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ANTIVIRAL ACTIVITY OF PERITONEAL EXUDATE CELLS OF MICE AGAINST INFECTION WITH HERPES SIMPLEX VIRUS TYPE 2

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ANTIVIRAL ACTIVITY OF PERITONEAL EXUDATE CELLS OF MICE AGAINST INFECTION WITH HERPES SIMPLEX VIRUS TYPE 2

By

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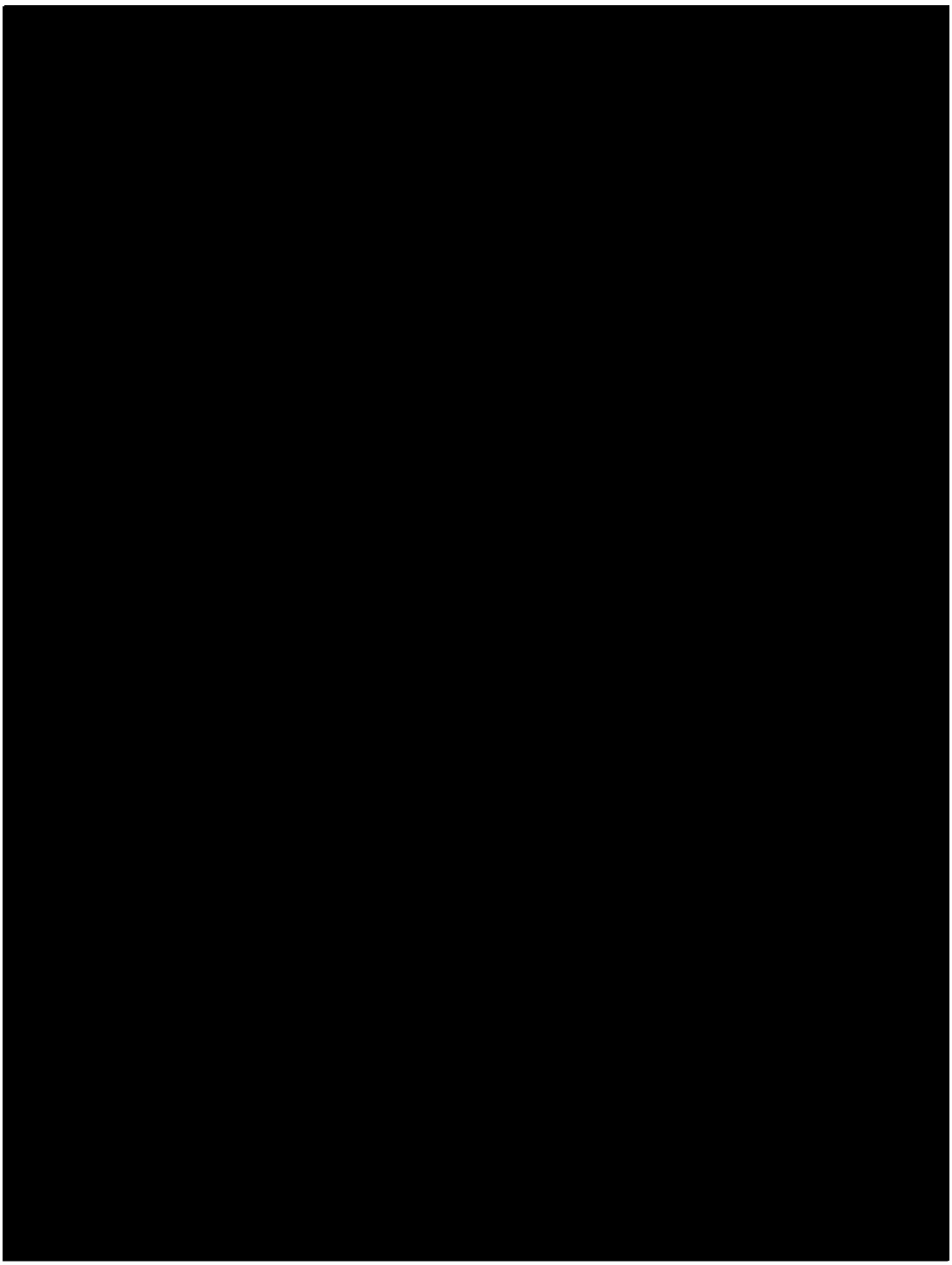

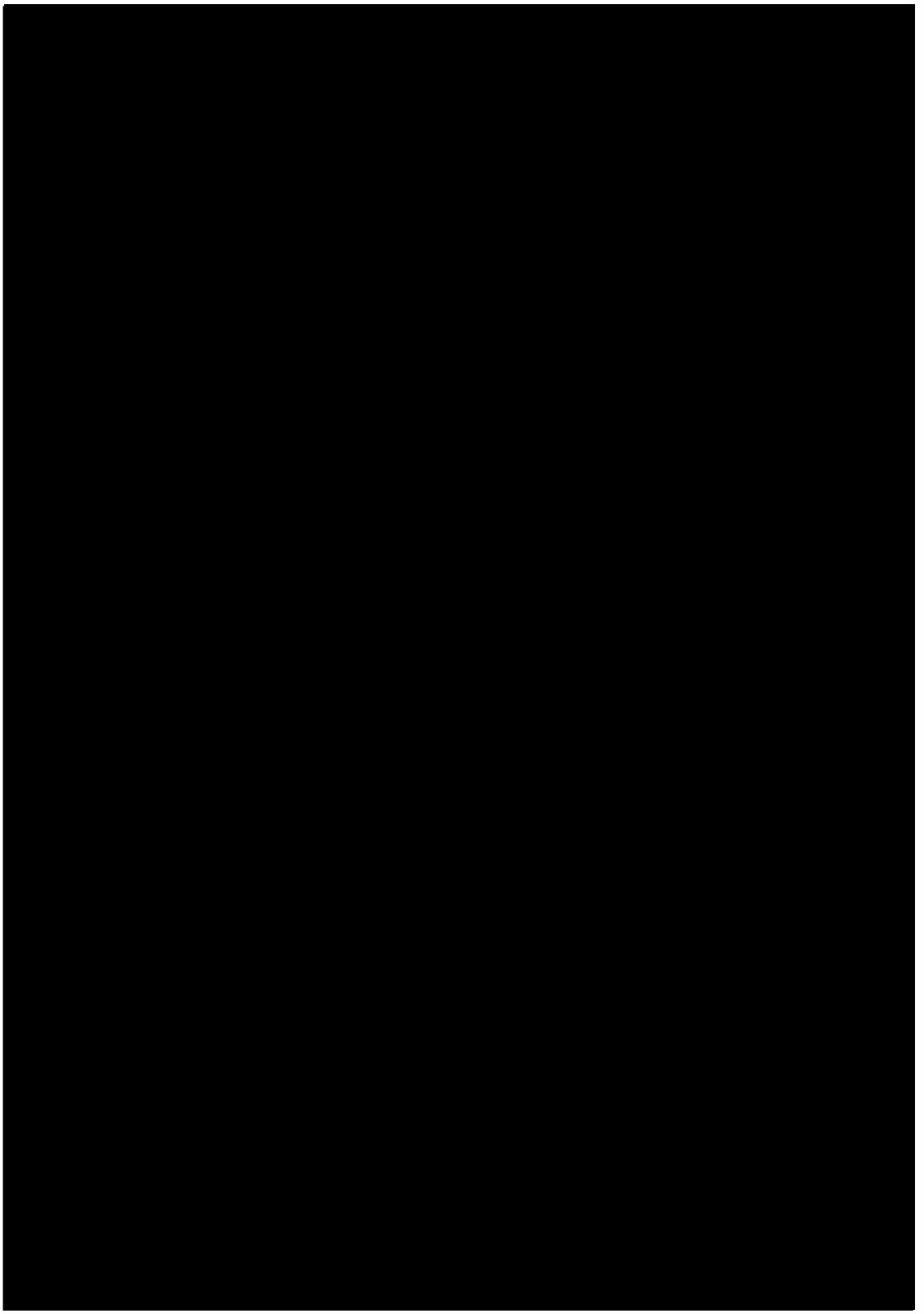

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INTRODUCTION

Herpes Simplex Virus and Cervical Carcinoma

Within the past decade, there has been increasing evidence which suggests that herpes simplex virus type two (HSV-2) is associated with cervical carcinoma. Because cancer of the cervix ranks as the second most common malignant disease of women, with 35 thousand new cases and 10,000 deaths per year, further investigation is warranted to determine if there is a causal relationship (Goldberg, 1976). Currently, most of the evidence which correlates HSV-2 with cancer of the cervix has been, for the most part, indirect: (i) there is an increased incidence of cervical anaplasia in women with cytologically detectable genital herpes infection, (ii) many patients with cervical carcinoma have a high titer of specific neutralizing antibody to HSV-2, (iii) HSV-2 has been isolated from a cell culture derived from one carcinoma in situ (Aurelian, 1976), (iv) HSV-2 membrane antigens were detected in a small percentage of cells from a culture not yielding infectious virus. In addition to the correlative studies, the transforming capacity of HSV-2 for human embryonic cells has been clearly demonstrated in vitro, and (vi) hamster cells transformed in vitro by the virus have oncogenic potential when injected into hamsters (Rapp and Reed, 1976). Lack of more direct evidence in relation to human carcinoma, however, such as the inability to demonstrate

hybridization of HSV-2 nucleic acid with that in the cervical carcinoma cell has left the issue of HSV-2 and cervical carcinoma cancer the subject of considerable controversy (zurHausen, 1976).

Latent Infection with Herpes Simplex Virus

In addition to the possible role of HSV-2 in initiating cervical carcinoma, the herpes simplex viruses of both type 1 and 2 are rather ubiquitous viruses which are common pathogens of man. Statistical evaluation has indicated that 70-80 percent of all individuals greater than 15 years of age have been infected with either HSV-1 or HSV-2. Moreover, at least one-third of individuals with antibody to HSV develop clinically apparent reactivation (Thong et al., 1975). HSV-2 is usually associated with lesions in the genital area and is second only to gonorrhea as the most prevalent form of venereal disease (Klein, 1976). In comparison to HSV-1, HSV-2 appears to be more neurotropic in experimental animal systems (Davis et al., 1973). Neonatal herpetic infections are often the result of mothers harboring genital HSV-2 infection and are usually severe or even fatal for the newborn.

The propensity for recurrent infections is also a property of the herpes simplex viruses. Latent HSV-1 and HSV-2 have been isolated from sensory and autonomic ganglia in situations which may manifest no clinical symptoms (Baringer, 1974). Recurrent infections of both HSV-1 and HSV-2 have been shown to occur not as a consequence of

re-infection but rather the maintenance of the virus in a noninfectious state (Stevens and Cook, 1971). The mechanisms by which the virus is reactivated to cause infection and the ability of the virus to reside in the host in a latent state are not well understood.

A number of investigators have demonstrated the existence of virus in the host between intervals of overt infection in the sensory ganglia innervating the area of infection (Stevens and Cook, 1976; Baringer, 1975). Baringer et al. (1975) reported recovery of HSV-2 virus from human sacral ganglia from patients who were known to have had a history of recurrent genital HSV-2 infection. Animal systems have also been established which demonstrate the presence of virus in ganglia following various routes of infection (Stevens and Cook, 1976; Hill, 1975).

In spite of all the available evidence concerning the existence of latent virus, there are currently no sufficient models for studying the mechanisms of maintenance and reactivation of virus under experimentally controlled conditions. There are several hypotheses which attempt to explain the mechanism(s) of latency, ranging from the biochemical state of the virus to the role of immunological mechanisms which may prevent the virus from being expressed in an infectious state (Stevens and Cook, 1974). The latter hypotheses involve the role of specific antibody and cell-mediated responses as mechanisms by which the virus is maintained in a quiescent state in the host (Stevens and

Cook, 1974; Lehner et al., 1975). Recurrences are explained by decreased levels of IgG or possible defects in the expression of humoral or cell-mediated immunity. The ability of the virus to become reactivated by such factors as trauma, sunlight, or stress may be explained by the fact these factors may contribute to the alteration of neurons such that specific antibody can no longer bind to the membrane and the virus is depressed and can proceed into the replicative cycles (Stevens and Cook, 1974).

Immunology of Herpes Simplex Virus Infection

With the increasing accumulation of data concerning the immune response to herpes simplex virus infection, it is now apparent that host resistance to this virus embraces a complex network of immunological interactions. These include specific humoral and cell-mediated immunological parameters as well as nonspecific factors. The relative contribution of each of these aspects remains to be elucidated.

