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Antibody-Mediated Recovery from Subcutaneous Herpes Simplex Virus Type 2 Infection

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Young adult mice infected with 3×10^6 plaque-forming units of herpes simplex virus type 2 (HSV-2) died within 9 to 12 days after spread of the virus from the sites of infection to the spinal cord and brain. Administration of HSV-2-neutralizing antisera prepared in syngenic mice or in rabbits inhibited spread of the virus from the footpad of infected animals and prevented death. A single intraperitoneal inoculum of antiserum (virus-neutralizing titer of 1:128) was effective in protecting mice when the antiserum was given at 8 to 48 h, but not at 72 h, after virus inoculation. Immune sera absorbed with HSV-2-infected cells no longer protected mice from subcutaneous infection, whereas absorption with noninfected cells had no effect. Thus, HSV-2-specific antibodies appeared to be responsible for the protection observed. If the mice were given a sublethal dose of irradiation (390 rads) at 24 h before antibody transfer, protection was no longer obtained. This suggested that the mechanism of protection probably was not solely due to in vivo neutralization of virus, but required the participation of a radiosensitive component which has not yet been defined.

The contributions of sensitized lymphocytes in immunity to herpes simplex virus (HSV) infections have been clearly established by adoptive transfer experiments (8, 17). However, the role of antibody in host defenses against HSV infections in vivo is poorly understood. In humans, it has been established that circulating antiviral antibody does not prevent virus recrudescences in individuals suffering from recurrent HSV infections (5, 7, 29). In addition, several investigators have reported that specific antibody given to experimental animals after HSV type 1 (HSV-1) inoculation does not protect the host from virus spread (10, 16, 20). Although it has been difficult to demonstrate a beneficial effect of specific antibody in vivo, studies in vitro have defined several mechanisms whereby antibody can reduce virus spread. These include antibody-dependent cell cytotoxicity (14, 21, 24-26, 27) and complement-dependent cell lysis (2).

The failure to protect laboratory animals from HSV-1 infection with specific antibody may be due to the circumstances in which HSV-1 infections are initiated. Because HSV-1 is not highly virulent in immunologically mature hosts (12, 13), most HSV-1 infections have been established by virus inoculation into brains of recipients or by peripheral infection of immunosuppressed or immunologically immature animals (10, 16, 20). Under these conditions, antibody may not have the capacity to initiate recovery. Unlike HSV-1, HSV-2 readily spreads to the central nervous system after subcutaneous infection of young adult mice. When this route of inoculation is employed, the effects of neutralizing antibody upon the course of HSV-2 infection can be examined by a nonneural route of infection in an immunologically competent host. We have found that passive administration of neutralizing antibody after subcutaneous HSV-2 infection inhibits spread of HSV-2 from the sites of inoculation. Our data also suggest that a radiation-sensitive component participates in the protective mechanisms because sublethally irradiated animals are not protected from subcutaneous HSV-2 infection by passive transfer of antibody.

MATERIALS AND METHODS

Cells and virus. Vero cells, obtained from Flow Laboratories, Inc., Rockville, Md., were maintained in 150-cm^2 plastic tissue culture flasks (Corning Glass Works, Corning, N.Y.) in minimum essential medium supplemented with antibiotics, 10% fetal calf serum, and $NaHCO_3$. HSV-2 strain 333 was obtained from Fred Rapp, Hershey, Pa. Virus stocks were prepared from plaque-purified virus. Monolayers of Vero cells were infected at a multiplicity of 0.1 plaque-forming unit (PFU) per cell, and the virus was harvested from cells 28 h later by three cycles of freezing and thawing. The supernatant was clarified and assayed on Vero cells as previously described (16).

Mice. Four- to five-week-old C_{57}/Black/10Sn mice (The Jackson Laboratory, Bar Harbor, Maine) were used in all experiments.

Assay of virus-infected tissues. Tissues to be assayed for virus were dissected from donor mice and homogenized in a Dounce tissue grinder (Bellco Glass, Inc., Vineland, N.J.) containing Hanks basal salt solution to give a 10% (wt/vol) suspension. The mixtures were frozen and thawed three times and centrifuged at 1,000 × *g* for 10 min at 22°C, and the supernatants were used for viral assays on monolayers of Vero cells.

