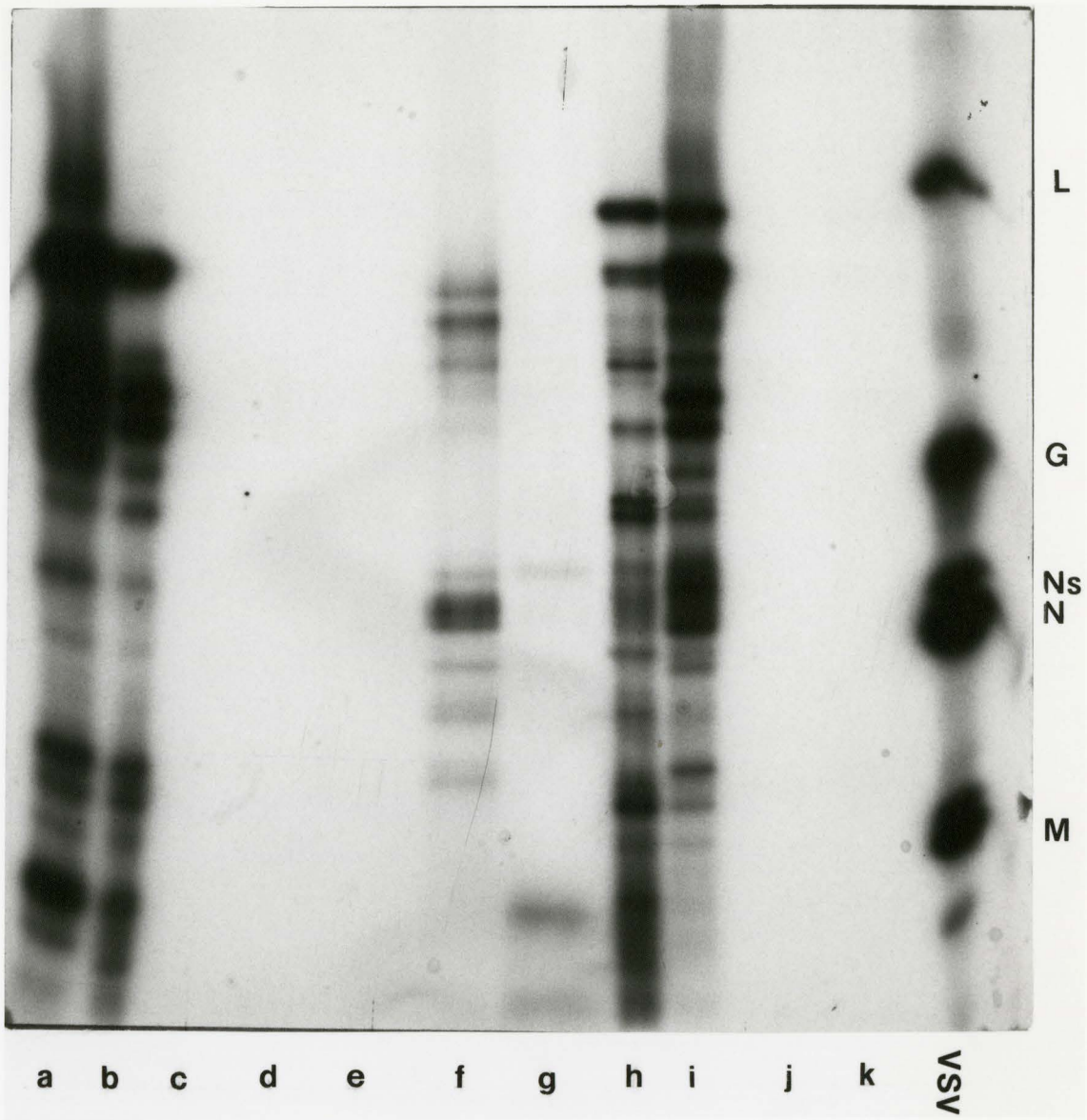

L
G
Ns
N
M
a b c d e f g h i j k VSV

Fig. 8: Immunoprecipitation of an HSV 2 early antigen with ascites fluid.

An early antigen was prepared according to the protocol described by Purifoy and Powell (1976) and as described in the Materials and Methods. Approximately 10 ul of ascites fluid or 5 ul of immune mouse serum was incubated with the antigen and Protein A-Sepharose beads. The precipitates were solubilized in elution buffer and then analyzed by SDS-PAGE. The dried gel was then exposed to Kodak XAR-5 film for 14 days to give this autoradiogram.

Lane (a) High speed supernatant antigen immunoprecipitated with immune mouse serum; (b) the high speed antigen; (c) clone 2-12; (d) clone 2-17; (e) clone 2-21; (f)clone αB11; (g) clone αC18; (h) the high salt cell extract prior to fractionation; (i) the pellet after centrifugation precipitated with immune mouse serum; (j) αE12; (k) clone 15βC3.


L
G
Ns
N
M
a b c d e f g h i j k VSV

runs using the ascites produced by our clones as the source of antiHSV antibody. Upon examination of these lanes, it can be seen that our ascites do not appear to recognize any of the early proteins present in the antigen preparation.

Three other clones: 2-12, 2-17 and 2-21 are shown in lanes c, d and e of Fig. 8. Like the other set of clones, these three antibodies also do not recognize any determinants in the early antigen preparation. Based on the results of these radioimmunoprecipitations and the data from the earlier screening assays, it can be concluded that the hybrids are negative producers of an antiHSV immediate early antibody.

Represented in Fig. 8 also is the immunoprecipitation runs of four monoclones produced in Dr. Bacchetti's laboratory. Monoclones αB11, αC18, αE12 and 15βC3 are found in lanes f,g,j and k respectively. The results of these runs will be discussed in tne subsequent section.

(VI) Characterization of established hybridomas secreting antiHSV 2 antibodies.

Several hybridomas secreting antibodies to HSV 2--induced proteins have been established in Dr. Bacchetti's laboratory. Essentially three different immunization protocols were used to produce antibodies against the immediate

early, early and late antigens of HSV 2 (Killington et al., 1981; Evelegh et al., manuscript in preparation). As a means to assist in the characterization of these monoclones, several immunoprecipitation experiments were performed in our laboratory.

Hybridomas 18αD4, 20βB5, 15βC3 and 17αC5 were obtained from mice immunized with the 18 hour cell lysate described by Killington et al. (1981). The results of the immunoprecipitation experiments with these antibodies and a ^{35}S-methionine labelled late antigen is illustrated in Fig. 9.

Hybridoma 18αD4, which secretes a mouse IgG1 and possibly IgM as determined by immunodiffusion analysis (personal communication by C. Sartori), precipitates a polypeptide of approximately 130 kilodaltons molecular weight. Based on this molecular weight, this polypeptide could possibly be ICSP 14 (VP 6) (Strnad and Aurelian, 1976; Bookout and Levy, 1980).

