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Role for Cell-Mediated Immunity in the Resistance of Mice to Subcutaneous Herpes Simplex Virus Infection

J. E. OAKES

University of Tennessee, Department of Microbiology, Knoxville, Tennessee 37916

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The role of cell-mediated immunity in the resistance of young adult mice to subcutaneous herpes simplex virus (HSV) type I infection was studied in mice receiving immunosuppressive doses of antilymphocyte sera (ALS) or antithymocyte sera (ATS). The effectiveness of these treatments to reduce cell-mediated responses was measured by their ability to prolong the life of allografts transplanted to ALS- or ATS-treated mice. It was found that subcutaneous infection of these mice with HSV resulted in spread of virus from the site of inoculation to the central nervous system. Neutralizing antibody could not be detected in the sera of ALS- or ATS-treated mice after HSV inoculation. Passive transfer of neutralizing antibody to ATS-treated mice did not restore resistance to subcutaneous HSV infection. However, adoptive transfer of HSV-sensitized spleen cells did provide significant protection against infection unless the spleen cells were treated with ATS prior to transfer. These experiments suggest that lymphocytes are involved in a cell-mediated response to subcutaneous HSV infection and demonstrate the importance of a noncompromised immune response in controlling spread of HSV from localized areas of infection.

Persistence in its natural host for long periods is a unique property of herpes simplex virus (HSV) pathogenesis. This persistence is often manifested as recurrent inflammatory lesions on cutaneous surfaces of individuals who have recovered from a primary infection. Since these infections can continually reappear in the presence of humoral (3, 4) and cell-mediated reactions against HSV (24), immunological mechanisms do not appear to play an important role in controlling activation of virus within the host. However, it has been observed that patients undergoing immunosuppressive therapy often suffer more severe attacks of recurrent herpetic infections which extend over larger areas of their body (25). Thus, the host's defense mechanisms may be a more decisive factor in limiting dissemination of virus from localized lesions than in preventing recurrent infections.

Experimental subcutaneous HSV infections in mice have also shown that a host can contain virus within localized areas unless the immune response has been compromised with immunosuppressive agents (20). Under these conditions, HSV leaves the site of infection and progresses to the central nervous system. The present study examines in further detail the influence of cell-mediated immunity in limiting spread of HSV after peripheral subcutaneous infection in young adult mice.

MATERIALS AND METHODS

Viruses. HSV type I (strain kos) (22) was kindly provided by W. E. Rawls (McMasters University, Hamilton, Ontario, Canada). Preparation and assay of viral stocks for plaque-forming units (PFU) on Vero cell monolayers were performed as previously described (20). Sindbis virus (26), a generous gift from the laboratory of A. Brown (Department of Microbiology, University of Tennessee), was passaged 14 times in suckling mouse brains and once in chicken embryo fibroblast cells. Final virus preparations were grown and assayed on baby hamster kidney cell monolayers.

Mice. Five-week-old SJL mice (Jackson Laboratory, Bar Harbor, Maine) or 4- to 6-week-old C_{57} black mice (Cumberland View Farms, Clinton, Tenn.) were used in all experiments. Mice to be immunosuppressed with irradiation were placed in groups of 18 in a circular lucite box on a revolving turntable 50 cm from the target of a 250-kV-power X-ray source (Maxitron 700, General Electric) operated at 20-mA tube current with a 1.13-mm Cu filter. The exposure dose rate delivered was 110 R per min as measured by a Victoreen R-meter (model 570 condenser R-meter, Victoreen Instrument Division, Victoreen, Inc., Cleveland, Ohio).