Role of Thymus-Dependent Cells

The initial suggestion that T-cells were important in resistance to herpetic infection, stems, in part, from observations that immunosuppressed or T-cell deficient patients are predisposed to a higher incidence and increased severity of herpetic infections (Montgomerie et al., 1969). Patients on therapeutic immunosuppression, such as transplant recipients, have been reported to have a higher

incidence of disseminated herpetic disease than do immunologically normal individuals (Rand et al., 1976; Korsager et al., 1975). Furthermore, in vitro, Rand and co-workers (1976) reported relatively good correlation between increased risk to herpetic infection in cardiac transplant patients and a depressed state of in vitro lymphocyte transformation to mitogens of specific antigens. The infection may be exogenous, although it has been presumed, in some instances to be attributed to being a result of reactivation of a latent infection (Korsager et al., 1975). Other immunodeficient states such as the Wiskott-Aldrich syndrome and certain lymphoreticular disorders also give rise to higher incidences and in many cases more severe herpetic diseases of both HSV-1 and HSV-2 than in patients with adequate immune responses (St. Geme, 1976; Rand, 1976).

Investigations using experimental animal models have confirmed that cell-mediated immunity is an important mechanism in resistance to infection of HSV types 1 and 2 (Lodmell et al., 1973; Ennis et al., 1974). Thymectomized, anti-thymocyte treated or cyclophosphamide treated mice showed a marked increase in susceptibility to the lethal consequences of HSV infection (Mori et al., 1967; Zisman et al., 1969; Oakes, 1975). This was also manifested in augmented levels of virus recovered from the brain and liver (Rager-Zisman and Allison, 1976). To lend further credence to the importance of T-cells, passively transferred

immune spleen cells restored normal resistance to infected, immunosuppressed animals. The protective capacity of these spleen cells was abrogated by pretreatment with anti-theta serum and complement to remove T-cells (Ennis and Wells, 1974).

The precise mechanism(s) by which T-cells are able to mediate this antiviral activity are unclear. Information obtained from in vitro studies have suggested several possibilities.

Production of interferon may well be involved in the antiviral activity. Lodmell and Notkins (1974) demonstrated that BCG immune lymphocytes stimulated in vitro with purified protein derivitive (PPD) mediated their activity against HSV-1 in a nonspecific manner through the production of interferon. Furthermore, Fujibayashi et al. (1975) demonstrated the production of interferon by HSV-immune lymphocytes cultured in vitro in the presence of HSV antigen-antibody complexes. These cells also showed an increased proliferative response. Unlike sensitized leukocytes, normal leukocytes did not produce interferon in vitro but did also exert an antiviral effect (Lodmell and Notkins, 1974). This effect, however, was only observable when the ratio of leukocytes to infected target cells was very high; thus, it was assumed to be a generalized nonspecific cytotoxic effect (Lodmell and Notkins, 1974).

A second antiviral mechanism which may be operative in T-cell-mediated resistance may be through a specific cytotoxic response exerted by immune murine T-cells against virusinduced antigens expressed on the surface of target cells. This reaction has been demonstrated in several viral systems in vitro, and appears, in some cases, to require certain identities in the histocompatibility region between target and effector T-cells (Blanden et al., 1976; Zinkernagel, 1975).

There have been relatively few definitive reports concerning the cytotoxicity of immune T-cells from HSV-infected animals or humans. Leukocyte-mediated cytolysis has been reported in the human against HSV-infected cells but rigorous characterization of the cells and the mechanism(s) involved has not been performed (Russell and Kaiser, 1976). Pfizenmaier et al. (1977) reported that HSV-immune cytotoxic T-cells could be generated by removing T-cells from lymph nodes draining a local site infected with HSV-1, followed by a seventy-two hour incubation in vitro. Conventional direct T-cell cytotoxicity assays against HSV-infected cells have been comparatively less successful thus far.

In spite of the lack of data available concerning HSV cytotoxicity, there have been several reports of direct T-cell cytotoxicity with herpes viruses other than herpes simplex. Rouse et al. (1977) demonstrated antiviral cytotoxicity with immune peripheral blood lymphocytes against

infectious bovine rhinotracheitis (IBR) virus or feline rhinotracheitis virus (Wardley et al., 1976). In contrast to the findings of Zinkernagel and Blanden, in studies with the bovine system, there was no apparent histocompatibility requirement between effector and target cells. The immune T-cells killed autologous as well as homologous infected cells (Rouse et al., 1977). The disparity between requirements or lack of requirements for genetic restriction may well be due to the nature of the histocompatibility systems among the different species.

Other mechanisms of antiviral activity attributed to the T-cell include production of various soluble factors, in addition to interferon. Peripheral blood lymphocytes from humans which were seropositive for HSV produced lymphotoxin as well as lymphocyte-derived chemotactic factor when exposed to HSV antigen in vitro (Rosenberg et al., 1974). Other factors such as migration inhibitory factors have also been reported following exposure of immune lymphocytes to HSV antigen (Snyderman, 1972). The significance of these soluble factors during viral infection in vivo has not been defined; thus it would be somewhat premature to establish a definitive role.

Humoral Immunity to Herpes Simplex Infection

The role of antibody in protection from primary and recurrent herpetic infection is somewhat paradoxical. The contradictions stem from the observations that persons subject to recurrent infections generally have a consistently

high titer of circulating antibody to HSV (Rand, 1976). The obvious question, therefore, is whether antibody to HSV plays a protective role against recurrent infection, as suggested by Stevens and Cook concerning the maintenance of latency (Stevens and Cook, 1975).

Antibody to HSV-1 and HSV-2 have been demonstrated to be of the IgM and IgG classes, generally speaking. There is a considerably high degree of cross-reactivity of HSV-1 and HSV-2 antibody as a result of the antigenic similarities shared by the two types. Consequently, infection by one type may give rise to antibody capable of reacting with both types 1 and 2 (Plummer et al., 1968). Although the precise mechanisms of virus-antibody interactions are not well understood, it is presumed that antibody may exert an antiviral effect in several ways. Neutralizing antibody may exert its effect by direct inactivation of extracellular virus, thus limiting the spread of virus to surrounding cells (Ennis, 1973). There may be antibody present capable of recognizing viral antigens on the surfaces of infected cells, and in the presence of complement, causing destruction of these cells (Brier et al., 1971). Whether these responses are of major importance in protection against recurrent infection is unclear. Various results, however, indicate that passive transfer of immune serum affords some protection to animals when given prior to primary challenge with virus (Breinig et al., 1978; Zisman and Allison, 1976). A shortcoming of antibody-mediated protection is

the fact that while HSV may spread extracellularly, it may also spread contiguously to adjoining cells through intracellular bridges, limiting the effect of antibody (Pavan and Ennis, 1977). This may at least, in part, account for the fact that patients with chronic T-cell suppression may have severe herpetic lesions, in spite of the presence of high levels of circulating HSV antibody (Lodmell et al., 1973).

Another role for specific neutralizing antibody to HSV has been suggested recently by Day et al. (1976). They reported that specific antibody characteristic of the IgE class could be found in rabbits sensitized with HSV. This antibody, like other classical IgE antibodies, may function to mobilize or activate cells associated with classic inflammatory response such as mast cells and basophils.

Thus, while antibody can prevent primary HSV infection, the protective role of antibody in recovery from primary infection, or in prevention of or recovery from recurrent herpetic disease, remains somewhat elusive. The accumulating evidence suggests, however, that while antibody may be protective, it is not necessarily essential for recovery from infection (Rand et al., 1976). A more comprehensive understanding of the mechanisms of latency and reactivation may help to define the relative importance of this aspect of the immune response.

Synergistic Action of Humoral and Cell-Mediated Responses

There is a preponderance of evidence suggesting that cooperation of cell-mediated and humoral parameters may well be an effective antiviral host defense mechanism. Antibody-dependent cell-mediated cytotoxicity (ADCC) has been demonstrated in vitro in several tumor as well as a variety of virus systems including several in the herpes virus group (Ralph, 1975; Moller-Larsen, 1977). The response involves the cooperation of antibody and effector cells in generating a specific cytotoxic resopnse against the appropriate target cell.

In the system of Shore et al. (1977) using human peripheral blood, characterization of the effector cell revealed an Fc-receptor bearing cell lacking surface Ig, lacking the ability to form E-rosettes, and lacking the ability to phagocytose carbonyl iron. Thus, the activity was ascribed to a killer cell (K-cell) (Perlmann et al., 1975; Shore et al., 1976; Shimizu et al., 1977; Melewicz et al., 1977). Characterization of this cell in the HSV system has been confirmed by others (Heron, 1977). There appears to be no requirement for previous sensitization for this cell to mediate a cytotoxic response in the presence of the appropriate antibody. There is a requirement for the presence of relatively low levels of specific antibody of the IgG class, which appears to attach directly to the target cell rather than directly activating the mononuclear K-cell (Shore et al., 1976). This antibody is capable of

crossing the placental barrier and cooperating with cord blood mononuclear cells in mediating ADCC. Shore et al. (1977) have proposed that this may be one way in which the neonate may be protected from herpetic infection in utero.

An interesting feature of ADCC in the HSV system is the rapidity with which this effector mechanism can recognize and subsequently destroy HSV-infected target cells in vitro. Reports have demonstrated that viral antigens expressed on the surface of infected target cells are recognized by ADCC effector cells as early as two hours after infection, and that significant damage to these targets can be produced within three hours after infection. By destroying the infected cells early during the course of infection, the release of mature viral progeny was markedly reduced, thus obviating contiguous as well as extracellular viral spread (Shore et al., 1977a). The degree of ADCC was dependent upon the degree of infectivity of the target cells; the higher the multiplicity of infection, the greater the cytotoxic response (Shore et al., 1976). Thus, it has been suggested that the phenomena of antibody-dependent cell-mediated cytotoxicity may be important mechanisms early during the course of infection and prior to the onset of specific cellular immune responses.

Role of the Macrophage in Resistance to HSV Infection

Johnson (1964) was of the first to report an age-related factor in resistance to extraneural HSV infection.

Resistance to intraperitoneal or intranasal HSV infection seemed to increase with increasing age. Investigation into the level at which the spread of extraneural HSV was inhibited revealed a correlation between acquisition of resistance and the maturation of macrophages. Infection with HSV of adult and suckling mouse macrophages in vitro demonstrated that the ability of virus to spread and infect surrounding cells was considerably greater in the cultures of infected immature macrophages than in macrophages from adults. The inability of age to alter the outcome of intracerebral infection with HSV was explained by the phenomenum of the blood brain barrier, which further supported the role of the macrophages in resistance to peripheral HSV infection.

Subsequent investigation by Stevens and Cook (1971) and Hirsch et al. (1970) described virus cell interactions in infected mouse macrophages. Confirming and extending the work of Johnson (1964), Hirsch and co-workers (1970) reported that restriction of HSV by mature macrophages was a result of improper viral assembly in the cell. Despite the fact that the entire spectrum of viral components in the macrophage was apparently produced, the number of infectious virions decreased as a result of the inability to assemble the virions, a property unique to the adult macrophage. The exact mechanism of intrinsic restriction of the macrophage is still not clear, however, and remains a subject of considerable interest.

In further support of the role of the macrophage in resistance to viral infection, Starr et al. (1976) demonstrated that an agent capable of activating macrophages could confer protection against HSV infection in the neonatal host. Administration of BCG six days prior to an intraperitoneal challenge of HSV-2 significantly protected neonatal mice from lethality as compared to normal controls. Several mechanisms have been proposed. The possibility exists that BCG activates macrophages (or matures the "immature" macrophages of the neonate) which subsequently limit viral spread in vivo. Lodmell et al. (1973) have reported the ability of immunologically activated macrophages to decrease HSV-2 spread in vitro, which provides correlative evidence to support this hypothesis. Whether or not activating agents act directly on the macrophage or stimulate the production of substances (lymphokines) which subsequently activate macrophages in vivo has not been resolved.

Attempts have also been made to determine the role of macrophage resistance to HSV infections by eliminating or impairing macrophage function in vivo. Zisman et al. (1969) demonstrated that intraperitoneal pretreatment of young adult mice with silica significantly decreased resistance to an intraperitoneal challenge with HSV-1. Enhancement of mortality was also noted in mice pretreated with anti-macrophage serum, although it should be noted that the antiserum preparation was relatively crude and partially

toxic to cell populations other than macrophages. Prolongation of skin allografts have been achieved by pretreatment with silica (Pearsall et al., 1968). Clearance of colloidal carbon from the reticuloendothelial system was depressed following silica treatment as well as the ability to transfer tumor immunity by immune macrophages (Pearsall et al., 1968). Treatment of macrophages in vitro by silica has been shown to inhibit the phagocytic ability of macrophages to a marked degree (Miller and Zarkower, 1974).

Other compounds such as dextran sulfate and trypan blue have also been shown to impair macrophage function. While Allison (1966) has proposed that the mechanism of action of silica ultimately involves autolysis of the macrophage, it has been suggested that trypan blue and dextran sulfate do not destroy the cell, but rather inhibit lysosomal enzyme activity, thus impairing phagocytic function (Hibbs, 1975; Hahn and Bierther, 1974). In work with the effect of dextran sulfate on resistance to Listeria infection, Hahn (1974) demonstrated a marked increase in the number of organisms recovered from the liver and spleen of mice infected systemically and treated with dextran sulfate, and subsequently enhanced mortality. Electron microscopic examination of macrophages from mice treated in vivo with the compound showed marked morphological changes (Hahn and Bierther, 1974). Using trypan blue as an inhibitor of macrophage function, Hibbs demonstrated that the antitumor activity of macrophages could be blocked by

pretreatment of mice with this compound (Hibbs, 1975). The effects of these macrophage inhibitors on viral infections have not been determined.