Preparation and assay of hyperimmune serum. Rabbit anti-HSV-2 hyperimmune serum was prepared by multiple intravenous inoculation of partially purified virus as described by Hampar et al. (11). The serum was harvested by cardiac puncture, heat inactivated at 56°C for 30 min, and stored at 60°C until use. Mouse hyperimmune serum was obtained by immunizing mice with 10^3 PFU of HSV-2 in the right rear footpad. After 2 weeks, the animals were rechallenged with 3×10^6 PFU of HSV-2 in both rear footpads. This resulted in an inflammatory reaction at the site of infection which cleared up in about 1 week. Mice were bled from the retro-orbital plexus at 10 to 12 days after the second virus challenge. The sera were pooled, heat inactivated at 56°C for 30 min, and stored at 60°C until use.

Sera were measured for neutralizing antibody by a plaque reduction method (22). Sera and virus dilutions were made in minimal essential medium without fetal calf serum. Approximately 300 PFU of HSV-2 per ml was added to equal volumes of twofold dilutions of the sera. The mixtures were incubated at 37°C for 1 h and then assayed for infectious particles. Each dilution was assayed in triplicate. 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 when compared with control plates. The proportional "distance" was estimated by the method of Reed and Muench (23). Anti-salmonella antibodies were prepared by parenteral injection of *Salmonella* O antigen into rabbits (4). The serum from several rabbits was pooled, and the titer was determined by bacterial agglutination (4). A 1-ml quantity of a 1:320 dilution of antiserum agglutinated 10^9 Formalin-treated cells per ml.

Passive transfer of hyperimmune sera. Mice were passively immunized by intraperitoneal administration of 0.5 ml of an appropriate serum. To standardize the various anti-HSV-2 hyperimmune serum preparations used in the passive transfer studies, all serum preparations were diluted to contain a neutralizing antibody titer of approximately 1:128 before passive transfer. This titer was the highest titer of antibody obtainable in actively immunized mice by our procedure.

Absorption of hyperimmune serum with HSV-2-infected cells. A 5-ml quantity of a 1:4 dilution of rabbit HSV-2 immune serum (initial titer, 1:128) in minimal essential medium without fetal calf serum was mixed with either 2×10^8 HSV-2-infected Vero cells or noninfected cells. A 1:4 dilution of hyperimmune serum (initial titer, 1:128) protected 90% or more of the mice inoculated subcutaneously with 3×10^6 PFU of HSV-2 (data not shown). Hyperimmune serum–HSV-2-infected cell mixtures were incubated at 37°C for 1 h. The cells were then removed by centrifugation. The resulting supernatant was reabsorbed with a second preparation of 2×10^8 infected cells for 4 h at 4°C before centrifugation at 1,000 × *g* for 10 min to pellet the cells. The supernatants were then transferred to polyallomer centrifuge tubes (1 by 3.5 mm; Beckman Instruments, Inc., Fullerton, Calif.) and centrifuged at 30,000 rpm for 1.5 h in a Beckman 50.1 rotor to pellet any antigen-antibody complexes or unneutralized virus. Hyperimmune sera and noninfected cell mixtures were treated similarly. The sera were carefully removed from the centrifuge tubes with a Pasteur pipette so as not to disturb the pellet. The serum preparations were used for passive transfer experiments.

Irradiation of mice. Mice were placed in groups of 12 in square Lucite boxes 80 cm from the target of an AECL model 8 irradiator containing ^{60}Co emitting 6,268 Ci and were exposed to 170 rads/min for 2.2 min. This dose did not produce any symptoms of irradiation damage in the animals.