Assay of antilymphoid sera. Rabbit anti-mouse lymphocyte sera (ALS) or antithymocyte sera (ATS) and normal rabbit sera (NRS) were purchased from Microbiological Associates, Bethesda, Md. The immunosuppressive activity of the preparations was measured by their ability to prolong skin allografts exchanged between C_{57} and SJL mice. Skin from the

donor or recipient areas was lifted into a conical tent with curved hemostats and cut off adjacent to the instrument. Any panniculus adiposus adhering to the grafts was removed by gentle scraping. Skin from the right subscapular area of the thorax on recipient mice was transplanted to the opposite side as a control. Skin from the donor was then transplanted onto the vacated graft bed on the right side of the recipient. Steri-strip skin enclosures (3M Co.) were placed over the grafts to secure them in place. A corban elastic bandage (3M Co.) was then wrapped around the thorax to form a "corset." At 12 days post-operative, the corban and steri-strip were removed. Donor grafts on NRS-treated animals rapidly contracted into dark-brown scabs. Rejection was considered complete when the scab could be removed by gentle rubbing. Grafts on ALS- or ATS-treated animals did not form a scab but were gradually replaced by recipient epithelium. Rejection in these animals was considered complete when recipient skin replaced the transplant. The autografts appeared normal 20 to 25 days after grafting and were not rejected.

Preparation and assay of neutralizing antibody. Rabbit anti-HSV hyperimmune serum was prepared by multiple intravenous inoculation of partially purified virus as described by Hampar et al. (9). The serum, harvested by cardiac puncture, was filter sterilized, heat inactivated (56 C for 30 min), and stored at −60 C until use. Mouse serum was obtained by bleeding from the retroorbital plexus, but the serum was not heat inactivated since early anti-HSV antibodies are complement dependent (33).

Sera were measured for neutralizing antibody by a plaque-reduction method (21). Sera were diluted twofold in Hanks balanced salt solution (HBSS). HSV preparations, diluted in Eagle basal medium with Hanks salts plus 2% fetal calf serum to approximately 500 PFU/ml, were added to 0.5 ml of diluted sera. The mixtures were incubated at 37 C for 1 h and then assayed for the number of PFU remaining. Three plates were used per dilution. Controls consisted of 0.5 ml of sera obtained from nonimmunized animals. The antibody titer was expressed as the reciprocal of the highest dilution of serum which caused a 50% reduction in plaques when compared to control plates. The proportional "distance" was estimated by the method of Reed and Muench (23).

Cell transfer of HSV immunity. Immune spleen cell donors were immunized subcutaneously in the right rear footpad with 5×10^5 PFU of HSV. Two to four weeks later, both rear footpads were injected with approximately 0.05 ml of 10% saline. The edematous areas were abrased with an emory board 6 h later to remove the stratum corneum. One drop of virus suspension from a 26-gauge needle (1×10^5 PFU/ml) was placed on the abrased area and rubbed in with the shank of the needle. This method of immunization resulted in an acute inflammatory infection at the site of inoculation. Control mice were treated with HBSS in place of virus. Nine to twelve days later, spleens were removed from the mice and extruded aseptically from their capsules. After being minced with scissors, the material was filtered through sterile cotton gauze to remove clumps of tissue. Cells were washed and resuspended in HBSS to 10^8 cells/ml. Adherent cells were depleted by placing 25 ml of the suspension in glass 32-ounce (ca. 0.95 liter) prescription bottles at 37 C in 5% CO_2 for 90 min. Nonadherent cells were decanted after gentle rotation of the flask. The cells were washed and resuspended in HBSS. Differential counts of spleen cell preparations showed approximately 95% lymphocytes, 4% monocytes, and 1% segmented cells. Several samples of donor spleen cells were also frozen and thawed twice and were found not to contain HSV as determined by isolation attempts on Vero cells. In some experiments bone marrow cells were transferred in addition to spleen cells. These were harvested from both femurs of SJL mice by aspiration with HBSS from a 27-gauge needle (30). The number of viable cells was determined by hemocytometer counts using the trypan blue exclusion test. Recipient mice received viable cells intravenously.

Sindbis virus-sensitized spleen cells were harvested 9 to 12 days after mice were inoculated subcutaneously in rear footpads with 5×10^6 PFU of Sindbis virus. The cells were prepared as above for transfer to recipient mice.