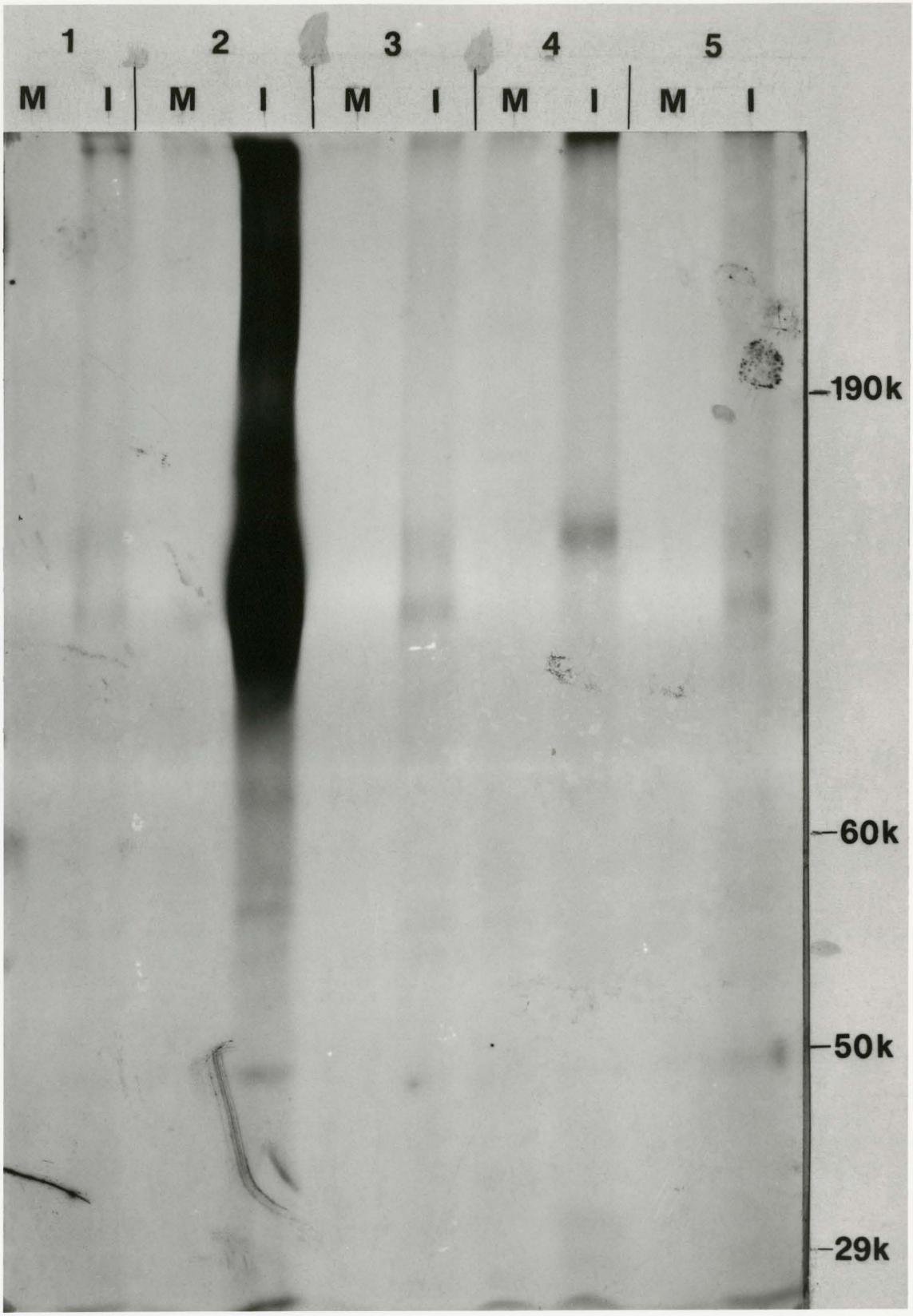
Monoclone 20βB5 which secretes a mouse IgG2 (personal communication by C. Sartori) precipitates three bands. The upper two bands range in molecular weight from 120 to 150 kilodaltons. The faster migrating band is approximately 50 kilodaltons molecular weight.

Recent studies have shown that monoclone 15βC3 precipitates a major band (155,000) and a minor band (39,000)

Fig. 9: Immunoprecipitation studies of established antiHSV 2 hybridomas.

Cell lysates of mock or infected VERO cells, labelled with ^{35}S-methionine were prepared as described in the methods. After immunoprecipitation with Protein A-Sepharose beads, the immunoprecipitates were prepared for SDS-PAGE analysis on a 9% acrylamide-DATD gel.

Lane (1) cell lysates immunoprecipitated with normal mouse serum; (2) 17αC5--ascites; (3) 18αD4--ascites; (4) 15βC3--ascites; (5) 20βB5--culture fluid.


1
2
3
4
5
M
I
M
I
M
I
M
I
M
I
190k
60k
50k
29k

(Evelegh et al., manuscript in preparation). As can be seen in Fig. 9, only one band of approximately 150,000 molecular weight is precipitated by 15 C3. This polypeptide is thought to be ICSP 9 based on it's molecular weight (Evelegh et al., manuscript in preparation). Immunofluorescence assays show that 15βB3 reacts with surface antigens on HSV 2-infected unfixed cells. It has also been noted that 15βB3 shows reactivity to both HSV 1 and 2 antigens as indicated by positive fluorescence on both HSV 1 and 2-infected fixed cells (Evelegh et al., manuscript in preparation).

Monoclone 17αC5 was used as a positive control in all the immunopreipitations for its immunoprecipitation profile is well documented (Balachandran et al., 1981). The pattern seen in Fig. 9 is in close agreement with those published observations. In our gel system, 17αC5 immunoprecipitates a series of polypeptides ranging in molecular weight from 40 to 150 kilodaltons. Evelegh et al., (manuscript in preparation) have shown that 17αC5 precipitates a 129,000-MW polypeptide which was shown to be ICSP 11/12, by cross--adsorption experiments with an antibody specific for ICSP 11/12.

The three clones, αE12, αB11 and αC18 were obtained from mice immunized with either the 2 hour cell lysate (αB11 and αC18) or with the 8 hour cell lysate (αE12). The immunoprecipitation profiles of these antibodies with a

labelled early antigen is illustrated in Fig. 8.

As can be seen in Fig. 8 ∝E12 shows a negative response on this particular antigen. Evelegh et al. (manuscript in preparation) have shown that ∝E12 precipitates a glycosylated polypeptide of 65 kilodaltons molecular weight. This polypeptide was further identified as gF or gC-2 by cross-adsorption with a gC-2 specific antibody---17∝A2.

The negative response obtained here probably reflects a low level of gC-2 present in the antigen preparation. Glycoprotein gC-2 behaves like a late protein in that detectable amounts are not found before 3 to 5 hours of infection and only reach maximal synthesis with the onset of viral DNA synthesis (Balachandran et al., 1981).

Hybridoma ∝C18 appears to precipitate two polypeptides, a 76 kilodalton-MW polypeptide and a 28 kilodalton-MW protein. Work done by Evelegh et al. (manuscript in preparation) show that ∝C18 precipitates a phosphoprotein of 76,000-MW. It has been suggested that this antibody recognizes ICSP 28, a 73,000-MW phosphoprotein described by Bookout and Levy (1980).

In Fig. 8, it can be seen that ∝B11 precipitates a series of polypeptides ranging in molecular weight from 40 to 150 kilodaltons. Additional studies by Evelegh et al.

(manuscript in preparation) show that αB11 precipitates three specific polypeptides, 107 kilodaltons, 48 kilodaltons and 24 kilodaltons molecular weight. These polypeptides have yet to be characterized and assigned a function.

DISCUSSION

(I) Immunization Protocol

The route and schedule of the immunization are two determining factors in the success of sensitizing splenic lymphocytes. Killington et al. (1981) have found that the most successful protocol in the production of monoclonal antibodies to HSV 2 consisted of a series of boosters, either intraperitoneally or via the footpad, after the initial challenge to the footpad with inactivated virus.

Koprowski et al. (1977) as well as Flamand et al. (1980), were able to raise hybrids to viral antigens with two single intraperitoneal injections of inactivated virus. In the same fashion, Zweig et al. (1979) successfully produced monoclonal antibodies to HSV 1 nucleocapsids using a series of weekly intraperitoneal boosters to sensitize the mice.

Evelegh and coworkers (manuscript in preparation) have successfully produced monoclonal antibodies to early antigens of HSV 2. After the initial injection of an infected cell lysate intraperitoneally, the mice received weekly boosters, two subcutaneously and one intraperitoneally. Three days after the last booster, the mice were sacrificed, spleen cells removed and fused to the mouse myeloma cells.