RESULTS

Effect of passive transfer of HSV-2-neutralizing sera on primary subcutaneous HSV-2 infection. Mice were inoculated subcutaneously in the right rear footpad with 3×10^6 PFU of HSV-2. The virus was allowed to absorb to tissues and initiate infection for 8 h. At the end of this period, groups of infected mice received either rabbit HSV-2-neutralizing sera, rabbit anti-salmonella sera, or normal rabbit sera. A fourth group of animals received syngenic mouse HSV-2-neutralizing sera. The remaining animals received normal mouse sera or were left untreated. All mice in groups receiving normal sera, anti-salmonella sera, or no treatment died from infection, whereas 90% or more of the mice receiving rabbit or mouse HSV-2-neutralizing sera survived (Fig. 1). All of the surviving mice remained free of clinical symptoms during 6 weeks of observation.

When tissues were removed from unprotected mice at time of their deaths, virus was found in the peripheral nervous system, central nervous system, and liver in mice receiving normal sera (Table 1). Virus was not detected in any tissues of animals passively immunized with HSV-2-neutralizing sera. These experiments indicate that HSV-2-neutralizing sera prevented spread of HSV-2 from subcutaneous sites of infection. Syngenic mouse or xenogenic rabbit neutralizing sera were equally effective in transferring antiviral immunity. Because rabbit sera were easier and less expensive to prepare, further investigations utilized rabbit sera instead of mouse sera.

Fate of HSV-2 antibodies in blood of mice after passive immunization. The appearance

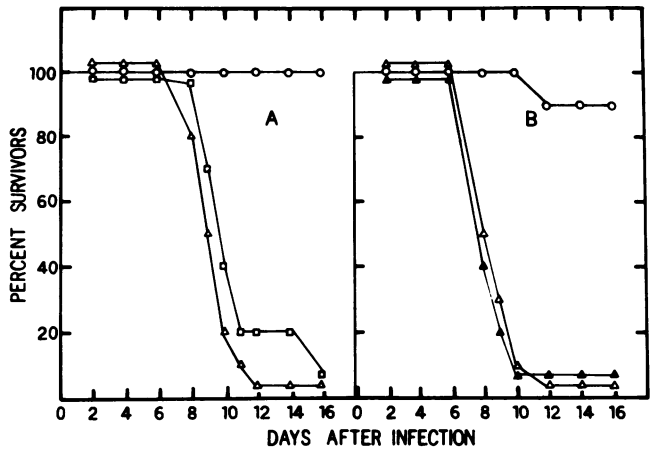

FIG. 1. *Effect of rabbit HSV-2-neutralizing serum (A) or mouse HSV-2-neutralizing serum (B) on survival of mice after subcutaneous inoculation of 3 × 10⁶ PFU of HSV-2 at 8 h before intraperitoneal transfer of 0.5 ml of HSV-2-neutralizing antisera. There were 10 mice per group. (A) Symbols: △, normal rabbit serum; ○, rabbit anti-HSV-2 serum; □, rabbit anti-salmonella serum. (B) Symbols: △, normal mouse serum; ○, mouse anti-HSV-2 serum; ▲, no treatment.*

TABLE 1. *Virus titer in tissues of mice given normal rabbit serum or rabbit HSV-2-neutralizing serum at 8 h after subcutaneous infection with 3 × 10⁶ PFU of HSV-2*[a]

Tissue	PFU/G of tissue in mice receiving:	
	Normal sera[b]	Neutralizing sera
Footpad[c]	3.3×10^3	0
Sciatic nerve[c]	5.0×10^2	0
Spinal cord[c]	2.5×10^5	0
Brain[c]	5.0×10^3	0
Liver[c]	2.0×10^2	0
Kidneys[c]	0	0
Blood[d]	0	0

[a] Tissues were removed when animals receiving normal sera were near death at 9 days after infection. Tissues were removed from animals receiving neutralizing sera at the same time. Tissues were homogenized in a Dounce homogenizer, frozen and thawed three times, and assayed on Vero cells for HSV-2.

[b] Average of four mice from each group.

[c] Sensitivity, ≥100 PFU/g of tissue.

[d] Sensitivity, ≥50 PFU/ml of blood.

and physiological fate of HSV-2-neutralizing activity in the blood of mice after passive transfer of rabbit HSV-2-neutralizing sera were investigated. Mice passively immunized with rabbit HSV-2-neutralizing sera were bled from the retro-orbital plexus at selected intervals, and the amount of neutralizing activity was determined (Table 2). It is apparent that neutralizing activity was present in the circulatory system soon after transfer of rabbit antisera. The decrease in neutralizing activity in these animals 13 days after transfer was probably due to physiological degradation and immune elimination of the rabbit antibody.