Sensitivity of immune spleen cells to ATS. The sensitivity of immune spleen cells to ATS was tested by suspending them at a concentration of 10^8 cells/ml in ATS diluted 1/2 in phosphate-buffered saline (0.5 M, pH 7.2) for 30 min at 37 C. The cells were then washed and resuspended in a 1:10 dilution of reconstituted guinea pig serum (Colorado Serum Co., Denver, Colo.) for 20 min at 37 C. The cells were then washed twice in HBSS and counted in a hemocytometer. This technique reduced the cell numbers from the original preparation by 33% as determined by hemocytometer counts with added trypan blue. Control cells were treated with NRS in place of ATS. Mice were inoculated intravenously with 3×10^8 trypan blue-excluding cells from the final wash.

RESULTS

Effect of ATS or ALS on allograft survival. Five-week-old SJL mice were given five intraperitoneal injections of 0.4 ml of ALS or ATS on alternate days. Normal rabbit serum was given as a control. After the second injection, skin from C_{57} mice was transplanted onto treated SJL mice. The mean survival time of these skin grafts is shown in Table 1. The routine of ATS or ALS treatment increased the mean survival time over NRS-treated mice by approximately 10 days. This was highly significant as determined by the Student t test ($P < 0.01$).

Effect of ALS or ATS on subcutaneous HSV infection. Employing the same preparations and administration schedule, groups of 10 mice each were treated with ALS, ATS, or NRS and inoculated subcutaneously with HSV in one rear footpad after the second treatment. As shown in Table 2, less than 10% of the mice in the NRS-treated group died from infection, whereas 80% or more of the ALS- or ATS-treated

TABLE 1. *Effect of ATS, ALS, or NRS on survival of allografts*[a]

Expt	Group[b]	Mean survival time ± standard deviation
1	ATS	24.3 ± 2.7
	NRS	14.6 ± 1.2
2	ALS	26.7 ± 3.4
	NRS	15.8 ± 1.5
3	ALS	24.8 ± 3.4
	NRS	14.1 ± 2.1

[a] Mice received five intraperitoneal injections of 0.04 ml of ATS, ALS, or NRS on alternate days. Skin was transplanted after the second treatment.

[b] Ten animals per group.

TABLE 2. *Percent survival of 5-week-old mice after subcutaneous inoculation into one rear footpad with 1 × 10⁶ PFU of HSV after treatment with ALS, ATS, or NRS*[a]

Expt	No. of animals/ group	% Survival after treatment with:		
		NRS	ALS	ATS
1	10	90	10	20
2	10	100	20	0

[a] Mice were treated with five intraperitoneal injections of 0.4 ml of ALS, ATS, or NRS on alternate days. Virus was inoculated 6 hours after the second treatment.

mice died. Clinical signs of infection in this group began with a flaccid paralysis in the inoculated foot 3 to 4 days postinfection. The paralysis increased in severity until both rear legs became paralyzed 2 to 3 days later. The affected mice began dying 9 days after HSV infection.

In another experiment, 10 mice were treated with ATS and again inoculated subcutaneously with HSV. Five moribund mice from this group were sacrificed on day 9, and their blood, liver, brain, spinal cord, and inoculated foot were removed. The tissues were individually homogenized in a Dounce tissue grinder (Bellco Biological Glassware and Equipment, Vineland, N.J.) and assayed for infectivity on Vero cells. As shown in Table 3, virus could be detected only in the central nervous system and inoculated foot.

These observations suggest that antilymphoid sera increase the susceptibility of the central nervous system to invasion by HSV from peripheral sites of infection. After spreading to the central nervous system, virus multiplies in the nervous tissue, killing the host in about 10 days.