It is thus apparent in the literature that each antigen represents an unique problem in terms of what schedule and route is the most suitable one. The schedule used in this project, as outlined in Table one, was not successful. As suggested by the results of Killington et al. (1981), sensitization of the immune system via the footpad perhaps would have been a better choice than intraperitoneal injections. Also, based on the results of Zweig et al. (1979), perhaps the use of frequent boosters would have been sufficient to sensitize the mouse. The third alternative, a combination of changing the route as well as the schedule perhaps is necessary to sensitize the mouse to the immediate early antigen. This is evident in the success obtained by Evelegh and coworkers (manuscript in preparation) in producing monoclones to the early antigens. In all cases, the immune response to the immunization protocol should be monitored by following the polyclonal response in the serum. This was not done in the original immunization which is perhaps a contributing factor to our lack of success in producing monoclonal antibodies to the immediate early antigens of HSV 2.

(II) ELISA

The Elisa (enzyme-linked immunosorbent assay) has been used both as a qualitative as well as a semi-quantative

in the study of antibody-antigen interactions. Engvall and Perlmann (1972) developed a solid phase assay using antigen-coated tubes that could detect antibody levels at nanograms per milliliter.

Witkin et al. (1980) have developed the Elisa system as a screening tool for the detection of antibodies to mouse mammary tumor virus (MMTV). Human sera from breast cancer patients show an immunological response to MMTV antigens (Witkin et al.,1980). As a result, the assay could be used in the early detection of breast cancer.

There are two inherent problems with the Elisa system which may contribute to the uninterpretable results alluded to earlier. Firstly, there is the problem of nonspecific reactivity such as that obtained with normal mouse serum which apparently enhances the enzymatic activity. To overcome this problem, O'Sullivan et al. (1979) suggest that the sample be diluted to reduce the nonspecific reaction.

The second problem involves the necessity of coating the wells or tubes with sufficient antigen. Witkin et al. (1980) have shown that in their system, between the range of 0.1 ug to 10 ug antigen per well, the antibody response is proportional to the antigen concentration. Perhaps the low readings obtained with the immune serum in our system would indicate that little antigen was present in

the wells. As a result, higher stock solutions of antigen should be used to coat the wells. To monitor the protein concentration, a Lowry determination or spectrophotometric measurements should be done to give an accurate estimate of the total protein content present in the antigen preparation.

(III) Characterization of established antiHSV 2 hybridomas.

Immunoprecipitation of infected-cell lysates with the hybridomas show an unique pattern for each clone tested. Hybridoma 18αD4 and 15βC3 each precipitate a single band, of 130,000 and 150,000 daltons respectively.

The precipitates of 20βB5 and 17αC5 show a multiple pattern of bands. This result is not an unusual one. Killington et al. (1981) suggest that multiple bands may represent precursor--products or proteins with similiar antigenic determinants or functional complexes. Balachandran et al. (1981) report that two hybridoma antibodies to HSV 2 glycoproteins react with the glycosylated precursors as well as their products. Zweig et al. (1980) also have shown that antibodies generated by a hybridoma to HSV p40, a nucleocapsid protein, also react with a larger polypeptide (80,000 daltons) which is neither a precursor protein nor a doublet of the 40,000 protein. As a result, the reason for multiple bands is as unique as the pattern itself.

The presence of multiple bands may also reflect an inaccuracy in the cloning procedure of that particular clone. To check whether the clone is a true single clone or a mixed clone, it should be cloned a second time. The resulting progeny should then be rescreened by radioimmunoprecipitation. If the original clone is a single clone, the progeny should produce similar immunoprecipitation patterns to the mother clone. However if the original clone is still a mixed clone, the progeny will then produce different and unique patterns compared to the immunoprecipitation pattern of the parental clone.

The antibodies produced by the hybridoma 17∝C5 immmunoprecipitates a polypeptide of approximately 129,000 molecular weight which has been shown by Evelegh *et al.* (manuscript in preparation) to be ICSP 11/12: the major DNA binding protein in HSV 2-infected cells. ICSP 11/12 has been observed to have a stronger affinity for single-stranded DNA than for double-stranded DNA (Powell *et al.*, 1981). The direct reaction of purified ICSP 11/12 on polydeoxyadenylic acid-polydeoxythymidylic acid helix is to reduce the melting point which might suggest that ICSP 11/12 may play a role in viral DNA synthesis (Powell *et al.*, 1981).

As discussed in the results, 17∝C5 coprecipitates several minor bands with ICSP 11/12. It has been suggested in the literature that ICSP 11/12 occurs in association with

other proteins. Mutants in ICSP 11/12 show a destabilization in the viral DNA polymerase and alkaline DNase (Littler et al., 1983). As a result, 17 C5 may prove to be a useful tool in discerning the functional units of this protein complex.

LaThangue and Chan (1984) have made use of a monoclonal antibody to HSV DNA-binding proteins, T18, to prepare immunoadsorbent columns as a means to purify a specific DNA-binding protein. Once purified, the protein was further characterized in terms of DNA affinity, molecular weight and cellular location. This information may be used by the authors as a means to further understand the controls involved in viral gene expression.

(IV) Summary

The attempts to produce monoclonal antibodies to the immediate early antigens of HSV 2 proved to be unsuccessful. With hindsight and current experience, the author can suggest sone alterations in procedure which would perhaps give a successful result.

The first area of concern is the composition of the antigen used in the protocol described. As alluded to earlier in the results, the injected antigen was at no time analyzed by the conventional means (i.e. SDS-PAGE) to determine what was truly injected into the recipient mice. As a result, it is difficult to be certain that the antigen was in fact the

correct temporal frame and contained immediate early proteins.

In addition to knowing the composition of the antigen, a semi-quantitative measure of the protein concentration is beneficial in determining the amount of antigen to be injected into the mouse. Currently, the author uses a range of 50-100 ug of protein per mouse per challenge. Perhaps the lack of a response reflects a low level of antigen present in the system. It is conceivable that insufficient antigen was introduced to stimulate the desired immune response.

Although the amount of antigen presented at a challenge is critical, how it is presented to the mouse is also a determining factor in obtaining a successful immunization. As discussed earlier, a variety of immunization schedules and routes appear to give successful results. In all the examples cited, the mice were given a series of regular boosters as a means to continually sensitize the spleen cells. In our protocol described in the results, long periods of time elapsed between sequential boosters. It would be advisable, based on the examples cited from the literature and on current experience, that the immunization schedule include frequent boosters (weekly) to the footpad, subcutaneously and intraperitoneally with and without Freund's adjuvant. In all cases however, the polyclonal response, as detected in the serum, should be monitored. A positive response reflects

a successful sensitization of the recipient mouse.

The third area of concern is the method of screening for positive hybrids. It is extremely important to have a working, reliable assay early in the growth of mixed hybrids. Fast growing non-secretors may overgrow slower positive clones which are subsequently lost. The Elisa was adopted as the first screening assay since it is fast, simple and has the capacity to screen many clones simultaneously As described earlier in the results, the Elisa was not totally reliable in our hands. Earlier in the discussion, it was mentioned that the Elisa is dependent on efficient as well as sufficient binding of antigen to the plate wells. In a current protocol, the author binds 100-500 ng of protein (antigen) to the well which is enough antigen for a reliable assay. To remove any non-specific binding of serum proteins, the plates are washed twice with 1.5 M KCl after incubation with the test antibodies which drastically reduces the non-specific reactions. As suggested earlier, the protein concentration of the antigen should be monitored much more carefully to ensure enough protein is bound to the wells.

In summary, three areas in the overall protocol need changes as seen by the author. Firstly, the antigen concentration and composition should be carefully controlled. Secondly, the immunization schedule and route should be changed to include more and frequent boosters to sensitize

the mouse more extensively. The success of changes in these two areas should be reflected as a positive polyclonal response in the serum which should be monitored under any circumstances.