TABLE 2. *Neutralizing circulating antibody titer in mice after passive transfer of rabbit HSV-2-neutralizing antiserum*

Time after passive transfer	Neutralizing antibody titer ($\log_{10}$)[a]
2 h	1.2
3 days	1.8
6 days	1.8
13 days	0.9

[a] Represents average of pooled sera from three mice. The neutralizing titer was determined as described in the text.

Absorption of hyperimmune serum with HSV-2-infected cells. If HSV-2-specific antibody can protect the host from primary subcutaneous HSV-2 infection, it should be possible to remove specific antibody from neutralizing sera by absorption with HSV-2-infected cells but not with noninfected cells. After absorption of neutralizing serum with infected cells, passive transfer of immunity to subcutaneous HSV-2 infection was eliminated (Table 3). Absorption with noninfected cells had no effect on the ability of serum preparations to passively transfer immunity. These results suggest that the protective factor in hyperimmune serum is HSV-2-specific antibody.

Relationship between protection and time after infection that mice were passively immunized. A total of 50 mice were inoculated in the right rear footpad with 3×10^6 PFU of HSV-2. At 8 h after infection, 10 mice were passively immunized with 0.5 ml of rabbit

anti-HSV-2 serum. The remaining animals were divided into groups of 10 and passively immunized at 24-h intervals postinfection. Groups of mice given passive antibody at 8 and 24 h postinfection were significantly protected from subcutaneous HSV-2 infection (Table 4). When passive antibody was given at 48 h postinfection, 20% of the animals survived the infection. However, passive immunization at 72 and 96 h postinfection was not effective in reducing mortalities.

Requirement for a radiation-sensitive component in addition to humoral antibody for resistance to subcutaneous HSV-2 infection. It has been reported previously that passive transfer of specific antibody to immunosuppressed mice infected with HSV-1 does not prevent ingression of virus into the central nervous system from peripheral sites of infection (16). It was therefore of interest to determine whether passive antibody could protect immunosuppressed mice infected subcutaneously with HSV-2.

Before these experiments were initiated, it was important to establish whether sublethal doses of irradiation interfered with the immune response to HSV-2. This was tested by measuring humoral antibody in irradiated and non-irradiated mice after subcutaneous HSV-2 infection (Fig. 2). A humoral antibody response was

TABLE 3. *Absorption of protective factor from hyperimmune serum with HSV-2-infected cells*[a]

Serum absorbed with:	No. of survivors[b] (%)
HSV-2-infected Vero cells	2/10 (20)
Vero cells	8/10 (80)

[a] Mice were infected with 3×10^6 PFU of HSV-2 in the right rear footpad. After 8 h, they were passively immunized with 0.5 ml of absorbed sera.

[b] Mice were observed for 6 weeks. All controls were dead by 12 days.

TABLE 4. *Relationship between protection and time after infection that animals were passively immunized*[a]

Time after infection (h)	No. of survivors[b] (%)
8	10/10 (100)
24	7/10 (70)
48	2/10 (20)
72	0/10 (0)
96	0/10 (0)

[a] Mice were subcutaneously inoculated with 3×10^6 PFU of HSV-2. At selected times postinfection, the animals were passively immunized with 0.5 ml of rabbit HSV-2-neutralizing antisera (titer, 1:128) by intraperitoneal inoculation.

[b] Mice were observed for 4 weeks.