Effect of ALS or ATS on neutralizing antibody. Three groups of 15 mice each were given a standard routine of ATS, ALS, or NRS treatment. A fourth group was untreated. After viral inoculation, three mice were randomly selected on alternate days from each group and bled from the retroorbital plexus. Their sera were pooled and assayed for neutralizing antibody. The amount of neutralizing antibody detected is illustrated in Fig. 1. Circulating antibody was present by day 7 postinfection in NRS-treated or untreated mice, but could not be detected in ALS- or ATS-treated mice. Thus, both ALS and ATS were effective in reducing the ability of the host to produce neutralizing antibody in response to a subcutaneous HSV infection.

Passive transfer of antibody. The importance of circulating antibody in controlling subcutaneous HSV infections could not be evaluated with antilymphoid sera since suppression

TABLE 3. *Number of PFU obtained from various tissues 9 days after subcutaneous inoculation of ATS-treated mice with 1 × 10⁶ PFU of HSV*

Tissue[a]	PFU/g (avg)
Foot	8.9×10^5
Spinal cord	1.4×10^6
Brain	2.5×10^3
Liver	0 (sensitivity ≥ 100 PFU/g)
Blood	0 (sensitivity ≥ 30 PFU/ml)

[a] Tissues from five mice were assayed.

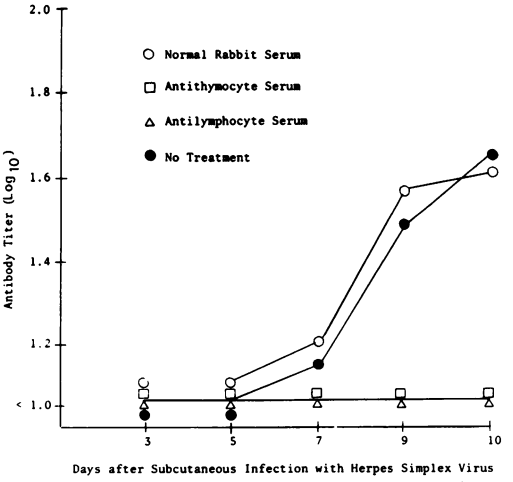

FIG. 1. *Kinetics of serum-neutralizing antibody formation in mice treated with ATS, ALS, or NRS and inoculated subcutaneously in the right rear footpad with 1 × 10⁶ PFU of HSV.*

of an antibody response to HSV accompanied both ATS or ALS treatment. Therefore, the influence of antibody on the course of subcutaneous HSV infection was directly determined by passively transferring sera from rabbits hyperimmunized to HSV to ATS-treated mice before HSV infection. Twenty mice received two 0.4-ml doses of ATS on alternate days. After the second ATS treatment, 10 of the mice received an intravenous injection of 0.4 ml of rabbit anti-HSV hyperimmune serum (HSV neutralizing titer = 1/640). Six hours later, all mice were inoculated subcutaneously in the rear footpad with 1×10^6 PFU of HSV. This was followed by three additional ATS treatments on days 2, 4, and 6 postinfection. Passive antibody did not significantly alter the outcome of HSV infection ($P < 0.01$, chi square test) (Fig. 2). The levels of passive antibody remaining in circulation during the 10-day experiment were determined by passively transferring an equal amount of neutralizing antibody to a group of 12 mice treated with identical doses of ATS. Two mice were removed from this group on alternate days and bled from the retroorbital plexus. Their sera were pooled and assayed for antibody. Neutralizing antibody remained in the circulatory system throughout the period required for HSV to spread from the footpad to the central nervous system (Fig. 3). The data also show that passive antibody transferred was comparable to the levels of antibody physiologically produced in untreated mice after subcutaneous inoculation. These results suggest that circulating antibody produced by the host in response to a subcutaneous HSV infection is not an important defense mechanism in preventing spread of virus to the central nervous system under these conditions.