Therapy Against Herpes Simplex Infection

There are currently no satisfactory therapeutic modalities available in the treatment of herpetic disease (Alford and Whitley, 1976). Chemotherapeutic agents such as cytosine arabinoside, iododeoxyuridine and others have limitations with respect to clinical value because of their relatively high levels of toxicity (Alford and Whitley, 1976). Most of the drugs currently administered in the treatment of herpetic disease are derivatives of purine and pyrimidine bases which act nonspecifically in that the site of action involves cellular DNA as well as viral DNA (Alford and Whitley, 1976). Thus, these drugs, in therapeutic doses, may markedly suppress actively dividing cells such as those of the myeloid series. Consequently, this mode of therapy may bear a two-fold effect - they may be beneficial with respect to antiviral activity, but at the same time may antagonize the development of immune mechanisms which may be important in the recovery from herpetic infection. Another form of therapy using photoinactivation of the virus by treatment of an active lesion with dye and light has been shown to be useful in the management of active lesions. However, this is not without limitations due to the fact that the combination of heterocyclic dye

and ultraviolet light may be mutagenic (Cusumano et al., 1975).

Another aspect to be considered in the development of an efficacious agent for the treatment of herpes simplex is that unlike most other viral diseases, the virus may establish latency in the host. While the mechanisms of latency have not yet been fully elucidated, it is unlikely that the chemotherapeutic agents currently available would ablate a latent infection without producing significant damage to the host.

An alternative approach to chemotherapy which has received increasingly widespread attention involves utilization of the immune system. This may be broadly classified into two methods of approach - development of a protective vaccine against herpes simplex which offers minimal risk to the host and modulation of the host response. As with the development of any vaccines suitable for human use, several parameters must be considered extensively. There are additional factors to consider in the preparation of a herpes virus vaccine, attributable to the latent and oncogenic potential of the virus. These two problems, in addition to the risk of introducing a potentially lethal infection into the host through vaccination, have almost completely eliminated the possibility of using a live virus vaccine, although a live virus vaccine generally affords a longer lasting immunity (Biggs, 1977; Hilleman, 1976). Potentially hazardous consequences may also be associated

with the use of a killed herpes simplex vaccine. Aside from the relatively poor preliminary results concerning the protective capacity of an inactivated preparation of the virus against primary and recurrent herpetic disease is the fact that some of the conventional methods of inactivation such as ultraviolet light exposure may enhance the oncogenic potential of the virus (Rapp and Reed, 1976).

Thus, it appears that the most practical herpes simplex vaccine would consist of a nucleic acid free subunit(s) which would be protective against the establishment of primary or recurrent disease with a minimal risk to the host. This approach, however, is not totally without problems, due to the relatively low efficacy of many subunit vaccines in addition to some problems intrinsic to the herpes viruses. Such problems include the difficulties associated with the purification of subvirion components. Components of the virion of HSV tend to be quite unstable and easily damaged during conventional purification methods and, thus, antigenic components may be damaged or altered. Development of appropriate purification techniques may accelerate the advent of a subunit herpes vaccine (Powell et al., 1975).

A second immunologic approach to dealing with herpetic disease is through immunomodulation, which involves the manipulation of host defense mechanisms through the administration of synthetic or naturally occurring agents (immunomodulators). Immunotherapy has, in the treatment of

certain malignant diseases, become a realistic therapeutic modality (Bluming et al., 1972). The question, however, as to whether these agents may have therapeutic value in the treatment of certain viral diseases has not been sufficiently answered. The mechanisms by which immunomodulators act has also not been completely delineated, and probably differs with the particular tumor or microbial system. However, nonspecific activation of macrophages has been demonstrated to be involved in many of the effects of certain immunomodulators such as pyran and C. parvum, especially antitumor activity (Kaplan et al., 1977; Morahan et al., 1977). Some evidence is also available concerning the role of immunomodulator activated macrophages in antiviral activity (Breinig et al., 1978).

Morahan et al. (1977) demonstrated the antiviral activity of pyran or Corynebacterium parvum activated peritoneal exudate cells in vitro. When mouse embryo fibroblasts were infected with vaccinia virus and incubated in the presence of activated peritoneal exudate cells, there was a significant inhibition in the number of plaque-forming units as compared to those cultures containing normal (resident) peritoneal cells or no peritoneal cells. Peritoneal cells stimulated with glycogen showed limited antiviral activity and, unlike pyran or C. parvum activated peritoneal cells, did not possess cytotoxic activity against osteogenic sarcoma cells.

The macrophage activity was demonstrated to be nonspecific because activity was shown against three different viruses. In addition to pyran and C. parvum activated peritoneal cells, peritoneal cells taken from mice infected with vaccinia virus also possessed nonspecific antiviral. Rodda and White (1976) have also demonstrated nonspecific antiviral activity of peritoneal cells taken from mice previously infected with Semliki Forest virus and originally attributed this to macrophages. However, a recent paper (MacFarland et al., 1977) reported that the cells were probably really natural killer cells.

The mechanism(s) by which these activated peritoneal cells are able to prevent the growth of virus in susceptible cells is not well understood. Other questions yet to be resolved include a possible requirement for histocompatibility or species identity between effector and target cells, whether or not the effector cells recognize virus-infected cells or directly inactivate the virus, and whether the effector cells inhibit infection at extracellular or intracellular levels. It is tempting to suggest that the activated macrophage is the primary effector cell responsible for the antiviral activity in these peritoneal cell populations. However, the heterogeneity of the population is such that separation of these cells into subpopulations is needed to identify definitively the effector cell(s).

The Research Plan

The objectives of the present research can be broadly classified into two categories: (1) the role of the adherent peritoneal cell (macrophage?) in nonspecific resistance to HSV-2 infection in vivo and in vitro, and (2) demonstration of one aspect of specific cell-mediated immunity to HSV-2 infection in vivo. Although the mononuclear phagocyte system and cellular immune system are recognized as having distinct functions in the network of immune responses, they are not entirely separate entities. It has been well established that the macrophage is necessary in many T-cell-mediated functions, both during the inductive phase of cellular immunity and in the effector stages (Yano et al., 1977; Erb et al., 1975). The aspect of cellular immunity studied in this research has involved the cooperative effect between macrophages and T-cells in the delayed-type hypersensitivity response. The role of the macrophage was also investigated alone, in the absence of T-cells.

The macrophage, because of its ability to evoke a state of nonspecific resistance, seems to be a likely candidate for being an important effector cell early during the course of initial infection, prior to the onset of specific humoral and cellular functions. Therefore, one aspect of this research has dealt with the role of macrophages early during the course of HSV-2 infection, and the

use of an in vitro model for investigating the antiviral activity of peritoneal exudate cells. The second aspect of the research dealt with the establishment of a system to investigate the appearance of a specific delayed-type hypersensitivity response in vivo following infection.

MATERIALS AND METHODS

Mice

Mice used for all investigations were of the BALB/c strain. This strain has been demonstrated to have a comparatively high susceptibility to HSV (Lopez, 1975) and has been used in previous and ongoing research in our laboratory (McCord and Morahan, 1975). The mice ranged from five to eight weeks of age except in those experiments requiring suckling mice, which ranged in age from one to seven days. The mice were maintained on Laboratory Chow and water ad libitum. Infected mice were housed in a room separated from normal uninfected mice.

Virus

The initial virus was obtained from Dr. Robert W. Tankersly. The virus was confirmed to be type 2 by the indirect hemagglutinin assay performed through the courtesy of Dr. John Stewart, Center for Disease Control, Atlanta, Georgia (McCord and Morahan, 1975).

A virus pool was prepared by infection of a subconfluent (75% confluent) monolayer of HEP-2 cells (approximately 10^7 cells/125 cm^2 bottle) with approximately 5×10^5 plaque-forming units (PFU) of the initial isolate. The virus was adsorbed for one hour at 36^oC followed by the addition of EMEM (Eagle's minimal essential medium containing Earle's balanced salt solution - antibiotics, L-glutamine) and 10% heat inactivated fetal calf serum. The

cells were incubated at 36°C until cytopathic effect (CPE) was noted to be in 75% of the cells.

Virus was harvested by freeze-thawing the infected cultures at -70°C followed by low speed centrifugation to remove cellular debris. The supernatant fluid was removed, dispensed into 1 dram vials and stored at -70°C. The titer was 5 x 10 PFU/ml when titered on HEP-2 cells. This pool was designated 1A since it represented the first passage of the original isolate. For all experiments in vitro and in vivo, pools 4B and 5B of HSV-2 were used. They represented the fourth and fifth pools, respectively, of virus made from pool 1A.

Media

All cell cultures were maintained in Eagle's Minimal Essential medium (MEM) with Earle's balanced salt solution (EMEM), 10% heat-inactivated fetal calf serum, 2mM glutamine, and no antibiotics unless otherwise noted.

Virus titrations for PFU were carried out in a methyl cellulose overlay medium. The methyl cellulose medium consisted of 1% methyl cellulose, MEM with Hanks' balanced salt solution, 2% fetal calf serum, 2mM glutamine, 200 units/ml of penicillin, 100 µg streptomycin/ml and 1 µg/ml of amphotericin B (Fungizone).

Cells

Vero cells: African green monkey kidney cells, Vero (courtesy of Dr. Byron K. Murray) were used for all virus

titrations. Vero cells (2 x 10^5) were seeded into 16 mm wells (24-well Linbro plates). Twenty-four hours later, the confluent monolayers were infected with 0.1 ml of serial dilutions of virus and virus allowed to adsorb for one hour. The infected cells were then overlayed with 1% methyl cellulose medium and incubated at 37°C in a 5% CO_2 95% air atmosphere. Forty-eight to seventy-two hours later, the cells were fixed by adding a 10% formalin solution and stained with crystal violet. Plaque-forming units were subsequently enumerated. The cells were used at passage levels 1-20.

Mouse embryo fibroblasts: Primary mouse embryo BALB/c fibroblasts were seeded into 25 cm^2 Falcon flasks and maintenance medium added. Once the monolayers were confluent, the cells were trypsinized and 1 ml placed into 16 mm wells at a concentration of 4 x 10^5 cells/well for use in antiviral assays twenty-four hours later.

HEP-2 cells: HEP-2, a continuous cell line originally derived from a laryngeal epidermoid carcinoma was used for the preparation of HSV-2 antigen. These cells (courtesy of Dr. B. K. Murray) were serially passed in 75 cm^3 Falcon flasks and used when the cells were approximately 75% confluent. The cells were used at passage levels 10-30.

Preparation of HSV-2 and HEP-2 Antigen

HEP-2 cells were seeded into 75 cm^2 flasks as described above and infected with HSV-2 at a multiplicity of

infection of 0.01. The virus was adsorbed for two hours at 37°C followed by washing the monolayers once with Hanks' balanced salt solution to remove unadsorbed virus. The cultures were replenished with EMEM containing glutamine, and antibiotics but without fetal calf serum and incubated at 34°C.

When cytopathic effect was determined to be approximately 70%, the maintenance medium was removed and 1 ml of sterile distilled water added to each flask. The monolayer was removed with a rubber policeman, the cells collected, disrupted by homogenization and centrifuged at 4°C 1,000 rpm for ten minutes. The supernatant fluids were removed and stored at -70°C. Portions of each pool were titrated on Vero cells. Preparation of the control HEP-2 antigen involved the same procedure as discussed above, but without infection of the cells with HSV-2.

The antigen preparations were inactivated by exposure to ultraviolet light (General Electric germicidal lamp G15T8, 15 watt) at 160 ergs/minute/mm^2 for six to eight minutes and re-titered to determine the presence of any infectious virus. If infectious virus was still present in the viral antigen preparation, the antigen was heat-inactivated at 56°C for ten minutes.

Inoculation of Mice With Virus

Mice were inoculated with varying dilutions of virus by one of three routes of inoculation. Those inoculated intravenously (i.v.) received a total volume of 0.2 ml of

virus dilution in the lateral tail vein. Mice inoculated intraperitoneally (i.p.) received a total volume of 0.2 ml of the virus dilution in the peritoneal cavity. Mice inoculated by the vaginal route received 0.03 ml of the virus suspension directly into the vaginal area using a small plastic catheter connected to a 0.5 ml syringe (Breinig et al., 1978).

Recovery of Virus From the Vaginal Area

Titers of virus in the vaginal area of infected female mice were assessed by taking swabs from the area at various times after infection. Small cotton swabs on toothpicks were moistened with viral diluent and inserted into the vagina. The swab was then placed into 1.0 ml of diluent. Titers of virus were expressed at the $\log_{10}$ PFU/ml of vaginal suspension.