Lastly, a working reliable and fast screening assay is a necessity in the early stages of monoclonal selection. The changes suggested earlier in the discussion for the Elisa may provide for the means to develop the assay into a more reliable one than in the past attempts.

A certain amount of luck, patience and persistence is needed for a successful fusion. If one protocol does not work, try a second but changing (a) the antigen composition i.e purified versus cell lysate; (b) the amount of antigen injected per booster; (c) the schedule of the immunization and (d) the route of sensitization.

APPENDIX

The technique of "protein blotting" has become a very widely used tool in protein analysis. Combining the resolving power of polyacrylamide electrophoresis and the sensitivity and specificity of immunochemistry, the technique can facilitate the identification and function of proteins in question. The technique involves basically the transfer of proteins from a gel onto an immobilizing matrix, such as a nitrocellulose filter. It is the filter that is then used in the immunochemical studies.

Three approaches have evolved to answer the problem of protein elution. The first is based upon the blotting technique devised by Southern (Southern, 1975). The gel is placed in a reservoir filled with a transfer buffer. On the gel is placed a filter and layers of adsorbent paper. The flow of the fluids is sufficient to elute the proteins towards the filter where they are trapped (Schaltmann and Pongs, 1980).

The second method, described by Bowen *et al.* (1980), is dependent on the diffusion of the proteins out from the gel onto the filters. The gel is sandwiched between two filters, two foam pads and two steel support screens. The sandwich is then placed into a tank filled with transfer

buffer for several hours. During this time, proteins will diffuse out from either side of the gel and are trapped by the filters (Bowen et al., 1980).

Presently, the most popular mode of transfer is that of electroelution. The gel and filter are again arranged into a sandwich configuration between two Scotch-Brite pads and two nonconductive supports. This is placed in a transfer box filled with buffer. Within the box, the electrodes are arranged in such a manner to provide a homogeneous electric field over the gel resulting in an even transfer (Towbin et al., 1979).

The use of protein transfers has centred around two primary objectives: (i) to illustrate protein-protein or protein-ligand interrelationships and (ii) to use the immobilized state as an intermediate step in further biochemical or immunological analysis.

Bowen et al. (1980) used protein transfers as a tool to study DNA-protein interaction. Hela nuclear, as well as, Hela nuclear matrix proteins were separated by PAGE and transferred to nitrocellulose filters. Radioactive probes, such as ^{32}P-DNA, ^{125}I-RNA, and ^{125}I-histone, were used to detect DNA-protein, RNA-protein and protein-protein interactions.

The sensitivity of immunochemical analysis of protein

transfers has been useful in the detection of specific and low quantity proteins in cell extracts. Symington et al. (1981) have detected specific Adenovirus-12 viral proteins in infected-KB cells by treating transfers with antiserum raised against Ad-2 virions and with anti-72K serum (72K is a DNA-binding protein encoded by the Ad-2 DNA). Levels as low as one nanogram of protein at a concentration of one part in 10^5 cell proteins were detected using the protocol described by Symington.

Glass et al. (1981) have extended the use of immunochemical analysis of protein transfers to detect tissue--specific antigens. In comparing chromatin from Novikoff ascites hepatoma and normal rat liver, a hepatoma-specific nuclear antigen (mwt=94,000 kd) as well as common antigens could be detected.

Smith and Summers (1981) developed a radioimmunoassay, using the protein transfer as the immobilizing step, to establish the antigenic relatedness amongst 17 different species of baculoviruses.

Radioimmunoassay and protein transfers have been used to study antibody specificity. Renart et al. (1979) have shown that a rabbit anti-T serum reacts specifically with a 85,000 Mwt polypeptide (large T) and 20,000 Mwt polypeptide (small T) in SV40-infected monkey cells. Antiserum to SV40

polypeptides, anti VP1 and anti-VP3, were shown to react specifically, with no cross-reactivity to their respective antigens.

As evident in the literature, protein transfers have become a very versatile tool in studying protein-protein interactions. Our goal was to refine the technique of protein transfer and immunochemistry to the state of becoming a reliable technique for screening of monoclonal cultures.

Methods

(i) Transfer of Proteins onto Nitrocellulose Filters from SDS-gels. (Bowen et. al, 1980)

To transfer proteins passively from SDS-gels onto nitrocellulose filters, the method described by Bowen et al. (1980) was essentially followed. Gels were placed in a urea containing buffer (10 mM Tris-HCl pH 7.0, 0.1 M dithiothreitol, 2 mM NaEDTA, 50 mM NaCl, and 4M urea) to allow the SDS to dissolve from the gel. The gels were then arranged in a sandwich configuration, between two nitrocellulose filters followed by two foam pads and then screen supports on both sides, as described by Bowen. The sandwich was then placed into a container filled with the transfer buffer (10 mM Tris-HCl pH 7.0, 50 mM NaCl, 2 mM NaEDTA, and 0.1 mM dithiothreitol). After 12-18 hours, the buffer was changed, adding fresh buffer to the container. The transfer

was then completed 24 hours later for a total transfer time of 36-48 hours. The gels were then stained with Coomassie blue (0.1%) to determine the extent of transfer.

(ii) Electrophoretic transfers of proteins from SDS-gels to nitrocellulose filters. (Towbin et al., 1979).

Electrophoretic transfer of proteins from SDS-gels to nitrocellulose was completed according to the procedure described by Towbin et al. (1979). The nitrocellulose filters to be used in the transfer are soaked for a few minutes in water. The wet filter is then placed on a Scotch-Brite pad which is supported by a plastic support. The gel is then layered upon the filter. Care was taken in this step to ensure that there were no air bubbles present between the gel and the filter. The second Scotch-Brite pad as well as the second support was then placed on top of the gel. The layers were then bound together with a few rubber bands.

The gel sandwich was then placed in a transfer box filled with transfer buffer (25 mM Tris- 192 mM glycine pH 8.3, 20% v/v methanol). It is important to note that the nitrocellulose filter must be facing the anode in order for transfer to occur onto the filter. An electric current of 225 mA was then applied for 2 hours. After the transfer time, the gel was fixed and then stained with Coomassie blue

to establish the efficiency of transfer.

(iii) Staining of transfers with amido black.

To determine the extent of transfer of proteins onto the nitrocellulose filters, the sheets were stained with amido black (0.1% amido black in 45% methanol/45% water/10% acetic acid) for 2-3 minutes. After a 30 second rinse in distilled water, the transfers were washed with destainer (90% methanol/8% water/2% acetic acid) three times. The final wash was with distilled water prior to blotting dry the filters. Completely dried filters were then exposed on film for autoradiography.

(iv) Immuno-enzyme staining of transfers (Towbin et al, 1979).

To stain the transfers immuno-enyzmatically, the following procedure was used. The nitrocellulose sheets were soaked for a minimum of 2 hours at 39.5^{o}C in a 3% BSA-saline (0.9% NaCl/10 mM Tris-HCl pH 7.4) solution. After five washes in saline, they were incubated in the test sera diluted accordingly in a saline-3% BSA-10% NBCS solution overnight (16-20 hours) at 37^{o}C. They were then rinsed 5 times, 30 seconds for each wash, with saline. The transfers were then incubated for 2.5 hours with horseradish peroxidase-conjugated IgG diluted in the BSA/NBCS/saline buffer. After

further washes with saline, the transfers were placed for 20-30 minutes into a freshly made substrate solution containing 25 ug of o-dianisidine/ml. To stop the staining reaction, the transfers were washed in distilled water and then allowed to dry completely before autoradiography and or photography.

Results

(i) Electrophoretic Transfer of proteins onto nitrocellulos filters.

To transfer proteins electrophoretically onto nitrocellulose, a simplified transfer box was constructed. The box essentially contained only two platinum electrodes. The first was attached across the bottom of the box in an S--shape configuration. The second was attached as a single strand to the top.