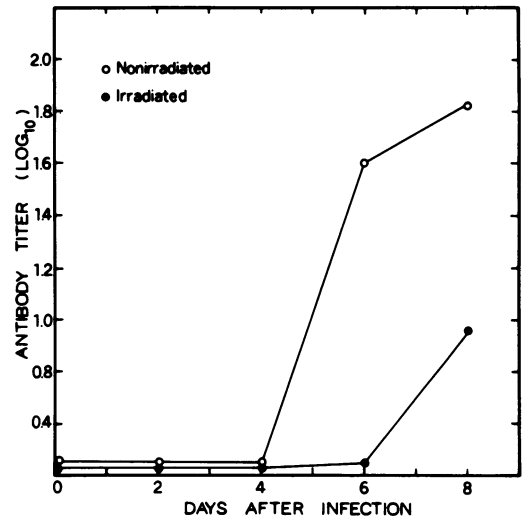

FIG. 2. *HSV-2-neutralizing antibody in blood of normal and irradiated mice after subcutaneous inoculation with 3×10^6 PFU of HSV-2 in the right rear footpad. Each point represents the pooled sera from three animals.*

detected by day 6 postinfection in mice with primary HSV-2 infection. Levels of circulating antibody continued to increase until death of the host at day 9 postinfection. The titer of neutralizing activity at this time was comparable to the neutralizing activity in the sera of mice passively immunized with rabbit HSV-2-neutralizing sera (Table 2). In immunosuppressed animals, antibody did not appear until 6 days after infection. In addition, the quantity of neutralizing antibody in sera of these animals was nearly 1 $\log_{10}$ lower than in sera of non-immunosuppressed hosts. The data suggest, therefore, that sublethal doses of irradiation can effectively reduce the humoral immune response of the host to subcutaneous viral infection.

Irradiated mice were then used as recipients in passive transfer experiments. Mice were irradiated with 390 rads. After 24 h, 30 mice were subcutaneously infected with HSV-2. Ten mice received hyperimmune serum or normal serum at 8 h after infection, and 10 mice received no further treatment. An additional control included 20 non-immunosuppressed mice subcutaneously infected with HSV-2, of which 10 also received passive antibody or normal rabbit serum at 8 h postinfection. Passive antibody protected non-immunosuppressed mice from subcutaneous HSV-2 infection as noted earlier (Fig. 1). In immunosuppressed animals, passive antibody delayed mortality of infected animals but did not significantly increase the number of mice surviving infection (Fig. 3).

The foregoing results indicate that passive neutralizing antibody does not protect the immunosuppressed host from subcutaneous virus infection. Therefore, it was of interest to determine the effect of neutralizing antibody on the spread of HSV-2 from the footpad to the central nervous system in immunosuppressed mice. Irradiated and non-irradiated mice were passively immunized with 0.5 ml of rabbit neutralizing antibody at 8 h after subcutaneous HSV-2 infection. At regular intervals, mice from both groups were sacrificed, and pertinent tissues were assayed for virus (Fig. 4). HSV-2 remained at the site of infection in immunosuppressed mice for 10 days. Virus was not detected in the spinal cord until day 10 postinfection. Appearance of HSV-2 in the brain and liver occurred at about the time that animals began to die. No virus was detected in any of the tissues in non-irradiated mice passively immunized with neutralizing antibody when they were examined on days 2 and 3 postinfection (data not shown). Thus, HSV-2 spread from sites of infection in the presence of neutralizing antibody in immunosuppressed hosts, whereas specific antibody prevented spread of virus in non-immunosuppressed animals. These results suggest that a radiation-sensitive component in the recipient is required in addition to passive antibody for resistance of the host to subcutaneous HSV-2 infection.

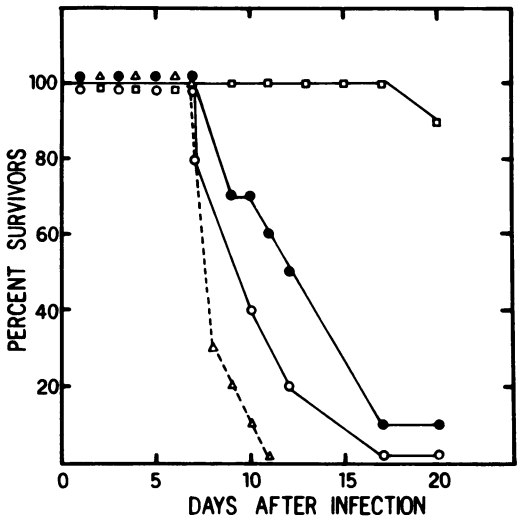

FIG. 3. *Effect of whole-body irradiation on survival of mice after subcutaneous inoculation of 3 × 10^6 PFU of HSV-2 at 8 h before intraperitoneal transfer of 0.5 ml of rabbit HSV-2-neutralizing antisera. The mice were irradiated 24 h before virus infection. There were 10 animals per group. Symbols: ●, irradiation and immune serum; □, no irradiation and immune serum; △, irradiation and normal rabbit serum; ○, no irradiation and normal rabbit serum.*