Drugs

Pyran copolymer, lot XA 124-177 was received courtesy of Dr. D. Breslow, Hercules, Inc., Wilmington, DE. The drug was prepared by dissolving 2.5 mg/ml in 0.9% NaCl solution and adjusting the pH to 7.0 - 7.2 with 0.1 N NaOH. Oyster glycogen No. 2 (Sigma, St. Louis, MO) was prepared by dissolving 2.5 grams in 100 ml of distilled water. Mice were injected i.p. with 0.5 ml of the glycogen solution five days prior to harvesting peritoneal cells.

Silica No. 12 (particle size 2-10 μm) was obtained courtesy of Dr. Benkert (Dörentroper Sand-und Thonwerke

GMbH, Dörentroper, West Germany) and prepared by suspending the agent in 0.9% NaCl at a concentration of 80 mg/ml. Immediately prior to use the suspension was sonicated for two 5-second bursts. Mice were inoculated i.p. with 0.5 ml of the suspension (80 mg/ml, 2,000 mg/kg) two hours prior to viral infection. Dextran sulfate (M.W. 500,000, Serva, Heidelberg, Germany) was prepared by dissolving the compound in 0.9% NaCl at a concentration of 1 mg/ml. Mice received 50 mg/kg of the solution i.p. 24 hours prior to use. Trypan blue was obtained courtesy of Dr. John Hibbs, VA Hospital, Salt Lake City, UT, at a final concentration of 10 mg/ml. Mice were injected subcutaneously with 2 mg of the trypan blue 24 hours prior to use (50 mg/kg) followed by 1 mg (25 mg/kg) i.p. 2 hours prior to infection.

Peritoneal Exudate Cell Harvesting

Mice were treated with either pyran or glycogen as described above, or left untreated. On day 0, peritoneal cells were collected in the following manner. Mice were sacrificed using chloroform, secured to a dissection board, the peritoneum was exposed, and 10 ml of cold EMEM without fetal calf serum was injected into the cavity. The fluid was subsequently aspirated. The cell suspension was centrifuged at 1,000 rpm at 4^{o}C, washed one time and resuspended in EMEM containing 10% fetal calf serum.

Identification of Mononuclear Phagocytes

Wright's staining: Mononuclear phagocytes were determined morphologically using the conventional Wright's differential stain technique (Fisher Scientific, Pittsburg, PA). Smears were made from peritoneal exudate cells and fixed for five minutes with methanol. Wright's stain was added for three minutes. An equal portion of distilled water was subsequently added to the slide with gentle mixing, and allowed to stand for six minutes. Slides were rinsed and enumerated for the presence of cells of the monocytic series.

Latex particle ingestion: Peritoneal cells (5×10^6/ml) suspended in EMEM containing 20% fetal calf serum were placed in 12 x 75 mm plastic tubes and 1 ml of a suspension of latex particles (Difco, Detroit, MI) was added to the cells. The tubes were placed on a rotor for thirty minutes at a speed of 25 rpm at room temperature. Cells were centrifuged for ten minutes at 1,000 rpm, resuspended in 0.4 ml medium and smears made. Slides were stained with Wright's stain as described above. Cells ingesting greater than eight latex particles were considered to be phagocytic and, thus, termed "latex positive."

Stain for nonspecific esterase: Macrophages were also characterized on the basis of the presence of a nonspecific esterase enzyme activity according to the procedure of Yam et al. (1971). The reagents used were as follows:

Phosphate buffer (0.15 M, pH 7.4) 8.9 ml

Hexatozized pararosanilin, 0.6 ml, Sigma Chemical, St. Louis

Alpha-napthyl acetate, 10 mg per 0.5 ml ethylene glycol monoethyl ether, Sigma Chemical, St. Louis

4% sodium nitrate solution, 0.6 ml, Sigma Chemical, St. Louis

Buffered formalin acetone mixture pH 6.6

1% methyl green solution, Sigma Chemical, St. Louis

Smears made of PEC were fixed for 20 seconds in the buffered formalin acetone mixture at $4^{o}C$ and allowed to air dry for thirty minutes. All other reagents except the methyl green solution were added to a Columbia jar and the final pH adjusted to 5.8 - 6.5 with 1N NaOH. The fixed smears were placed into the Columbia jars and allowed to incubate in the mixture for 45 minutes at room temperature. The smears were then washed in distilled water and counter-stained with 1% methyl green stain for 1-2 minutes, washed and air-dried and mounted onto slides with Permount. The presence of the esterase activity was noted as the appearance of red granules within the cytoplasm of the cell, and termed esterase positive.

Basic Assay for Delayed Type Hypersensitivity in Mice

Mice were infected i.v. or intravaginally with an LD_{50} inoculum of HSV-2. At various times following infection, the mice were challenged in one hind footpad with 0.05 ml of UV light - inactivated HSV-2 antigen (original titer 5×10^{7} plaque-forming units/ml). Control HEP-2 antigen was injected

into the contralateral footpad. Uninfected mice were treated in a similar manner. At twenty-four hours after antigen inoculation, footpad swelling was quantitated by measurement with a caliper and compared with footpad measurements prior to inoculation with antigen. Measurements of footpad swelling in infected animals were compared with those of the uninfected group. Swelling in the footpad receiving the HSV-2 antigen was also compared with the contralateral foot receiving control antigen. Histological examination was performed to determine the nature of the inflammatory response.

I^{125}-albumin Assay for Delayed Type Hypersensitivity

Another method to assay for delayed hypersensitivity in mice to HSV-2 involved a modification of the assay reported by Paranjpe (1972). The assay involved inoculation of antigen into the footpads as previously discussed. Twenty-four hours following antigen challenge, 0.2 ml of I^{125}-labelled human serum albumin (Mallinckrodt, St. Louis, MO), specific activity 0.1 mc/ml was injected i.p. Two hours following injection of the albumin, both feet were amputated just below the hackle joint and placed into a gamma counter to determine the amount of radioactivity. A stimulation ratio was derived by comparing the amount of radioactivity in the hind foot challenged with HSV-2 antigen with the foot receiving control antigen of each individual mouse.

$$SI = \frac{\text{cpm in left hind footpad}}{\text{cpm in right hind footpad}}$$

Basic Assay for Antiviral Activity of Peritoneal Exudate Cells In Vitro

Vero or secondary mouse embryo fibroblasts were seeded in 16 mm Linbro plates as discussed previously. Twenty-four hours later, the confluent monolayers were infected with 30-50 PFU of HSV-2 and virus adsorbed for two hours. Following virus adsorbtion, various concentrations of peritoneal cells were added to the infected controls and allowed to adhere for two hours. At this time, nonadherent cells were removed by washing the monolayers three times with Hanks' balanced salt solution and then EMEM or methyl cellulose overlay media containing 10% fetal calf serum and antibiotics were added. Cultures were incubated for forty-eight to seventy-two hours at 36^{o}C in 5% CO_2 atmosphere. Supernatant fluids were removed at daily intervals and titered on Vero cells to determine levels of extracellular virus. The monolayers were fixed with formalin and stained with crystal violet for plaque enumeration. In each experiment, in addition to the test PEC group, there was also a group of cells receiving virus alone, and a group that received normal PEC (from mice inoculated i.p. with 0.2 ml of physiological saline at the same time as virus was inoculated). Toxicity controls were performed to determine any toxic effect that the peritoneal cells were exerting on uninfected target cells.

Peritoneal Cell Transfer

Donor mice were either treated with pyran or glycogen or left untreated. At day 0, peritoneal cells (PEC) were

harvested as described previously and resuspended to a final concentration of 1.5×10^7 PEC/ml. This cell suspension (0.2 ml) was injected i.p. into one to seven day old suckling mice. Twenty-four hours later, the suckling mice were challenged with HSV-2 intraperitoneally and mortalities recorded.

Virus Neutralization

Serum suspected of containing HSV-2 antibody was heat inactivated for 30 minutes at 56°C. Serial two-fold dilutions of the serum were incubated with a constant dilution of virus (approximately 40 PFU) and 20 hemolytic units of guinea pig complement (Colorado Serum Co., Denver, CO) for 30 minutes at 37°C. Each dilution (0.1 ml) was subsequently titrated on Vero cells to determine reduction in PFU. The titer of specific neutralizing antibody was expressed as the reciprocal of the dilution causing 50% plaque reduction as compared to cultures containing no antibody. Control rabbit anti-HSV-2 serum of known titer (1480 units $\pm$ 188) was always run as a control (Breinig et al., 1978).

Preparation of Pathological Sections

Pathological sections of the footpad were prepared and stained with hematoxylin-eosin courtesy of Dr. Michael Snodgrass.

Statistical Analysis

Significant differences ($p<0.05$) among control and experimental groups were determined by using the Student

t-test. The LD_{50} of the virus was determined using the method of Reed and Muench (1938) with Yates correction factor.

RESULTS

A. Cell-Mediated Immunity During HSV Infection Demonstrated by Delayed-Type Hypersensitivity

Characteristics of the delayed-type hypersensitivity (DTH) response to HSV-2 antigen. A footpad swelling assay was developed to measure the in vivo DTH aspect of the cell-mediated immune response that developed in mice infected i.v. with an LD_{50} of HSV-2. The specificity of this assay was demonstrated by the ability of mice infected 14 days previously with HSV-2 to respond significantly to the HSV-2 antigen, but not to control antigen (HEp-2) (Table 1). The HEp-2 antigen was chosen as the control antigen since the virus used for inoculation was propagated in vitro in HEp-2 cells. Thus, the use of HEp-2 as a control antigen would detect responses which may have occurred as a result of in vivo sensitization with HEp-2 specific antigen.

Four groups of mice, two groups of uninfected and two groups of HSV-2 infected mice received either HSV-2 antigen or control antigen in the left hind footpad while the right hind footpad remained uninoculated (Table 1). A stimulation ratio was derived by dividing the counts per minute obtained from the inoculated food with the counts per minute of the uninoculated foot in each individual mouse. The only group that showed a significant response in either the footpad swelling or the radioisotopic DTH assay was the group of mice infected with HSV and challenged with the HSV antigen.

TABLE 1

Comparison of Footpad Swelling and Radioisotopic Assay to Measure Specificity of Delayed Type Hypersensitivity Response[a]

Infection of Mice	Footpad Challenge With	Increase in Footpad with[b] (mm ± SE)	MSR ± SE[c]
None	HEP-2 Ag	1.02 ± 0.12	0.03 ± 0.02
	HSV-2 Ag	1.14 ± 0.05	0.10 ± 0.03
HSV-2	HEP-2 Ag	1.05 ± 0.09	0.05 ± 0.02
	HSV-2 Ag	1.74 ± 0.18[d]	0.40 ± 0.04[d]

[a] BALB/c mice were untreated or infected intravenously with HSV-2 which caused 50-70% mortality. Fourteen days later right hind footpads were inoculated with the antigen indicated. Twenty-four hours later footpad thickness of the rear footpads was measured, followed by inoculation with ^{125}I-albumin intraperitoneally. After 2 hours, feet were excised just below the hackle joint and amount of radioactivity analyzed in a gamma counter.

[b] Increase in footpad thickness/width between inoculated right and uninoculated left footpad. There were 10 mice per group.

[c] MSR = mean stimulation ratio

$$\frac{\text{counts per minute in right hind footpad}}{\text{counts per minute in left hind footpad}}$$

[d] $p<0.05$ of inoculated compared to uninoculated footpad or compared to HSV-infected mouse footpad challenged with HEP-2 Ag, or uninfected mouse footpad challenged with HEP-2 or HSV-2 antigen

Table 1 also demonstrates that the radioisotopic assay correlated well with measurement of footpad swelling measured 24 hours after antigen challenge. There were no significant increases in footpad thickness or stimulation ratios in the footpads of normal mice inoculated with HSV-2 or control antigen or HSV infected mice inoculated with control antigen. Thus, in subsequent experiments, groups were consolidated such that one group of uninfected animals and one group of infected animals would receive HSV-2 antigen in the left hind footpad and control antigen in the right hind footpad.

A second aspect of the system was to determine the optimal time for peak responsiveness following antigen challenge. The appearance of the optimal response following antigen challenge in the footpad was typical of the usual DTH response (Table 2). The mean stimulation ratio was 1.11 at 6 hr, maximal (1.58) at 24 hrs, and waned (1.12) by 48 hrs after antigen challenge. Again, uninfected mice showed no significant response during the 6-48 hour period. Statistical analysis of variance revealed that there were no statistical differences in the footpad response among the groups of normal animals among the various experiments. Thus, the uninfected animals from each experiment were pooled to provide a combined control group for comparison with the various groups of mice infected intravenously with HSV-2.

TABLE 2

Optimum DTH Response of Mice to HSV-Antigen Challenge 14 Days After IV Infection[a]

Infection of Mice	Hours After Antigen Challenge (MSR ± SE)[b] 6	24	48
None	1.00 ± 0.07	1.10 ± 0.05	1.09 ± 0.07
HSV-2	1.11 ± 0.11[d]	1.58 ± 0.08[c]	1.12 ± 0.08[d]

[a] BALB/c mice were either untreated or infected i.v. with HSV-2 which caused 50-70% mortality. Fourteen days later their right hind footpads were inoculated with control HEP-2 antigen and the left hind footpads inoculated with HSV-2 antigen. Twenty-four hours after antigen challenge, mice were inoculated intraperitoneally with ^{125}I-albumin. Two hours later the left and right hind footpads were removed just below the hackle joint and counted in a gamma counter.