Transfer of sensitized spleen cells to HSV-infected mice. The previous experiments demonstrated that passive antibody cannot restore resistence of young adult mice to HSV infection after treatment with antilymphoid sera. This suggests that a cellular response is of greater importance than an antibody response in recovery of the host after infection. To further investigate the influence of a cell-mediated response on the course of infection, HSV-sensitized spleen cells were transferred to mice before virus inoculation. Mice were immunosuppressed with 450 R of X irradiation prior to cell transfer to increase their susceptibility to HSV infection (20). Six hours later, groups of mice received intravenously normal spleen cells or HSV-sensitized spleen cells. Bone marrow cells were given to one-half of each group in addition to spleen cells to replace any monocyte precursor cells which may have been damaged by the X irradiation dose (31). The bone marrow provided a potential population of monocytes which have been shown to be required in many cell-mediated responses (13, 14). Spleen cells from Sindbis virus-inoculated mice were also transferred to irradiated mice to serve as an additional control. The spleens were harvested from these mice at the time a maximum immune response to Sindbis virus has been re-

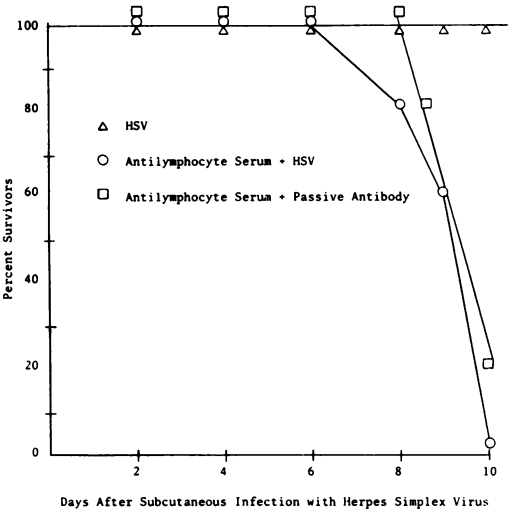

FIG. 2. *Survival of mice treated with ATS and inoculated subcutaneously with 1×10^6 PFU of HSV 6 h after passive transfer of 0.4 ml of rabbit HSV-neutralizing antibody. There were 10 mice per group.*

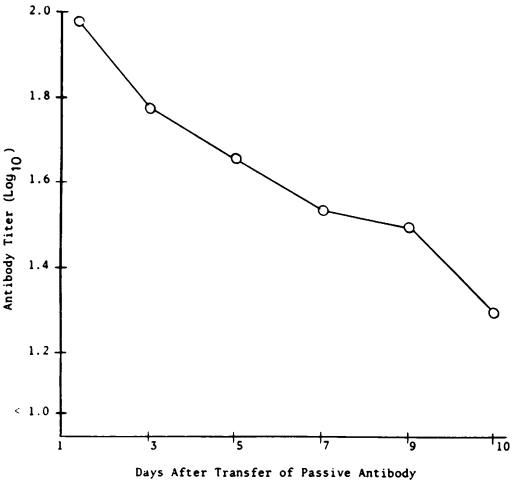

FIG. 3. *Amount of HSV-neutralizing antibody in the blood of mice treated with ATS and passively immunized with 0.4 ml of rabbit HSV-neutralizing antibody.*

ported to occur in spleens of footpad-infected mice (8). Twenty-four hours after cell transfer, all groups received 1×10^6 PFU of HSV subcutaneously in the right footpad. The results in Table 4 show that HSV-sensitized spleen cells significantly protect mice against subcutaneous HSV infection. Bone marrow cells were not required as helper cells to augment protection provided by a single transfer of immune spleen cells. The reaction was specific since Sindbis virus-sensitized spleen cells afforded no protection against subcutaneous HSV infection. Anti-viral protection is dependent upon the number of immune spleen cells adoptively transferred to susceptible mice (Fig. 4).

Immune spleen cells were also treated with ATS prior to their transfer into irradiated recipients to deplete the lymphocyte population. ATS treatment of spleen cells capable of protecting mice against HSV infection eliminated the potential of these cells to protect susceptible mice against subcutaneous inoculation (Table 5).