To test the system, ^{35}S-methionine labelled VSV-infected cell extracts were run on 10% polyacrylamide gels. The gels were then placed into the sandwich apparatus Towbin (1979) described. This was then placed in the transfer box filled with transfer buffer. A constant voltage of 5V/cm (30V or 225mA) was then applied for 1 1/2 to 2 hours.

Although the results of the electroblotting are not presented here, the efficiency of transfer varied greatly

from one attempt to another. Several transfers contained "bald spots" resulting from air bubbles trapped between the gel and the filter. Not all the bands within a run were equally transferred with an equal efficiency. Transfer of high molecular weight bands was considerably less efficient than the lower molecular weight material. Since consistency between successive trials was difficult to achieve, a second mode of transfer, that of diffusion was attempted.

(ii) Passive Transfer of proteins onto nitrocellulose from SDS-gels.

To test the efficiency of passive transfer of proteins onto nitrocellulose from SDS-gels, the following experiment was performed. Once again, labelled extracts from VSV-infected cells were run on 10% acrylamide gels. The gels were processed then according to the protocol described by Bowen et al. (1980). Transfer times ranged from 36 to 48 hours. After this time, the gels were stained with Coomassie blue or prepared for autoradiography. The transfers likewise, were dried and prepared for autoradiography.

Comparing the autoradiograms of the gel and it's respective transfer illustrated the fidelity of transfer that can be achieved with this technique. The filters were exact replicas of their parental gel. Bald spots, that were so evident on the electrophoretic transfers, were not

present in these transfers. All the bands, from the high molecular weight proteins to the lower ones were transferred equally well during the allotted transfer time period. As the method produced highly reproducible transfers between consecutive attempts, it was the method adopted in the remainder of the protein transfers.

(iii) Immunostaining of Protein Transfers.

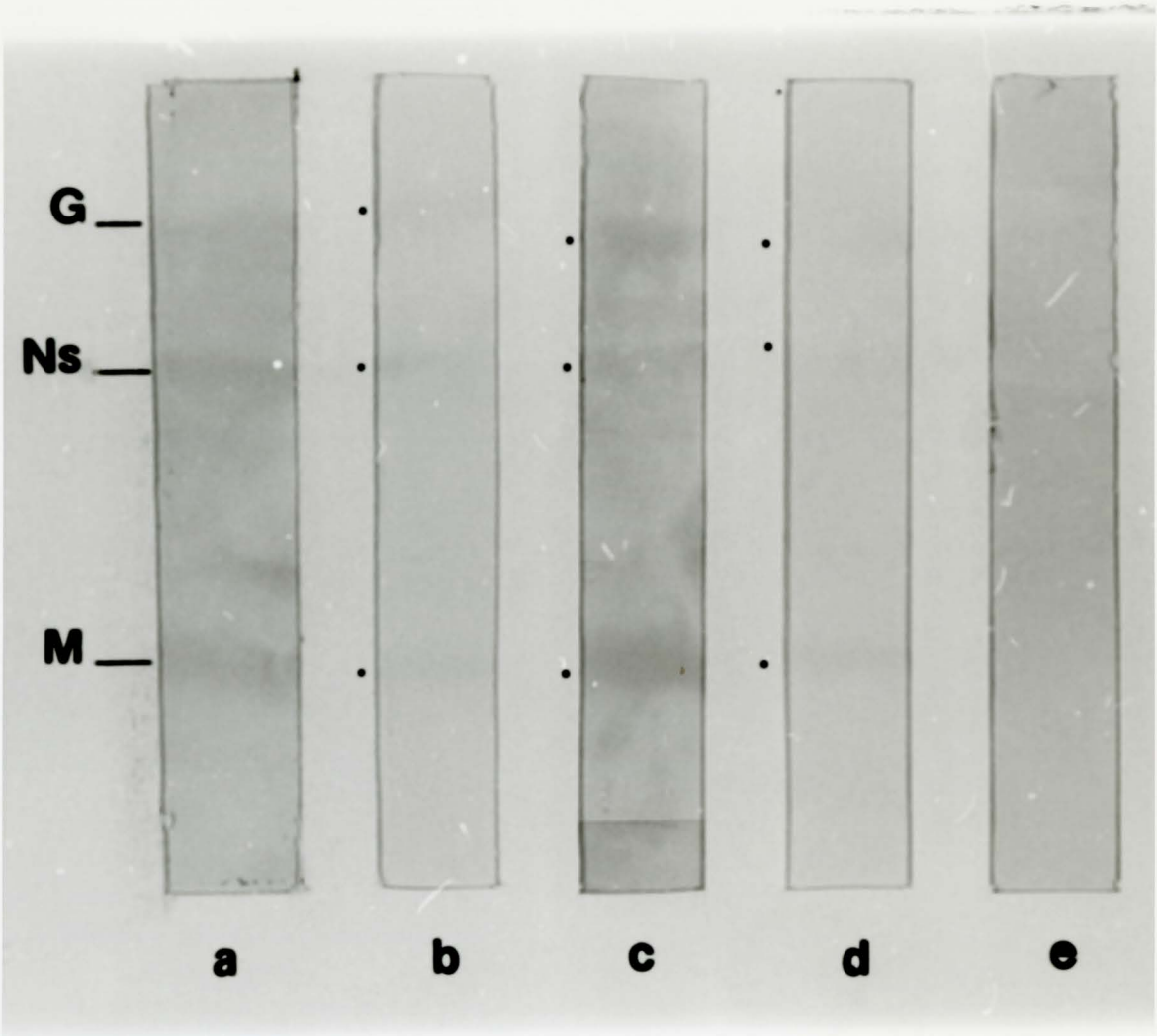
To establish the effectiveness of protein transfers as a screening tool for monoclonal or polyclonal antisera, the transfers were stained immunoenzymatically with a known rabbit antiVSV serum as the test antibody. After exposure to one of the three rabbit serum dilutions (neat, 1/5, 1/10), the transfers were incubated with HRP-conjugated goat antirabbit IgG as described in the methods. The coloured transfers were then dried and prepared for autoradiography.

As is depicted in Fig. 10, three of the five VSV proteins, M, Ns and G proteins are detected using this method. Earlier immunoprecipitation studies with this antisera and labelled infected cell extracts showed that the antisera was capable of precipitating M, N, NS and G. Examination of the autoradiograms of these transfers show that N protein is well represented in comparison to NS or G protein. The lack of response of this sera with N can possibly be attributed to a low antiN titre which is below the minimum titre

Fig. 10: Immunostaining of VSV Proteins with Rabbit antiVSV serum.

Transfers of ^{35}S-methionine labelled VSV proteins were incubated in a BSA/saline solution for 1.5 hours at 37°C. This was followed by an incubation in antiVSV serum for 20 hours at 37°C. After washing, the transfers were incubated for 2 hours in the second antibody HRP-goat antirabbit IgG. The transfers were washed and then placed in the substrate solution. Twenty minutes in substrate solution was sufficient to give enough colour to distinguish the bands.

Lane (a) Neat rabbit antiVSV; HRP-goat antirabbit IgG 10^{-2}; (b) neat rabbit antiVSV; HRP-goat antirabbit IgG 10^{-3}; (c) rabbit antiVSV 1/5; HRP-goat antirabbit IgG 10^{-2}; (d) rabbit antiVSV 1/5; HRP-goat antirabbit IgG 10^{-3}; (e) rabbit antiVSV 1/10; HRP-goat antirabbit IgG 10-2.


G
Ns
M
a
b
c
d
e

required for this assay.