[b] MSR = mean stimulation ratio

$$\frac{\text{counts per minute in left hind footpad}}{\text{counts per minute in right hind footpad}}$$

[c] $p < .05$ as compared with uninfected mice at either 6, 24, or 48 hours.

[d] N.S. = not significant as compared with uninfected mice at either 6, 24, or 48 hours after antigen challenge.

FIGURE 1A: Histological section of a hind footpad from an uninfected mouse 24 hours after receiving HSV-2 antigen in the footpad.

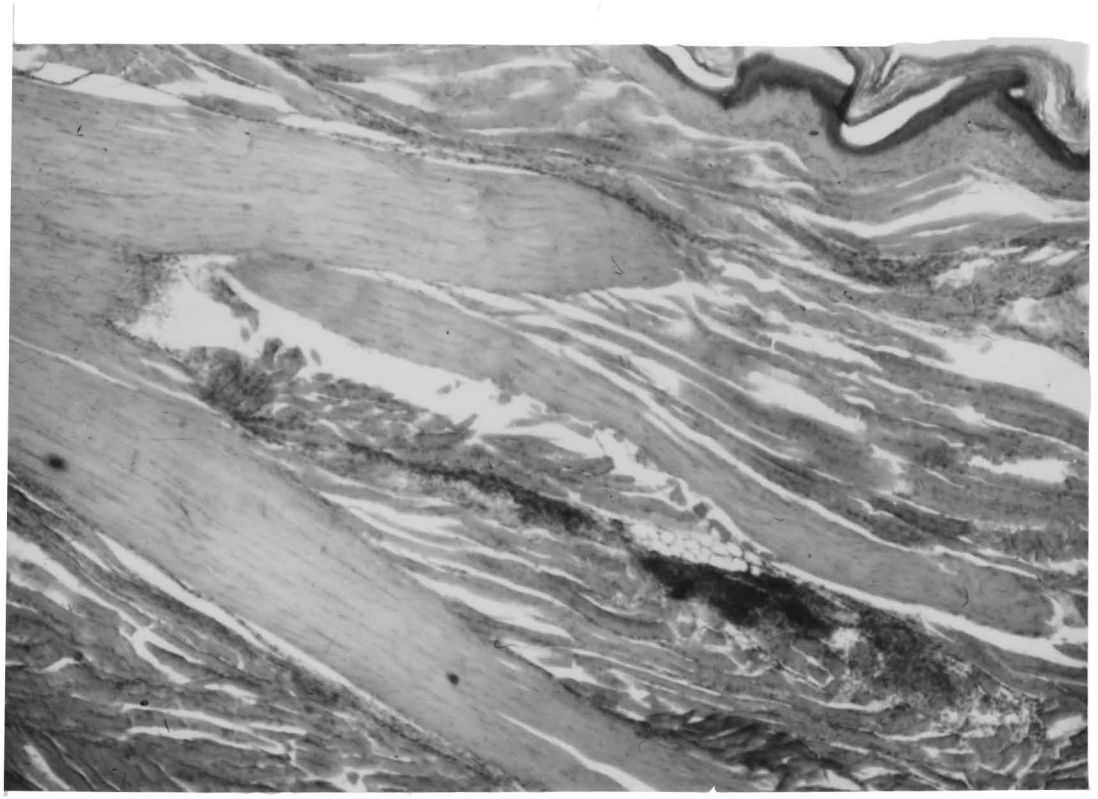
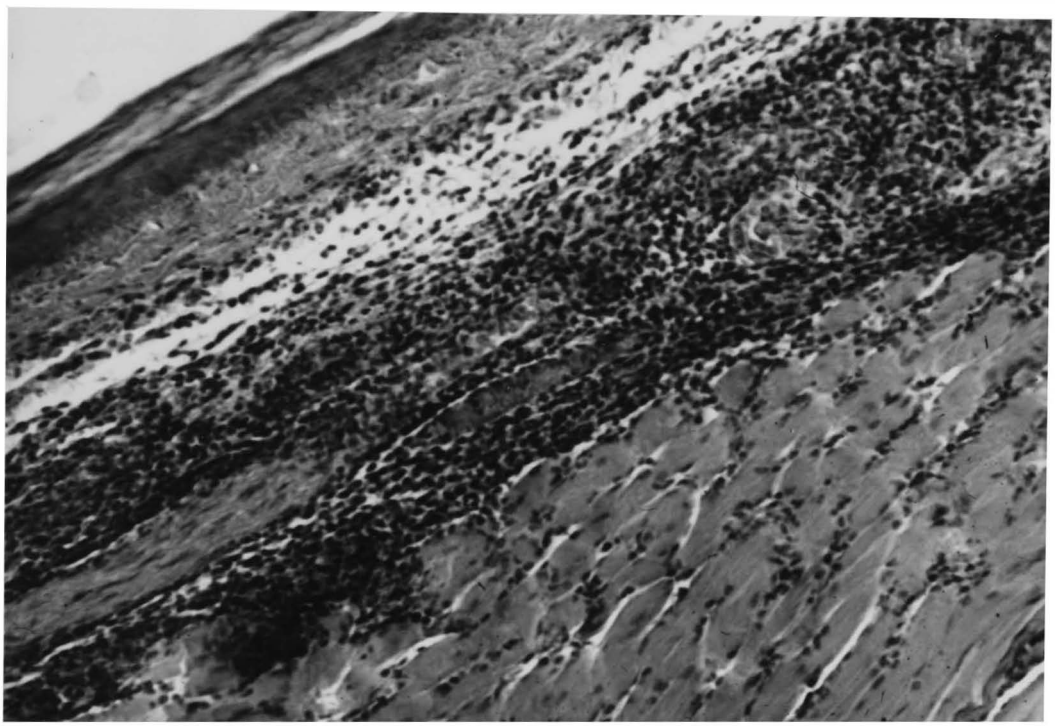


FIGURE 1B: Histological section from the hind footpad of a mice infected intravenously on day -6 and challenged with HSV-2 antigen in the footpad on day 0. Twenty-four hours later pathological sections were made. Note the dense cellular infiltrates.



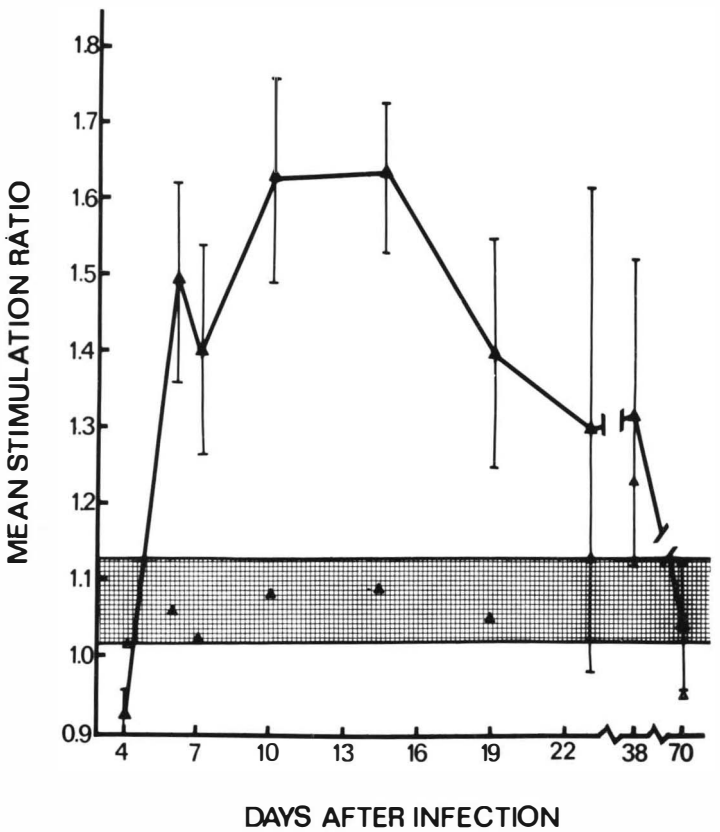
Histological examination was also performed on footpad sections of both uninfected and infected mice to determine the nature of the inflammatory response 24 hours following antigen challenge. Figure 1A represents a section from an uninfected mouse receiving HSV-2 antigen in the footpad. There is little evidence of cellular infiltration or necrosis of tissue. In contrast, however, in a mouse previously infected there was a comparatively larger degree of cellular infiltration in the footpad receiving HSV-2 antigen (Fig. 1B). This cellular infiltrate was composed primarily of mononuclear cells. Thus, the histological data provided support for the presence of a specific DTH response in the footpads of infected mice.

Kinetics of the DTH response in mice infected intravenously with HSV-2. Once the optimal conditions for the assay were established, the kinetics of the DTH response were determined following i.v. infection of BALB/c mice infected intravenously with HSV-2 (Fig. 2). A significant DTH response was not observed earlier than 4 days after infection. By day 6 a response was observed. The mean response was significantly elevated over control levels on days 7, 10, and 14, began to decline by 19 days after infection, and virtually disappeared by day 70.

A criterion was established to determine what was considered to be a positive stimulation ratio in an individual animal. A tolerance interval was established such that a stimulation ratio of 1.32 or greater in an individual mouse

FIGURE 2: Kinetics of the appearance of the delayed type hypersensitivity response following intravenous infection with HSV-2. BALB/c mice were infected with HSV-2 and at various times after infection the DTH response measured using the radioisotopic footpad method as previously described. The shaded area represents the DTH responses of normal uninfected mice while the connected lines represent patterns of DTH responsiveness in HSV-2 infected mice at various intervals following infection.

$$\text{MSR} \pm \text{S.E.} = \frac{\text{CPM in left hind footpad (HSV-2 Ag)}}{\text{CPM in right hind footpad (HEP-2 Ag)}}$$


MEAN STIMULATION RATIO
1.8
1.7
1.6
1.5
1.4
1.3
1.2
1.1
1.0
0.9
4
7
10
13
16
19
22
38
70
DAYS AFTER INFECTION

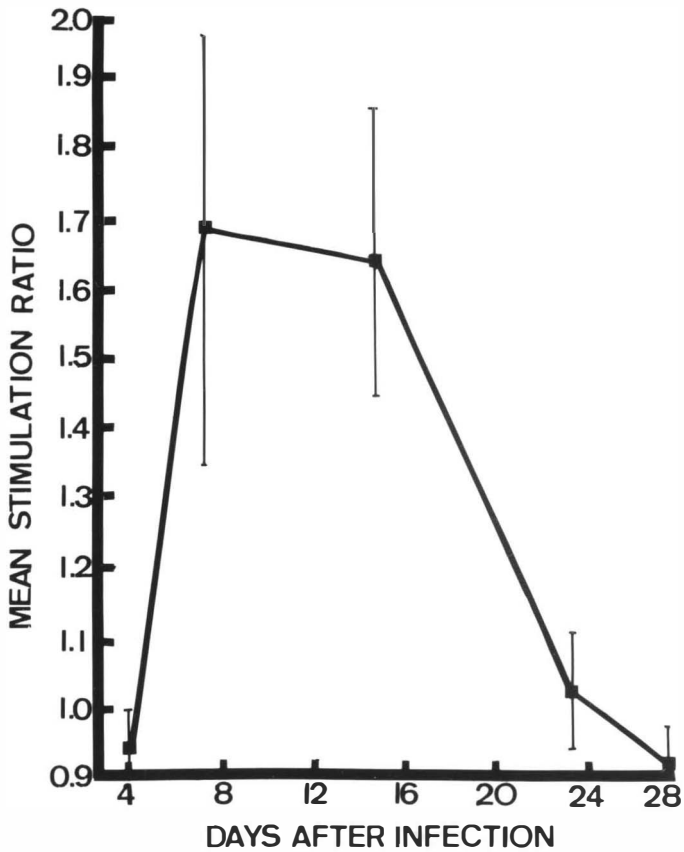
was considered to be a positive DTH response. In terms of percentages of infected mice giving a positive response, 80% of the mice showed a positive response by day 6, while by day 19 only 50%, and on day 70 none of the mice elicited a positive DTH response.

Kinetics of the DTH response following intravaginal infection with HSV-2. Experiments similar to the intravenous infection were performed with mice infected intravaginally with HSV-2 (Fig. 3). Similar to the intravenous infection, no response was detected as early as 4 days post-infection. A positive response appeared by day 7, and was maintained with animals responding maximally on day 14, but became undetectable by day 23. It appeared that the DTH response following vaginal infection was characterized by a more transient response than that following systemic infection.

Attempts to correlate a positive DTH response with survival during HSV infection. Attempts were made to determine if a correlation existed between the presence of a DTH response and survival of the animal. In these experiments, footpad swelling was used to determine the presence or absence of a DTH response on day 6 following intravenous infection. The individually marked animals were then followed for survival (Table 3). There was no apparent correlation between a positive DTH response on day 6 and survival.