DISCUSSION

Resistance and recovery of young adult mice to HSV infection was initially associated with the host's macrophages (10, 11). These cells were shown to play an important role in preventing spread of virus within infected tissues by inhibiting the multiplication of ingested virus (29). However, it soon became apparent that lymphocytes in addition to macrophages were involved in immunity to some forms of HSV infection. For instance, it was found that adoptive transfer of sensitized lymphocytes protected recipients from intravenous viral challenge (6), whereas both macrophages and lymphocytes were required to limit spread of virus after intraperitoneal inoculation (34). These observations suggested that under some conditions, at least two populations of immunologically competent cells are involved in defense against HSV. Certainly the interactions involved between lymphocytes and macrophages in restricting spread of virus from subcutaneous

TABLE 4. *Adoptive transfer of immunity to irradiated mice against HSV with immune spleen cells*[a]

No. of cells transferred	Survivors/total no. of mice infected	
	Expt 1	Expt 2
Immune spleen cells (5×10^8)	9/10	8/10
Immune spleen cells (5×10^8) + 3×10^8 bone marrow cells	9/10	
Nonimmune spleen cells (5×10^8)	1/10	2/10
Nonimmune spleen cells (5×10^8) + 3×10^8 bone marrow cells	2/10	
Bone marrow cells (3×10^8)	0/10	
Sindbis virus-immune spleen cells (5×10^8)	1/10	
None	0/10	1/10

[a] Bone marrow was given intravenously after mice received 450 R. Spleen cells were transferred intravenously 24 h later followed by subcutaneous inoculation of 1×10^6 PFU of HSV in the right rear footpad. Mice were observed for 3 weeks. There were no deaths after 14 days.

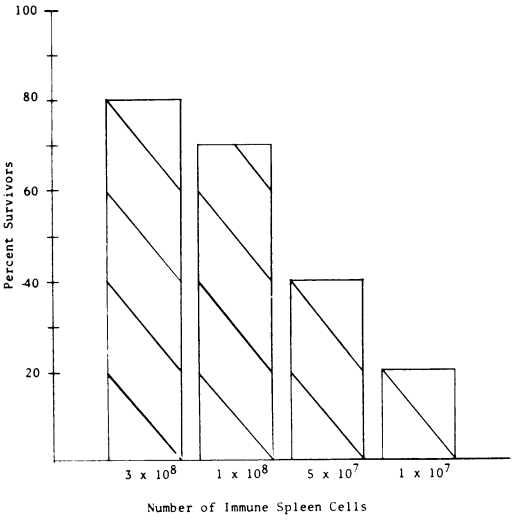

FIG. 4. *Relationship between number of cells adoptively transferred and resistance of irradiated mice to subcutaneous HSV infection. Mice were treated with 450 R 24 h before adoptive transfer of spleen cells. Six hours later the right rear footpads were inoculated with 1×10^6 PFU of HSV. There were 10 mice per group.*

TABLE 5. *Adoptive transfer of immunity to irradiated mice against HSV with immune spleen cells treated with NRS or ATS*[a]

Treatment of immune spleen cells before transfer	Survivors/total no. of mice infected[b]
NRS + complement	8/10
ATS + complement	2/10
No treatment	9/10

[a] Mice were irradiated with 450 R 24 h before intravenous transfer of 3×10^8 treated immune cells. Subcutaneous inoculation of 1×10^6 PFU of HSV in the right rear footpad followed 24 h later.

[b] Mice were observed for 3 weeks. There were no deaths after 12 days.

areas of infection are not clearly understood even though the skin and mucous membranes are common sites of both primary and recurrent herpesvirus infections (18).

In this investigation, antilymphoid sera was initially used to determine the contributions of lymphocytes in controlling subcutaneous HSV infection. Mackaness has shown that a population of specifically sensitized lymphocytes is required to trigger a cell-mediated response to an infection (16). Antilymphoid sera will selectively deplete this population of antigen-reactive recirculating lymphocytes, thereby limiting the potential of the host to mediate a cellular response (12). In the present study, both ATS and ALS were used to treat infected mice since there has been some claim of the superiority of one type of serum over the other (32). However, both ATS and ALS were effective in reducing a cell-mediated response in these experiments as measured by allograft rejection.