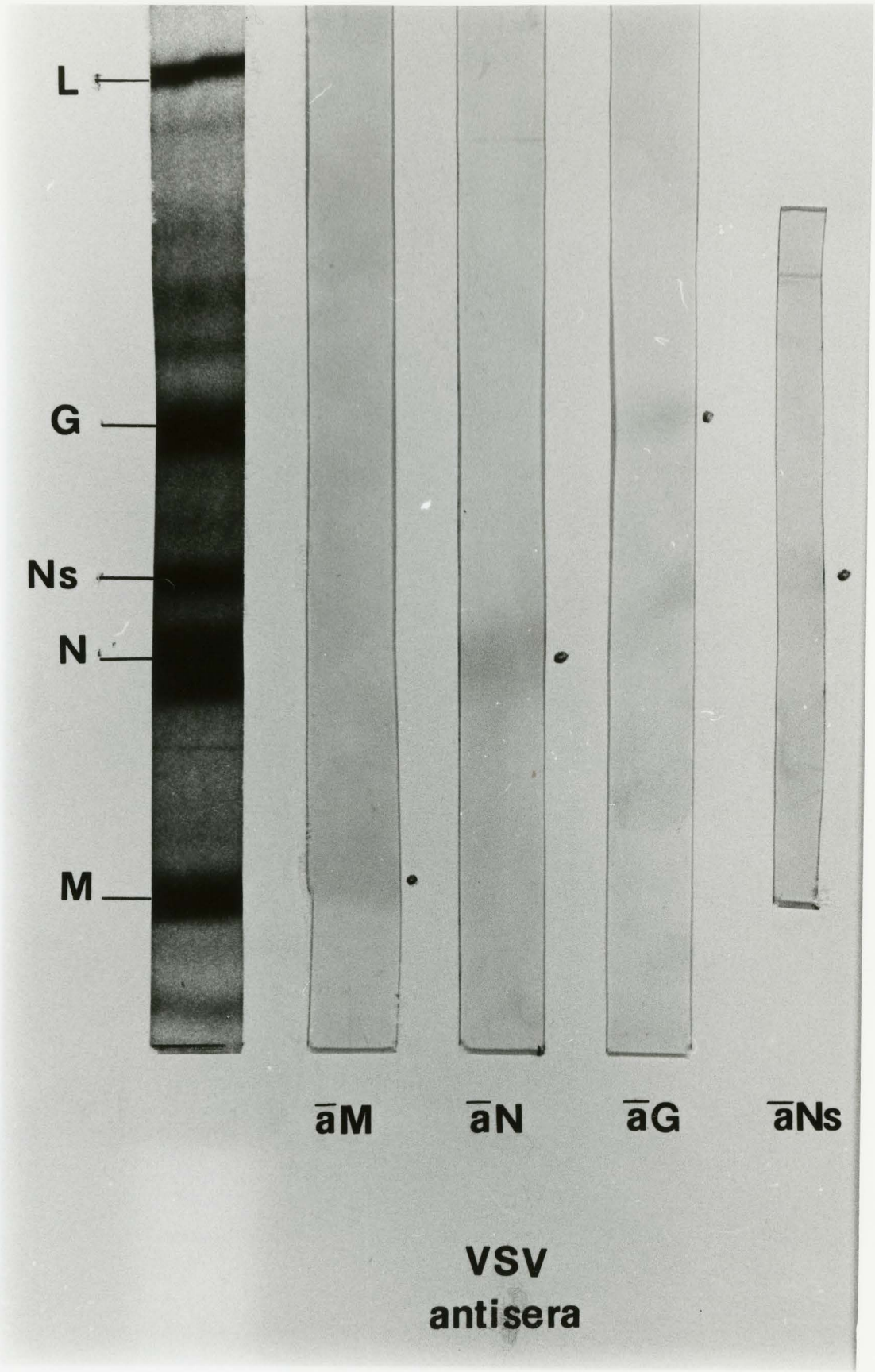
As a follow up to this experiment, monospecific antisera (antiM, antiNs, antiN and antiG) was used to replace the polyclonal rabbit serum as the test antibody. Fig. 11 illustrates the results of such an experiment. In the photograph, it is very difficult to discern the bands stained immunoenzymatically with the monospecific antisera. However, the original transfers do show that the monospecific antisera react specifically with their respective antigen.

The colour intensity of the stained bands varies between the different antisera tested. This variation could be attributed to one of two factors or perhaps both. Firstly, the antibody titre of each specific sera is different. A higher titre will produce a much stronger signal than a lower titre of antibody.

Secondly, the amount of protein present on the transfer of each viral protein varies. A quick visual examination of the autoradiograms of the transfers suggest that N protein is the more abundant protein as indicated by the darker band on the X-ray film. The other proteins, M, G, and NS are found in lesser amounts and in that order. As a result, the interaction between N and antiN antibody (which produces a much stronger signal than the NS and antiNS interactions) could be possibly attributed to the amount of protein

Fig. 11: Immunostaining of VSV Transfers with Monospecific antiserum.

Transfers of labelled VSV-infected cell extracts were incubated with 5 ml of monospecific antiserum overnight at $37^{o}C$ with the HRP-goat antirabbit IgG at a dilution of 10^{-2}. Twenty minutes in the substrate solution was sufficient to distinguish the bands. The transfers were dried and exposed on Kodak XRP-1 film overnight.


L
G
Ns
N
M
āM
āN
āG
āNs
VSV
antisera

present on the transfer.

Discussion

In order for electrophoretic transfer of proteins to be an effective mode of transfer, the problems associated with trapped air bubbles between the gel and the filter must be circumvented. The trapped bubbles provide areas of high resistance and hence reduced transfer efficiency resulting in the bald spots so evident in many stained transfers. To eliminate this problem, the gel must be fitted closely to the filter within the gel sandwich and throughout the entire transfer period (Geroshoni and Pallade, 1983). Perhaps a different support design, something of a heavier material would provide for a tighter fit between the two objects.

The commercially available electroblotting apparatus is constructed such that the gel sandwich stands vertically between the two electrodes. This allows for the bubbles to travel upwards and parallel to the filter rather than through the sandwich as it does in the horizontal apparatus. This construction then reduces the problems in transfer efficiency that are attributed to air bubbles.

Electroelution of proteins is apparently dependent upon their molecular weight. High molecular weight species are the less efficient to transfer, creating a misrepresentation

of the original gel pattern. Geroshoni and Pallade (1983) describe three approaches to overcome this problem. Briefly they are: (i) use of a reversible gel linker followed by gel polymerization; (ii) limited protease digestion to smaller transferable units; and (iii) addition of a detergent such as SDS to the transfer buffer.

In the system used, this artifact created by the dependency of transfer on molecular weight did not prove to be a serious problem. All the bands appeared to transfer reasonably well whenever the air bubble problem was resolved.

Since electroeluting proteins did not give consistent results between trials, the mode of transfer via diffusion was adopted. This method is a very simple yet an efficient way to transfer proteins. Bowen et al. (1980) claims that the overall efficiency of transfer between the two filters is approximately 75%. Although exact measurements were not taken, comparisons of the gel strips before and after transfer, stained with Coomassie blue, showed a relative decrease in overall protein content after transfer.

It is a longer process compared to electroelution, which could be considered a negative characteristic if time is of importance. However, the lengthy transfer time permits the higher molecular weight proteins to be transferred more effectively. These higher molecular weight molecules

may also be transferred electrophoretically using a longer transfer time, such as overnight. In doing so, care must be taken to regulate the current and temperature of the system. The buffer tends to heat up and will effectively "cook" the gel, destroying it. As a result, the longer transfer times are done at a low voltage and at $4^{o}C$.

The immunostaining of the transfers provided an insight into the applicability of protein transfers in screening polyclonal and or monospecific antisera. The results depicted in Figs. 10 and 11, illustrate the capability of the antibodies to interact specifically with their respective antigens despite the immobilized state of the antigen. The immobilization of the antigen allows the antigen-antibody interaction to be detected easily without necessitating the formation of precipitates as in radioimmunoprecipitation. As Towbin et al. (1979) suggests, monovalent antibodies such as monoclonal antibodies can be screened and characterized using this method provided that some information is known about the antigen in terms of its electrophoretic pattern in SDS-gels.