FIGURE 3: Kinetics of the appearance of the delayed type hypersensitivity response following intravaginal infection with HSV-2. BALB/c mice were infected with HSV-2 and at various times after infection the DTH response measured using the radioisotopic footpad method as previously described.

$$\text{MSR} \pm \text{S.E.} = \frac{\text{CPM in left hind footpad (HSV-2 Ag)}}{\text{CPM in right hind footpad (HEP-2 Ag)}}$$


2.0
1.9
1.8
1.7
1.6
1.5
1.4
1.3
1.2
1.1
1.0
0.9
MEAN STIMULATION RATIO
4
8
12
16
20
24
28
DAYS AFTER INFECTION

Approximately an equal proportion of animals demonstrating a positive DTH response survived (8/13) as succumbed (5/13) to the HSV infection.

B. Role of Macrophage in Resistance to HSV

Effects of macrophage depletion in vivo on susceptibility of mice to i.v. infection with HSV-2. We compared the effect of administration of three different agents, known to impair macrophage function, on the resistance to systemic (i.v.) or local (intravaginal) infection with HSV-2.

Mice were pretreated with one of the three macrophage inhibitory agents (silica, trypan blue, or dextran sulfate) as described in Table 4. The drugs administered in the absence of infection with HSV-2 were not toxic to the animals at the given dosages.

Characterization of the effects of the treatments on esterase positivity and latex particle ingestion of the peritoneal cells revealed that silica treatment resulted in the greatest decrease in percentage of esterase and latex positive cells (31% in controls vs 8% in silica treatment). All three agents also caused an increase in the percentage of polymorphonuclear neutrophils in the peritoneal cavity, probably due to a nonspecific inflammatory response. In terms of cell yields the largest observed effect was seen with trypan blue, which caused a 2-fold decrease in cell number as compared to control mice inoculated with saline.

TABLE 3

Lack of Correlation of the Delayed Type Hypersensitivity Response on Day 6 With Survival After Intravenous Infection with HSV-2[a]

	Delayed Type Hypersensitivity Response			
	Positive		Negative	
Experiment	Survived	Dead	Survived	Dead
1	2	3	2	1
2	6	2	11	1
Total	8/28	5/28	13/28	2/28
% Age	29%	18%	46%	7%

[a]Mice were infected intravenously with HSV and on day 6 inoculated in the left hind foodpad with HSV-2 antigen and in the right hind footpad with HEP-2 antigen. Twenty-four hours later hind footpads were measured with a caliper.

TABLE 4

Effects of Macrophage Inhibitory Agents on Peritoneal Cell Populations[a]

Treatment	Regimen: Dose (mm/kg)	Regimen: Route	Regimen: Time (hr)	Number of Peritoneal Cells ($x10^6$)	Differential (%)[b]: Mono	Differential (%)[b]: Lymph	Differential (%)[b]: PMN	Percent[c] Esterase + Latex Positive Cells
Saline	-	i.p.		2.1	$29^{+}_{-}1$	$65^{+}_{-}1$	$6^{+}_{-}1$	$32^{+}_{-}2$
Silica	2000	i.p.	-2	2.0	$7^{+}_{-}3$	$23^{+}_{-}3$	$69^{+}_{-}1$	$8^{+}_{-}2$
Dextran Sulfate	50	i.p.	-24	3.0	$27^{+}_{-}6$	$23^{+}_{-}2$	$50^{+}_{-}5$	$15^{+}_{-}1$
Trypan Blue	50 and 25	s.c. i.p.	-24 -2	1.0	$15^{+}_{-}2$	$62^{+}_{-}4$	$22^{+}_{-}5$	$20^{+}_{-}1$

[a]Mice were treated with the macrophage inhibitory agents at the doses and times indicated prior to obtaining peritoneal cells. The peritoneal cells were then removed and analyzed for number and type of cells present.

[b]Mono = monocyte; lymph = lymphocyte; and PMN = polymorphonuclear leukocyte as determined by microscopic examination of Wright's stained smears.

[c]As determined by detection of nonspecific esterase enzyme according to the method of Yam et al. and latex particle ingestion.

On day 0, mice were inoculated i.v. with various dilutions of HSV-2. The LD_{50} was calculated using the method of Reed and Muench (1938). There was generally a ten-fold or greater increase in the LD_{50} in each of the treated groups as compared to the untreated groups (Table 5).

Effect of macrophage inhibitory agents on vaginal HSV-2 infection. Mice were treated with the agents as described above but infected vaginally with HSV-2 (Table 5). In contrast to the results obtained with i.v. infection, silica, trypan blue, and dextran sulfate had no effect on the susceptibility of mice to vaginal infection as compared to untreated controls. Titers of virus recovered in the vaginal secretions were also monitored in mice infected by this route. All mice showing evidence of virus in vaginal secretion eventually succumbed. Figure 4 illustrates the $\log_{10}$ geometric mean titer of the treated groups and controls. No significant differences were observed in titers of vaginal virus in the treated versus untreated groups. Thus, the lack of effect on lethality by treatment with these compounds correlated with the lack of effect on local virus growth in the vaginal area. In some surviving mice, serum was tested for neutralizing antibody activity. Antibody levels were not markedly affected by treatment with silica, trypan blue, or dextran sulfate.

In vivo antiviral activity of peritoneal exudate cells. Johnson (1964) reported resistance to infection with HSV increased with increasing age. This age factor was found to

TABLE 5

Effect of Treatment of Mice With Macrophage Inhibitory Agents on Intravenous or Vaginal HSV-2 Infection[a]

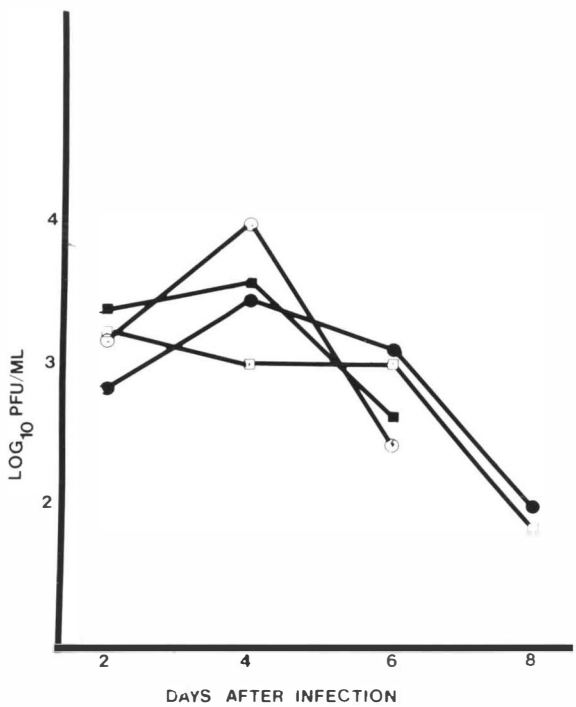
Infection	Mouse Strain	LOG_{10} LD_{50}/0.2 ml			
		Saline	Silica	$DxSO_4$	Trypan Blue
I.V.	BALB/C	4.4	5.5 (1.1)[b]	ND[c]	ND
	BALB/C	2.9	4.2 (1.3)	3.8 (0.9)	4.4 (1.5)
	BALB/C	1.6	3.6 (2.0)	2.9 (1.3)	2.7 (1.1)
	CDF_1	2.1	2.9 (0.8)	2.4 (0.3)	3.4 (1.3)
Vaginal	BALB/C	2.5	2.5 (0)	ND	ND
	BALB/C	1.1	0.8 (0.3)	0.9 (0.2)	0.7 (0.4)

[a]Mice were treated with the macrophage-inhibitory agents as described in Table 4 and infected i.v. or vaginally with dilutions of HSV-2, mortality recorded, and the 50% lethality endpoint (LD_{50}) calculated.

[b]Numbers in parentheses indicate LOG_{10} difference from control.

[c]ND, not determined.

FIGURE 4: Local replication of HSV-2 in the vaginal area. Mice were treated with the macrophage inhibitory agents as described in Materials and Methods, infected intravaginally with HSV-2, and vaginal swabs taken daily for determination of virus titers. Each value is the geometric mean titer of titers from several individual mice. O, Control; ●, Trypan blue treated; , Silica treated; , Dextran sulfate treated.


4
3
2
LOG10 PFU/ML
2
4
6
8
DAYS AFTER INFECTION

be attributable to the lack of a population of mature macrophages in newborn mice. Thus, the suckling mouse serves as an appropriate model of animals naturally deficient in macrophage function and is useful for determining the effect of macrophages on resistance following HSV infection.

The antiviral effect of pyran-activated or glycogen-elicited peritoneal exudate cells was assessed by determining the protective ability of passive transfer of these cells into suckling mice (Table 6). Mice 1-4 days of age received either peritoneal cells from pyran, glycogen, or saline-treated adult mice or were left untreated. On day 0 the neonatal mice were infected with HSV-2 and mortality recorded. There was a significant difference between the mortality of the group passively treated with pyran-activated PEC as compared with the untreated group or the group treated with normal peritoneal cells. Transfer of glycogen peritoneal exudate cells showed slight, but insignificant protection. There were no significant differences in the mean survival time of mice that died, which in the untreated group was 7.45 days. To determine whether or not the protective effect was a result of the transfer of residual pyran rather than peritoneal cells, two groups of suckling mice received either pyran or glycogen alone. Treatment of neonatal mice with the compounds alone at the doses indicated gave no significant protective effect.

TABLE 6

Protection Against HSV-2 Infection in Suckling Mice by Transfer of Peritoneal Exudate Cells

Treatment[a]	Infection with HSV	Number Dead/Total			Number Dead/Total	
		Exp. 1	Exp. 2	Exp. 3		
None	+	ND	4/10	7/11	11/21	(52%)
Normal PEC	+	8/11	8/18	11/15	27/44	(51%)
Pyran PEC	+	3/10	1/21	3/11	7/42	(17%)[c]
Glycogen PEC	+	3/11	5/12	7/16	15/39	(38%)[d]
Pyran[b]	+	ND	6/18	9/14	15/32	(47%)
Glycogen[b]	+	ND	3/8	5/10	8/18	(44%)

[a]Peritoneal cells were removed from adult BALB/C mice inoculated intraperitoneally with either pyran (25 mg/kg) on day 1, 0.5 ml of 2.5% on day 5, or nothing. The cells were harvested, washed and 3×10^6 cells in 0.2 ml were inoculated intraperitoneally into 4-8 day old suckling mice. The suckling mice were inoculated intraperitoneally with HSV-2 24 hours later.

[b]Suckling mice treated directly with immunomodulators on the same dose and schedule.

[c]$P<.02$ as compared to suckling mice receiving normal PEC or nothing.

[d]N.S.

Kinetics of the antiviral activity of peritoneal exudate cells from mice infected with HSV-2. The previous experiments demonstrated that macrophages were involved in resistance to systemic HSV infection, as evidenced by decreased resistance on inhibition of macrophage function and increased resistance on transfer of activated macrophages. The following experiments were designed to establish directly that macrophages with antiviral activity appeared during systemic HSV infection. A series of kinetic experiments established the appearance of the antiviral activity of peritoneal cells. Mice were infected i.p. on day -7 and days -4 through -1 with a dose of virus causing 50 to 70% mortality (Table 7). On day 0, peritoneal cells were harvested and assayed for antiviral activity. Yields of peritoneal cells as well as percent monocytes as determined using Wright's stain were determined at the different time periods. There was a slight increase (approx. 2-fold) in the number of peritoneal cells harvested at days 1-4 after infection as well as a slight increase in the number of cells of the monocytic series.

In mice infected on day -1, there was an increase in the number of HSV PFU over and above the control values. This was probably attributable to the presence of residual virus in the peritoneal cavity, which was subsequently transferred to the culture. There was little or no change noted in the virus yield from supernatant fluids as compared

TABLE 7

Time Course After IP HSV-2 Infection of

Antiviral Activity of Peritoneal Cells (PEC)

PEC Added (Days After HSV-2)	PEC Characteristics		Antiviral Activity[a]	
	No./Mouse ($\times 10^6$)	Percent Monocytes[b]	HSV-2 Plaques (PFU $\pm$ SE)	HSV-2 Yield ($\log_{10}$ PFU/ml)
None	--	--	37.4 $\pm$ 0.7	5.8
Normal	2.6	27	31.2 $\pm$ 2.3	5.7
Day 1	5.0	34	58.3 $\pm$ 3.2	5.6
Day 2	4.9	36	22.3 $\pm$ 2.1	5.5
Day 3	4.3	38	5.5 $\pm$ 1.4	<3.0
Day 4	3.6	33	15.8 $\pm$ 1.9	3.7

[a]Vero cells were infected with HSV-2 and 3 x 10^6 normal PEC or PEC from HSV-2 infected mice adsorbed, nonadherent cells removed with washing, and virus yields and PFU were determined at 3 days.