Mice immunosuppressed with ATS or ALS demonstrated an increased susceptibility to subcutaneous HSV inoculation. Evidence was found for virus spreading from the footpad to the central nervous system where it caused a fatal encephalitis. Mice treated with NRS presumably retained normal recovery mechanisms since treatment did not increase their susceptibility to HSV infection. It could not be concluded, however, that recovery was dependent upon a cellular response involving lymphocytes since antibody synthesis was also impaired following ATS or ALS treatment. Passive antibody was therefore transferred to ATS-treated mice to provide levels of circulating antibody normally produced after subcutaneous HSV inoculation. It was found that this antibody did not restore the resistance to infection that antilymphoid sera had eliminated. These results therefore suggest the major importance of a cell-dependent response in recovery of mice from subcutaneous HSV infection. However, they do not completely eliminate a role for antibody-mediated immunity. It is known, for example, that specific antibody in addition to complement can destroy infectious cells containing HSV-specific antigens on their surface (2). Mononuclear cells have also been observed to interact with antibody, augmenting their ability to kill virus-infected cells (27). These mechanisms could be operating in local tissues to influence the outcome of infection even though controlling the susceptibility of mice to the virus. Also, the HSV-neutralizing sera used for passive transfer experiments reported here may not contain the necessary cytophilic antibodies involved in these important reactions.

Direct evidence for participation of immunologically competent cells in recovery of mice from subcutaneous infection was obtained by adoptive transfer of HSV-immune cells to irradiated recipients. These cells were highly effective in protecting irradiated mice against a subcutaneous virus challenge when harvested from spleens of mice recovering from a subcutaneous HSV infection. The reaction was specific since protection could not be adoptively transferred with cells harvested from mice immunized to heterogenous Sindbis virus. The ability of ATS treatment to reduce the potential of immune spleen cells to transfer immunity into recipients identified the reactive cells as a species of lymphocyte. These findings indicate that sensitized lymphocytes are an important barrier in limiting spread of virus from initial sites of infection in this system and support the conclusions that cell-mediated immunity is involved in recovery of mice from subcutaneous HSV infection.

The precise mechanism by which sensitized cells protect recipient mice against a subcutaneous HSV infection is not understood. However, the results of several in vitro studies suggest that the host's immune response may be able to react to HSV-infected tissues at the site of inoculation to limit further spread of virus. Many viruses produce specific antigens on the surface of cells they infect. When lymphocyte populations from animals sensitized to a particular virus are then placed with these cells in culture, they respond to the surface antigens and destroy the infected cells (15, 16, 28). Sensitized mouse spleen cells have also been shown to control spread of HSV in cell culture (5), but the ability of these cells to protect irradiated recipients in vivo in the absence of bone marrow was not expected. Blood monocytes are required in many cell-mediated responses, where they infiltrate into the site of reaction to become the effector cell (16). Irradiation severely damages the ability of bone marrow to resupply these cells to the host (31). It is unlikely that the 10^8 spleen cells adoptively transferred in these experiments could have provided the necessary numbers of monocytes since in other systems the spleen has been shown not to be the source of monocytes during a cell-mediated reaction (7, 19). Blanden has also noted that macrophage-depleted sensitized spleen cells can elicit a protective effect against ectromelia virus infections in irradiated mice (1). Thus, the ability of immune spleen cells to react directly to a viral infection in irradiated hosts is not a unique property of HSV infection.

This study demonstrates the important role of a cell-mediated response in controlling spread of virus from subcutaneous areas of

infection. The ability of antilymphoid sera to both decrease host resistance to infection and eliminate adoptive transfer of immunity supports the concept that lymphocytes are the cells which initiate the cell-mediated response leading to recovery of the host in this disease.

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