Summary

As the results would indicate, protein transfers could prove to be a useful tool in screening monoclonal and polyclonal antisera. The simple and yet sensitive assay

will be greatly effective in establishing the relationship between the antibody and its respective antigen.

BIBLIOGRAPHY

ARSENAKIS, M. and B. Roizman. (1984). A PostαGene Function Turns off the Capacity of a Host Protein to Bind DNA in cells infected with Herpes Simplex 1. J. of Virology 49: 813-818.

BACCHETTI, S., Evelegh, M.J., Muirhead, B., Sartori, C.S., and D. Huszar. (1984). Immunological Characterization of Herpes Simplex Virus Type 1 and 2 Polypeptides Involved in Viral Ribonucleotide Reductase. J. of Virology 49: 591-593.

BALACHANDRAN, N., Harnish, D., Killington, R.A., Bacchetti, S. and W.E. Rawls. (1981). Monoclonal Antibodies to Two Glycoproteins of Herpes Simplex Virus Type 2. J. of Virology 39: 438-446.

BALACHANDRAN, N., Harnish, D., Rawls, W.E. and S. Bacchetti. (1982). Glycoproteins of Herpes Simplex Virus Type 2 As Defined by Monoclonal Antibodies. J. of Virology 44: 344-355.

BOOKOUT, J.B. and C.C. Levy. (1980). Comparative Examination of the Polypeptides of Herpes Simplex Virus: Types 1 and 2. Virology 101; 198-216.

BOWEN, B., Steinberg, J., Laemmli, U.K. and H. Weintraub. (1980). The detection of DNA-binding proteins by protein blotting. Nucleic Acids Research 8: 1-20.

CLEMENTS, J.B. and J. Hay. (1977). Review Article: RNA and Protein Synthesis in Herpesvirus-infected cells. J. Gen. Virology 55: 1-12.

COHEN, G.H., Ponce de Leon, M., Diggelmann, H., Lawrence, W.C., Vernon, S.K., and R.J. Eisenberg. (1980). Structural Analysis of the Capsid Polypeptides of Herpes Simplex Virus Type 1 and 2. J. of Virology 34: 521-531.

COMPTON, T. and R.J. Courtney. (1984). Virus-Specific Glycoproteins Associated with the Nuclear Fraction of Herpes Simplex Virus Type 1-infected Cells. J. of Virology 49: 594-597.

DIXON, R.A.F. and P.A. Schaffer. (1980). Fine-Structure Mapping and Functional Analysis of Temperature-Sensitive Mutants in the Gene Encoding the Herpes Simplex Virus Type 1 Immediate Early Protein VP175. J. of Virology 36: 189-203.

EASTON, A.J. and J.B. Clements. (1980). Temporal Regulation of Herpes Simplex Virus Type 2 Transcription and Characterization of Virus Immediate Early mRNA's. Nucleic Acids Research 8: 2627-2645.

ENGVALL, E. and P. Perlmann. (1972). Enzyme-linked immuno--sorbent assay, ELISA III. Quantititation of Specific Antibodies by Enzyme-labelled AntiImmunoglobulin in Antigen-coated Tubes. J. of Immunology 109: 129-135.

EVELEGH, M.J., Sartori, C. and S. Bacchetti. (1984). Production and Characterization of Monoclonal Antibodies

to 12 Different Polypeptides found in Herpes Simplex Virus Type 2 Infected cells. (manuscript in preparation).

FLAMAND, A., Wiktor, T.J. and H. Koprowski. (1980). Use of Hybridoma Monoclonal Antibodies in the Detection of Antigenic Differences Between Rabies and Rabies--related Virus Proteins. I. The Nucleocapsid Protein. J. Gen. Virology 48: 97-104.

FREEMAN, M.J. and K.L. Powell. (1982). DNA-Binding Properties of a Herpes Simplex Virus Immediate Early Protein. J. of Virology 44: 1084-1087.

GALLOWAY, D.A., Fenoglio, C.M. and J.K. McDougall. (1982). Limited Transcription of the Herpes Simplex Virus Genome When Latent in Human Sensory Ganglia. J. of Virology 41: 686-691.

GALLOWAY, D.A. and J.K. McDougall. (1983). The Oncogenic Potential of Herpes Simplex Viruses: Evidence For a 'Hit-and-Run' Mechanism. Nature 302: 21-24.

GEROSHONE, J.M. and G.E. Palade. (1983). Protein Blotting: Principles and Applications. Analytical Biochemistry 131: 1-15.

GILMAN, S.C., Docherty, J.J., Clarke, A. and W.E. Rawls. (1980). Reaction Patterns of Herpes Simplex Virus Type 1 and 2 Proteins with Sera of Patients with Uterine Cervical Carcinoma and Matched Controls. Cancer Research 40: 4640-4647.

GLASS, W.F., Briggs, R.C. and L.S. Hnilica. (1981). Identification of Tissue-Specific Nuclear Antigens Transferred to Nitrocellulose from Polyacrylamide Gels. Science 211: 70-71.

HONESS, R.W. and D.H. Watson. (1974). Herpes SImplex Virus--Specific Polypeptides Studied by Polyacrylamide Gel Electrophoresis of Immune Precipitates. J. Gen. Virology 22: 171-185.

HONESS, R.W. and B. Roizman. (1974). Regulation of Herpesvirus Macromolecular Synthesis. I. Cascade Regulation of the Syntheis of Three Groups of Viral Proteins. J. of Virology 14: 8-19.

HONESS, R.W. and B. Roizman. (1975). Regulation of Herpesvirus Macromolecular Synthesis: Sequential Transition of Polypeptide Synthesis Requires Virus Polypeptides. Proceedings of the National Academy of Sciences, USA 72: 1276-1280.

HUSZAR, D. and S. Bacchetti. (1981). Partial Purification and Characterization of the Ribonucleotide Reductase Induced by Herpes Simplex Virus Infection of Mammalian cells. J. of Virology 37: 580-588.

KILLINGTON, R.A., Newhook, L., Balachandran, N., Rawls, W.E. and S. Bacchetti. (1981). Production of Hybrid Cell Lines Secreting Antibodies to Herpes Simplex Virus Type 2.

J. of Virol. Methods 2: 223-236.

KOHLER, G. and C. Milstein. (1975). Continuous cultures of fused cells secreting antibody of predefined specificity. Nature 256: 495-497.

KOHLER, G. and C. Milstein. (1976). Derivation of specific antibody-producing tissue culture and tumour lines by cell fusion. Eur. J. of Immunology 6: 511-519.

KOPROWSKI, H., Gerhard, W. and C.M. Croce. (1977). Production of Antibodies Against Influenza Virus By Somatic Cell Hybrids Between Mouse Myeloma and Primed Spleen Cells. PNAS 74: 2985-2988.

KOPROWSKI, H., Steplewski, Z., Herlyn, D. and M. Herlyn. (1978). Study of Antibodies Against Human Melanoma Produced by Somatic Cell Hybrids. PNAS 75: 3405-3409.

LAEMMLI, U.K. (1970). Cleavage of Structural Proteins During the Assembly of the Head of Bacteriophage T4. Nature 227: 680-685.

LaTHANGUE, N.B. and W.L. Chan. (1984). The Characterization and Purification of DNA-Binding Proteins Present Within Herpes Simplex Virus Infected Cells Using Monoclonal Antibodies. Archives of Virology 79: 13-33.

LEMASTER, S. and B. Roizman. (1980). Herpes Simplex Virus Phosphoproteins (II) Characterization of the Virion Protein Kinase and of the Polypeptides Phosphorylated in the Virion. J. of Virology 35: 798-811.