[b]Differential count, Wright's stain.

to cultures without PEC. Cultures of HSV infected Vero cells and PEC from mice infected 2 days previously showed some activity, with a 41% reduction in the number of HSV PFU and a 2-fold reduction in HSV-2 yield. Cultures with PEC from mice infected 3 days previously demonstrated the optimal period for antiviral activity. There was an 86% reduction in the number of virus plaques as well as a greater than 2.8 $\log_{10}$ reduction in yield of virus. PEC from mice infected 4 days previously also showed significant antiviral activity with a 58% reduction of HSV PFU and a 2.1 $\log_{10}$ reduction in virus yield. By day 7 after HSV infection, however, the PEC response had waned and approached activity similar to that noted with normal untreated peritoneal cells.

Antiviral activity in vitro of nonadherent and adherent peritoneal exudate cells from HSV infected mice. These experiments were designed to characterize the cell(s) responsible for the antiviral activity on the basis of adherence properties. Vero cells were infected as described in Material and Methods and overlaid with peritoneal exudate cells from normal or HSV-2 infected mice (3 days after infection). Peritoneal cells were allowed to adhere on the infected target cell cultures for 2 hours. After 2 hours, nonadherent cells were removed, washed, adjusted to the appropriate cell concentration and incubated with other infected cell cultures. Cultures containing adherent effector cells were washed vigorously to remove any residual

nonadherent cells and prepared as described. Approximately 28-30% of the peritoneal cells were adherent.

Nonadherent cells from either normal or HSV-infected mice provided no reduction in virus plaque formation or in the virus yield in supernatant fluids (Table 8). Normal adherent cells provided some reduction in plaque formation and to a lesser degree in virus yield in supernatant fluids. Adherent cells from mice infected with HSV showed a significant reduction in the number of virus plaques as well as a 2.2 $\log_{10}$ reduction in virus in the supernatant fluids, suggesting that the population of cells responsible for the antiviral activity resided in the adherent fraction which contained at least 72% esterase positive cells.

Lack of species specificity of antiviral activity of peritoneal cells from mice infected with HSV-2. To determine whether or not a syngeneic system between effector and virus infected target cell would produce greater antiviral activity, the syngeneic mouse embryo fibroblast cell and the xenogeneic Vero cell line were infected with HSV-2. Peritoneal exudate cells from infected mice inhibited viral growth in both mouse embryo fibroblasts and Vero cells (Table 9). There was at least a 10-fold reduction (1.6 and 2.1 $\log_{10}$) in viral titers of supernatant fluids from Vero and mouse embryo cultures. In this experiment, normal cells provided some reduction in viral yields, but not to the degree as did cells from infected mice.

TABLE 8

Antiviral Activity of Adherent or Nonadherent Peritoneal Cells

From Normal Mice or Mice Infected with HSV-2

Treatment	Cells	% Esterase Positive	Inhibition of HSV-2[a] Plaque Formation (PFU ± SE)	Inhibition of HSV-2[a] Virus Yield (log_{10} PFU/ml)
None		--	49.0 ± 1.1	5.8
Normal	Adherent	68	28.3 ± 0.9	5.0
Normal	Nonadherent	1	49.0 ± 4.0[b]	>5.0
HSV-2	Adherent	72	0.2 ± 0.2[c]	3.6
HSV-2	Nonadherent	2	48.0 ± 2.0[b]	5.6

[a] 3×10^6 peritoneal cells were adsorbed to HSV-2 infected Vero cells for 2 hr. The residual nonadherent cells were recovered and incubated with duplicate HSV-2 infected Vero cells. The yields of virus and PFU were determined 3 days later. Approximately 28-30% of the total peritoneal cell population adhered within 2 hr.

[b] N.S.

[c] $P<.05$ as compared to control and normal PEC.

TABLE 9

Lack of Species Specificity of Antiviral Activity of Peritoneal Cells from HSV-2 Infected Mice

Peritoneal Cells[a] Added	Yield of HSV-2 In: Vero Cells Yield (log_{10}PFU/ml)	Vero Cells Reduction (log_{10})	Mouse Embryo Cells Yield (log_{10}PFU/ml)	Mouse Embryo Cells Reduction (log_{10})
None	5.4	--	4.3	--
Normal	5.0	0.4	3.2	1.1
HSV-2	3.8	1.6	2.2	2.1

[a] 3×10^6 peritoneal cells were adsorbed to HSV-2 infected Vero or secondary mouse embryo fibroblasts for 2 hr, the nonadherent cells removed with washing, and virus yield assayed 3 days later.

Nonspecificity of antiviral activity of adherent peritoneal exudate cells. It was of interest to determine whether the antiviral activity of peritoneal exudate cells harvested from HSV-infected mice was specific for HSV. Therefore, the antiviral activity of these cells was tested against cells infected with HSV-2 or an unrelated single-stranded RNA virus, vesicular stomatitis virus (Table 10). It was observed that cells from HSV-2 infected mice were capable of inhibiting HSV-2 and VSV growth as measured by PFU as well as reducing viral titers in supernatant fluids.

TABLE 10

Nonspecificity of Antiviral Activity of Peritoneal Cells From HSV-2 Infected Mice

Peritoneal Cell Source	Inhibition of Virus in Target Cells Infected With[a]			
	HSV-2		VSV	
	Plaques (PFU ± SE)	Virus Yield (log_{10}PFU/ml)	Plaques (PFU ± SE)	Virus Yield (log_{10}PFU/ml)
None	48.5 ± 1.1	4.0	32.5 ± 1.1	6.0
Normal	36.3 ± 3.3	4.2	20.1 ± 3.3	6.0
HSV-2	0	3.0	0.2 ± 0.2	4.8

[a]Vero cells infected with either HSV-2 or vesicular stomatitis virus were overlaid with 3×10^6 peritoneal cells for 2 hr, the nonadherent cells removed with washing, and plaques or virus yields determined 3 days later.

DISCUSSION

Based on observations of the increased severity of HSV in patients deficient in cellular immunity as well as in animal models, it has been shown that cell-mediated immunity (CMI) is an important defense mechanism. Ennis (1973) demonstrated that syngeneic immune spleen cells passively transferred into normal mice markedly protected them from a lethal challenge with HSV. Blanden reported similar results using a similar model in the mousepox system, with decreased titers of mousepox virus in the livers of mice receiving immune spleen cells (Blanden et al., 1971).

The delayed type hypersensitivity (DTH) represents one of the earliest immunologic systems as described by Jenner in 1801. The prototype for the delayed hypersensitivity reaction is credited to Koch who described a cutaneous reaction observed when guinea pigs, previously exposed to tuberculosis, were injected with a filtrate of the bacilli. Another distinguishing feature of the DTH response is its ability to be transferred into DTH (David, 1975) animals using lymph nodes, spleen or peritoneal exudates from DTH positive animals. Peak sensitivity occurs 24 hours following infection with the specific antigen into a sensitized host and wanes by 48 hours.

Several aspects of cell-mediated immunity have been demonstrated following infection with HSV-2. Morahan et al. (1977) showed an increase in *in vitro* blastogenesis

specific for HSV-2 antigens in mice previously infected with the virus. Syngeneic spleen cells from HSV-immune mice confer protection when passively transferred into naive mice. Correspondingly, depletion of T-cells *in vivo* results in an increased resistance to the infection (Morahan and McCord, 1975).

The delayed type hypersensitivity response to HSV-2 has not been previously reported in the mouse model. Thus, it was of interest to design a model for determining the kinetic patterns of the DTH response to HSV, and attempt to correlate patterns of DTH responsiveness to other aspects of cell-mediated immunity which have been previously reported. Using the isotopic footpad assay reported by Parajnpe and co-workers (1972), a model was established which demonstrated some interesting patterns relating to the route of infection.

The DTH response to the HSV-2 antigen was found to be maximal at 24 hours following antigen challenge in mice previously infected with HSV-2. The response was also specific to HSV-2 antigen. A response was not detectable earlier than 6 days following intravenous infection and persisted until approximately 40 days after infection in the surviving mice. There was no apparent correlation with survival, however, as evidenced by the fact that an equal proportion of DTH negative and DTH positive mice survived the initial infection. This may have been due to the fact that those mice which were presumed to be DTH negative were

not initially infected with a dose of virus sufficient to elicit a DTH response or cause a lethal infection. It would be of interest to determine if the presence of a DTH response in mice has any correlation to the establishment of latency in the host.

The DTH response was found to also be present in mice infected vaginally. These data suggest that some systemic sensitization occurred, although previous observations have reported that the virus replicates locally in the vaginal area, proceeds directly to the spinal cord, and ultimately to the central nervous system (Breinig et al., 1978). The lymph nodes proximal to the vaginal area and draining the area of the footpad may be similar and, thus, be responsible for the sensitization and the presence of the DTH response in the footpad. However, we have also shown that spleen cells from vaginally infected mice are sensitized to the HSV antigen as evidenced by blastogenesis (Brienig et al., 1978).

A point in contrast with the DTH response in vaginally and systemically infected animals was the fact that the DTH response in vaginally infected mice represented a more transient phenomenon as compared to systemically infected animals. Whereas the DTH response was maintained beyond 40 days after infection in intravenously infected mice, vaginally infected mice elicited a response which had waned by 21 days after infection. A possible explanation for the deficiencies in the longevity of the responses may be due

to the degree of sensitization. Systemic infection as observed through pathogenesis studies has been found in visceral as well as reticuloendothelial organs, prior to entry into the central nervous system.

Specific immune responses play a critical role in resistance to a variety of viral infections. Moreover, specific responses serve as the main defense mechanisms against re-infection. Following primary infection, however, nonspecific host defense mechanisms play a critical role, prior to the acquisition of specific immune responses. These nonspecific defenses consist of a variety of pre-existing factors including barriers preventing virus penetration, nonspecific antiviral factors and cells capable of nonspecific antiviral activity (Mogensen, 1979).

There have been several lines of evidence which suggest that macrophages are important effector cells in resistance to HSV infections as well as to a variety of other viral infections (Hirsch et al., 1970). Johnson (1964), using immunofluorescence techniques, demonstrated that young mice were more highly susceptible to HSV infection because of the lack of a population of mature macrophages. Mogensen (1978) also reported that macrophage-related age-dependent resistance plays a role in protecting BALB/c mice from intraperitoneal HSV-2 induced focal necrotic hepatitis. Stevens and Cook (1971) using a biochemical approach extended these observations to the finding that macrophages infected with HSV undergo an abortive infection due to

improper viral assembly. Moreover, Hirsch et al. (1970) demonstrated in vivo that resistance to HSV infection in adult mice could be abrogated by pretreatment of mice with silica, an agent known to be toxic for macrophages. Others have shown that innate resistance can be enhanced by treatment of mice with agents which modulate or potentiate macrophage function (Starr et al., 1976; Morahan et al., 1974). Adult, activated macrophages when passively transferred into neonatal mice markedly protected mice from HSV lethality (Breinig et al., 1978). Frank and co-workers (1978) demonstrated that HSV virus multiplication in macrophages differed markedly in susceptible or resistant strains. Macrophages from highly susceptible NMRI strains supported replication of HSV 1 and 2 to a much greater degree than did macrophages from highly resistant C57/Bl mice. These results extend the original observations of Lopez (1975) who reported that susceptibility varied dramatically among different inbred strains of mice.

Our results are concordant with those reported by Hirsch et al. (1970) concerning the effects of silica on depressing resistance to intraperitoneal HSV infection as well as with other viruses. We noted a significant increase in susceptibility when mice treated intraperitoneally with silica were subsequently infected by the intravenous route. This was in contrast to Hirsch et al. (1970) who administered the silica and virus by the intraperitoneal route.

In addition to the use of silica on susceptibility to systemic infection, two other macrophage inhibitory agents not previously reported to have been used in the HSV system were utilized for the basis of comparison of the efficacy of the three agents. Dextran sulfate (MW 500,000) has been shown to increase susceptibility to Listeria infection in mice (Hahn, 1974). Trypan blue has been shown to decrease resistance of mice to tumor allograft challenge in mice in addition to abrogating the protective effect of passive transfer of tumor immunity (Hibbs, 1974). As observed in the results, all three agents increased susceptibility of mice to systemic HSV infection.

Silica has been shown to decrease resistance to a number of viral agents including HSV-1, HSV-2, yellow fever virus and Coxsackie virus, as well as enhancing carcinogen-induced tumor growth (Zisman et al., 1969; Keller, 1976). The mechanism of action appears to be autolysis of the macrophage. Dextran sulfate appears to exert its inhibitory effect not by killing the cell but by inhibiting phagosomal-lysosomal fusion (Hahn and Bierther, 1974). Frank et al. (1978) reported recently that the effect of dextran sulfate on HSV infection was only evident over a short period of time and concluded that decreased resistance by this agent must be affecting an early stage of virus host interaction. Trypan blue also appears to mediate its activity through inhibition of lysosomal function.

Furthermore, it was of interest to determine if route of infection had any effect on whether or not these three macrophage inhibitory agents would increase susceptibility. Therefore, we administered the same drug regimens as with systemic HSV, but infected mice by the vaginal route. The lethality as a result of vaginal infection was not markedly affected as evidenced by virtually no differences in the LD_{50} or local virus replication in the vaginal area. Several possibilities might explain this lack of effect. The macrophage inhibitory drugs were administered by the intraperitoneal route and, thus, may primarily affect macrophages in the peritoneal cavity and proximal reticuloendothelial system (RES) organs such as the liver or spleen. These organs may be involved in controlling the spread following systemic infection, but not in vaginal infection, in which virus has been shown to replicate locally and proceed directly into the central nervous system (Morahan et al., 1977). A second possibility to be considered is that, assuming that macrophages are important, they reside in the vaginal area and were not markedly affected by intraperitoneal administration of the macrophage inhibitory agents or not inhibited early enough to affect viral growth in that area. Finally, the possibility exists that macrophages during vaginal infection are not as critically important as in systemic infection and that local factors, such as pH of the vaginal secretions, may be

more important in preventing viral replication in the genital area.

While these three agents have been reported to vary with respect to mechanism of action, they all decreased resistance to systemic HSV-2 infection to a similar degree. While all three agents affected systemic infection, none of the agents affected vaginal infection. Whether or not functions which require macrophages in an accessory role was not determined, although it was observed that none of the agents markedly affected virus-specific antibody to HSV-2. It seems evident, however, that macrophage dysfunction produced by various means may contribute significantly to increased viral dissemination and ultimately to increased susceptibility to systemic HSV infection.

The administration of pyran copolymer in vivo prophylactically has been shown to increase resistance to a number of viruses as well as tumors in mice (McCord et al., 1976; Morahan et al., 1974; Mohr et al., 1975). Although the precise mechanism of pyran's activity has not been completely elucidated, there are several lines of evidence which suggests that the macrophage is the prime target of pyran's activity. Several changes in macrophage function occur following the administration of pyran including increased phagocytosis and microbicidal activity (Morahan et al., 1974).

With respect to its antiviral activity, pyran also appears to mediate its activity via the macrophage (Brienig

et al., 1978). The antiviral activity displayed when pyran was administered prophylactically followed by systemic infection with systemic HSV is not due to humoral T-dependent responses such as increased neutralizing antibody activity. Treatment by pyran critically delayed the appearance and production of antibody as compared with untreated mice, suggesting that pyran's antiviral activity was mediated through a phenomenon present prior to the onset of specific humoral responses (Morahan et al., 1975). The incidence of a specific delayed type hypersensitivity response was found to be less frequent in pyran-treated mice than in untreated mice which survived a primary challenge of virus. Furthermore, Morahan and McCord (1975) demonstrated pyran still retained its antiviral activity in mice depleted of T-cells. Pyran-treated mice were also not resistant to a secondary systemic challenge of HSV, unlike untreated mice which survived a primary challenge of virus, providing further evidence that T-cells were not involved in pyran's antiviral activity (Brienig et al., 1978). Pyran does not appear to mediate its antiviral effect through interferon, nor does it directly inactivate the virus (McCord et al., 1976). Pyran-treated peritoneal cells also possess antiviral activity *in vitro* as evidenced by a significant reduction in the $\log_{10}$/ml yield of HSV-2 (Breinig et al., 1978). This phenomenon was attributable to the adherent cell population comprised primarily of esterase positive and latex positive macrophages.

Treatment of this population with silica almost completely abrogated the response (Brienig et al., 1978).

Thus, it was of interest in our studies to determine if syngeneic peritoneal cells from pyran-treated adult mice could protect neonatal macrophage-deficient mice from a systemic lethal HSV infection. Concordant with the findings of Hirsch et al. (1970), it was noted that normal peritoneal cells afforded no protection. In contrast, passive transfer peritoneal cells from pyran-treated mice when passively transferred into neonates significantly reduced the mortality rate. Glycogen-treated peritoneal cells afforded some protection although not to the degree which was observed by pyran cells. This suggests that glycogen modulates macrophage activity in some fashion but whether these cells are activated in the classic sense was not determined. It was obvious, however, that neither pyran nor glycogen exerted a direct antiviral effect by virtue of the fact that neonates treated with glycogen or pyran were not protected. Clearly, pyran modulated macrophage function by way of a cell transferred into the neonates.

Following the observations that transfer of macrophage *in vivo* markedly increased resistance to systemic HSV-2 infection, it was of interest to determine if the antiviral activity of peritoneal exudate cells against HSV could be detected in an *in vitro* situation. Thus, a system was designed to look at the inhibition of viral replication by peritoneal exudate cells from mice infected with HSV,

recognizing that the peritoneal exudate represents a population having a comparatively high percentage of macrophages. Since the peritoneal exudate was to be the source of effector cells, the peritoneal cavity was chosen as the route of infection, as this was likely to provide the most direct interaction between virus and effector cell. As a result of these studies, several observations were made.

Peritoneal exudate cells from infected mice were capable of significantly reducing viral replication *in vitro*. This effect appeared early during the course of *in vivo* infection, prior to the onset of specific humoral and cell-mediated responses (Morahan et al., 1977). The response was transient, appearing 2 days following infection *in vivo*, peaking at 3-4 days and waning by day 7, a time at which specific CMI and humoral responses become apparent. These results are similar to those reported by Rodda and White (1976) using a cytotoxicity assay in the Semliki Forest virus system, but the effector cell in that system was a nonphagocytic NK cell (MacFarlan et al., 1977).

The majority of the antiviral activity appeared to reside in the adherent population of cells, as evidenced by the fact that nonadherent cells alone provided no inhibition of viral replication, and removal of these cells from the total population did not change the degree inhibition of the remaining adherent cells. These adherent cells consisted primarily of cells resembling macrophages (65-80%) based on morphology, latex particle ingestion, and the

presence of the nonspecific esterase enzyme. Thus, it is tempting to suggest that the macrophage is the prime effector cell responsible for the observed antiviral activity. However, although the term "macrophage" is used interchangeably with adherent peritoneal exudate cell, it is recognized that peritoneal cells represent a very heterogenous population. The possibility exists that the antiviral activity resides in the part of the adherent population that do not resemble macrophages, such as those reported by Nathan et al. (1977). They described a cell representing a relatively small percentage of the adherent cell population which was esterase positive, nonphagocytic, lacking EAC receptors, and resistant to killing with anti-macrophage serum. These cells were more prevalent in BCG-treated mice which suggests that an activating mechanism may stimulate the production of these cells, which may or may not be analogous to our system. Furthermore, these cells were capable of killing tumor cells in vitro, a phenomenon which has also been observed with HSV cells from HSV-infected mice (Morse, personal communication).

Further characterization of the antiviral activity of adherent population of peritoneal exudate cells from HSV-infected mice revealed several other characteristics. The antiviral activity was dose responsive. As the number of peritoneal cells added to infected cultures was decreased, so did the degree of viral inhibition. A minimum ratio of 1:2 macrophage:target cells was required. The activity was

nonspecific with regard to virus species. Cells from HSV-infected mice inhibited *in vitro* a totally unrelated RNA virus, vesicular stomatis virus, as well as HSV-2. Moreover, there appeared to be no observable requirement for histocompatibility between effector and target cell since mouse peritoneal cells inhibited viral replication in xenogeneic Vero monkey kidney cells as well as in syngeneic mouse embryo fibroblasts. Thus, the response although nonspecific did not appear to be mediated by "classic" interferon, due to the fact that there was no species specificity involved with regard to target and effector cell.

The point must also be discussed concerning the antiviral activity of normal macrophages. Activity was observed in some cases when normal PEC were added to cultures. However, the antiviral activity was much more inconsistent and never achieved the amount of inhibition exhibited by PEC obtained from HSV-infected mice. It is possible that in the presence of virus-infected cells *in vitro* that normal macrophages were activated during the three day culture period. Alternatively, it is possible that macrophages are, to some extent, in a constant state of "activation" and are capable of nonspecifically eliciting an antiviral effect. It seems reasonable to state, however, that macrophages from HSV-2 infected mice undergo some degree of change or alteration which makes them much more effective in inhibiting viral replication. Whether this

change is analogous to "activation" as with BCG, pyran or other synthetic agents or stimulation as with glycogen was not determined. Preliminary experiments by Morse et al. (manuscript in preparation) have indicated that HSV PEC, like pyran-activated cells, are capable of killing tumor cells *in vitro*, a criteria which has been associated with classical macrophage activation.

There have been comparatively few reports concerning the antiviral activity of macrophages against HSV-2 infected target cells (extrinsic antiviral activity). Considerably more data are available regarding the intrinsic antiviral activity or the response of the macrophage directly to the virus. It has been shown that there are considerable differences in the relative susceptibilities among different inbred strains of mice to intraperitoneal HSV infection (Lopez, 1976). It was thus of interest to determine if the ability of the macrophage to inhibit or support viral replication *in vitro* could be correlated with relative resistance *in vivo*. The recent report of Lopez and Dudas (1979) using HSV, and Selgrade and Osborne (1974) using murine cytomegalovirus suggest that such a correlation does not exist in the genetic resistance shown by adult mice. This appears in contrast to Johnson's findings which showed that the high susceptibility of newborn mice to HSV-2 infection was correlated with the ability of macrophages to support viral replication. However, it should be recognized

that Johnson's system dealt with age-related resistance and Lopez's system involved the resistance of adult mice as genetically determined. Different mechanisms are likely to be operative.

No reports to date have been available concerning the extrinsic antiviral activity of adherent peritoneal exudate cells from mice infected with HSV-2. Lodmell et al. (1973) using HSV-1 infected rabbit kidney monolayers demonstrated that peritoneal exudates from rabbits injected with casinate were capable of inhibiting viral plaque formation in vitro. Much higher ratios of macrophages to target cells (50:1) than in the present study were necessary to produce the effect. It was suggested that this activity was a result of the ability of the leukocytes to inhibit formation of intracellular bridges by damaging infected cells and surrounding adjacent cells. Antibody was effective in prohibiting extracellular spread, but was unable to inhibit cell to cell spread of infection.

Subsequent to this work, Lodmell and co-workers (1974) using a similar system reported that cells from rabbits immunized in vivo with Mycobacterium turberculosis were capable of inhibiting viral growth in HSV-infected rabbit kidney monolayers in the presence of PPD. Similarly, leukocytes from HSV-2 sensitized animals were capable of inhibiting viral replication in vitro. It was suggested that interferon produced by sensitized leukocytes might be the mediator of this antiviral activity. Only at high

effector:target cell ratios did unsensitized leukocytes exert an antiviral effect through a generalized nonspecific effect, and little or no interferon was detectable in these cultures.

Morahan et al. (1977) reported extrinsic macrophage-mediated antiviral activity of peritoneal exudate cells from mice treated with pyran, C. parvum or infected with vaccinia virus. Similar to the findings of Lodmell et al. (1973, 1974), it was suggested that interferon might be involved in the antiviral activity of vaccinia PEC. In support of this, Kirchner et al. (1977) reported that C. parvum activated spleen and peritoneal cells were capable of producing an antiviral factor which was trypsin sensitive, species specific and virus nonspecific. Thus, it was determined to be interferon.

Several analogies may be drawn to the data presented from this work and the work of others. The most obvious similarity is the fact that in reports, the antiviral activity of the macrophages was nonspecific with regard to virus specificity. Secondly, the activity displayed by these cells was capable of restricting intercellular spread of virus and perhaps extracellular spread. Although this implies that the mechanisms of antiviral activity of the effector cells involved in the various systems may in fact be similar, the data on interferon involvement indicate that differing mechanisms must be operative.

Although the scope of this research did not encompass the mechanism(s) of adherent peritoneal cell (macrophage) antiviral activity, it is tempting to entertain several hypotheses. One aspect would be to determine how the macrophage interacts with the virus. Two likely situations exist. The macrophage may be directly interacting with virus and, following phagocytosis, results in destruction of the infectious virion. This would, thus, be occurring at the extracellular level, and would not account for inhibition of contiguous spread. Alternatively, instead of a direct macrophage-virus interaction, the effector cell may exert its activity through a macrophage-virus infected cell interaction. This could occur through a recognition of viral antigens on the surface of infected cells, followed by destruction of these cells resulting in an abortive viral replication cycle. This theory could account for inhibition of contiguous spread of virus. Other mechanisms operative on the target cell that render it nonpermissive for viral growth are, of course, also possible.

A second consideration with regard to macrophage-mediated antiviral involves the interaction of the macrophage and either the virus or virus-infected target cell. Preliminary observations suggest that direct physical contact between the macrophage and virus or infected cell may not be required (Morse, personal communication). This would suggest that the macrophage might mediate its activity

through elaboration of some type of factor. This factor, although we did not attempt to characterize it, appears not to be a "conventional" type of interferon, since interferon is classically thought to be species-specific. This was not the case in our system. Antibody also does not appear to be operative in this system since there was no apparent specificity toward the initial infecting virus. A more detailed analysis of the molecular events which occur during macrophage-virus interaction should provide a more comprehensive understanding of the mechanisms of macrophage-mediated antiviral activity.

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