LITTLER, E., Purifoy, D., Minson, A. and K. Powell. (1983). Herpes Simplex Virus Non-structural Proteins III. Function of the Major DNA-binding protein. J. of General Virology 64: 983-995.

MARSDEN, H.S., Stow, N.D., Preston, V.G., Timbury, M.C. and N.M. Wilkie. (1978). Physical Mapping of Herpes Simplex Virus-Induced Polypeptides. J. of Virology 28: 624-642.

MATIS, J. and J. Rajcani. (1980). Preparation of Immune Serum to Immediate Early and Early Polypeptides Specified by Herpes Simplex Virus Type 1. Acta Virol. 24: 105-113.

O'SULLIVAN, M.J., Bridges, J.W. and V. Marks. (1979). Enzyme immunoassay: a review. Annals of Clinical Biochemistry. 16: 221-240.

PARSONS, S.J., McCarley, D.S., Ely, C.M., Benjamin, D.C. and J.T. Parsons. (1983). Isolation and Partial Characterization of a Monoclonal Antibody to the Rous Sarcoma Virus Transforming Protein $pp60^{src}$. J. of Virology 45: 1190-1194.

PEREIRA, L., Wolff, M.H., Fenwick, M. and B. Roizman. (1977). Regulation of Herpesvirus Macromolecular Synthesis V). Properties of α polypeptides made in HSV 1 and HSV 2 infected cells. Virology 77: 733-749.

POWELL, K.L. and R.J. Courtney. (1975). Polypeptides Synthesized in Herpes Simplex Virus Type 2-Infected HEP-2 Cells. Virology 66: 217-228.

POWELL, K.L., Littler, E. and D.J.M. Purifoy. (1981). Nonstructural Proteins of Herpes Simplex Virus II. Major Virus-Specific DNA-Binding Proteins. J. of Virology 39: 894-902.

PRESTON, V.G. (1981). Fine-Structure Mapping of Herpes Simplex Virus Type 1 Temperature-sensitive Mutations Within the Short Repeat Region of the Genome. J. of Virology 39: 150-161.

PRESTON, V.G., Davison, A.J., Marsden, H.S., Timbury, M.C., Subak-Sharpe, J.H. and N.M. Wilkie. (1978). Recombinants Between Herpes Simplex Virus Type 1 and 2: Analysis of Genome Structures and Expression of Immediate Early Polypeptides. J. of Virology 28: 499-517.

PURIFOY, D.J.M. and K.L. Powell. (1976). DNA-Binding Proteins Induced by Herpes Simplex Virus Type 2 in HEp-2 Cells. J. of Virology 19: 717-731.

RAWLS, W.E., Bacchetti, S. and F.L. Graham. (1977). Relation of Herpes Simplex Viruses to Human Malignancies. Current Topics in Microbiology and Immunology 77: 71-95.

REAY, G.S. and N. Frenkel. (1983). Herpes Simplex Virus Mutants Defective in the Virion-Associated Shutoff of Host Polypeptide Synthesis and Exhibiting Abnormal Synthesis of α (Immediate Early) Viral Polypeptides.

J. of Virology 46: 498-512.

RENART, J., Reiser, J. and G.R. Stark. (1979). Transfer of Proteins From Gels to Diazobenzyloxymethyl-paper and Detection with Antisera: A Method for Studying Antibody Specificity and Antigen Structure. PNAS 76: 3116-3120.

ROIZMAN, B. The Herpesviruses pg. 769-848 in The Molecular Biology of Animal Viruses, Volume 2. editied by D.P. Nayak New York: Marrel Dekker Inc. 1978.

ROIZMAN, B., Norrild, B., Chan, C. and L. Pereira. (1984). Identification and Preliminary Mapping with Monoclonal Antibodies of a Herpes Simplex Virus 2 Glycoprotein Lacking a Known Type 1 Counterpart. Virology 133: 242-247.

SCHALTMANN, K. and O. Pongs. (1980). A simple procedure for Blotting of Proteins to Study Antibody Specificity and Antigen Structure. Hoppe-Seyler's Z. Physiol. Chem. 361: 207-210.

SECHER, D.S. and D.C. Burke. (1980). A Monoclonal Antibody for Large-Scale Purification of Human Leukocyte Interferon. Nature 285: 446-450.

SEKIZAWA, T., Openshaw, H., Wohlenber, C. and A.L. Notkens. (1980). Latency of Herpes Simplex Virus in Absence of Neutralizing Antibody: Model for Reactivation. Science 210: 1026-1028.

SMITH, G.E. and M.D. Summers. (1981). Application of a Novel Radioimmunoassay to Identify Baculovirus Structural Proteins That Share Interspecies Antigenic Determinants. J. of Virology 39: 125-137.

SMITH, J.W., Lowry, S.P., Melnick, J.L. and W.E. Rawls. (1972). Antibodies to Surface Antigens of Herpesvirus Type 1 and Type 2-Infected cells Among Women with Cervical Cancer and Control Women. Infection and Immunity 5: 305-310.

SOUTHERN, E.M. (1975). Detection of Specific Sequences Among DNA Fragments Separated by Gel Electrophoresis. J. Mol. Biol. 98: 503-517.

SPEAR, P.G. and B. Roizman. (1972). Proteins specified by Herpes Simplex Virus (V) Purification and Structural Proteins of the Herpesvirion. J. of Virology 9: 143-159.

STRNAD, B.C. and L. Aurelian. (1976). Proteins of Herpesvirus Type 2: (I) Virion, Nonvirion and Antigenic Polypeptides in Infected cells. Virology 69: 438-452.

SYMINGTON, J., Green, M. and K. Brackmann. (1981). Immunoautoradiographic Detection of Proteins After Electrophoretic Transfer from Gels to Diazo-Paper: Analysis of Adenovirus-Encoded Proteins. PNAS 78: 177-181.

TOOZE, J. The Molecular Biology of Tumor Viruses. New York: Cold Spring Harbor Laboratory, 1980.

TOWBIN, J., Staehelin, T. and J. Gordon. (1979). Electrophoretic Transfer of Proteins from Polyacrylamide Gels

to Nitrocellulose Sheets: Procedure and Some Applications. PNAS 76: 4350-4354.

WATSON, R.J. and J.B. Clements. (1978). Characterization of Transcription Deficient Temperature-Sensitive Mutants of Herpes Simplex Virus Type 1. Virology 91: 364-379.

WILCOX, K.W., Kohn, A., Sklyanskaya, E. and B. Roizman. (1980). Herpes Simplex Virus Phosphoproteins I). Phosphate Cycles On and Off Some Viral Polypeptides and Can Alter Their Affinity for DNA. J. of Virology 33: 167-182.

WITKIN, S.S., Sarkar, N.H., Good, R.A. and N.K. Day. (1980). An Enzyme-Linked Immunoassay for the Detection of Antibodies to the Mouse Mammary Tumor Virus: Application to Human Breast Cancer. J. of Immunological Methods 32: 85-91.

ZEZULAK, K.M. and P.G. Spear. (1983). Characterization of a Herpes Simplex Virus Type 2 75,000 Molecular Weight Glycoprotein Antigenically related to Herpes Simplex Virus Type 1 Glycoprotein C. J. of Virology 47: 553-562.

ZWEIG, M., Heilman, Jr., C.J., Rabin, H., and B. Hampar. (1980). Shared Antigenic Determinants Between Two Distinct Classes of Proteins in Cells Infected with Herpes Simplex Virus. J. of Virology 35: 644-652.

ZWEIG, M., Heilman Jr., C.J., Rabin, H., Hopkins, R.F.,

Neubauer, R.H. and B. Hampar. (1979). Production of Monoclonal Antibodies Against Nucleocapsid Proteins of Herpes Simplex Virus Type 1 and 2. J. of Virology 32: 676-678.