DISCUSSION

We have examined the effects of circulating antibody on the outcome of subcutaneous HSV-2 infection in young adult mice. When neutralizing antibody was administered to mice at 8 h after subcutaneous HSV-2 infection, it inhibited the spread of virus from the sites of inoculation. Administration of specific sera with high neutralizing activity was not required. Protection

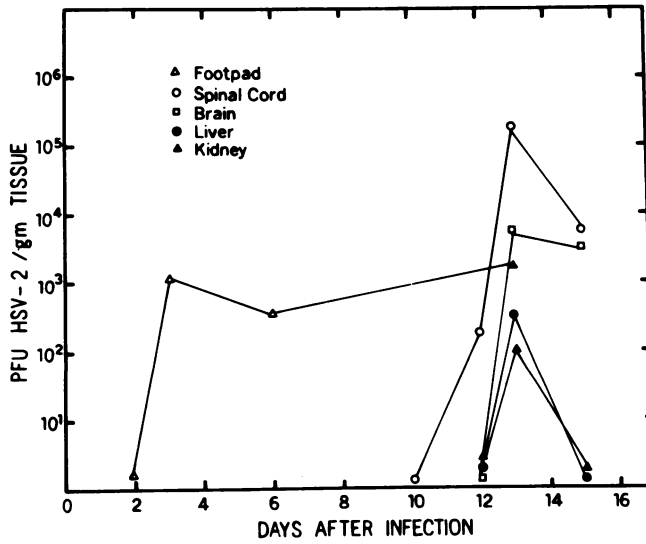

FIG. 4. *Pathogenesis of HSV-2 in irradiated mice after subcutaneous inoculation of 3 × 10^6 PFU of HSV-2 at 8 h before intraperitoneal transfer of 0.5 ml of rabbit HSV-2-neutralizing antisera. The mice were irradiated 24 h before virus inoculation. There were 10 mice per group.*

was observed in recipients whose sera had neutralizing activities near 1:64. This activity was comparable to the neutralizing activity found in the blood of untreated mice 9 days after subcutaneous HSV-2 infection. Nonspecific immune mechanisms rather than specific antibody could have been involved in the passive transfer of antiviral immunity in these experiments. An important nonspecific immune mechanism has been described which involves serum antibody and F_c receptors on the surface of HSV-infected cells (6). These receptors bind to the F_c portions of immunoglobulin G molecules, resulting in inhibition of virus replication. The reaction is nonspecific because antibody molecules of any specificity have the potential to bind by their F_c ends to infected tissues. We investigated this possibility by passively transferring rabbit anti-salmonella hyperimmune sera to mice after virus infection. Anti-salmonella hyperimmune sera had no effect on the outcome of infection, whereas anti-HSV sera protected 90% of the mice infected (Fig. 1). Furthermore, the protective factor in anti-HSV hyperimmune sera could be absorbed out with HSV-2-infected Vero cells but not with noninfected cells (Table 3). These two observations suggested, therefore, that specific antibody was involved in protection of the host from subcutaneous HSV-2 infection.

Recent evidence has accumulated from other laboratories which also suggests that specific antibody can affect the interaction of HSV with its host in vivo. Walz et al. have shown that passive immunization limited spread of HSV-2 along sensory nerves to dorsal root ganglia in nude mice after footpad inoculation (28). In another investigation, Stevens and Cook reported that rabbit immunoglobulin G specific for HSV-1 inhibited intraneural viral DNA and antigen synthesis when given to noninfected mice receiving a ganglionic transplant containing latent virus (27). A possible mechanism for the action of specific antibody in preventing virus spread has been suggested by in vitro studies. Pavan and Ennis reported that HSV-1 could be eliminated from infected tissue cultures by specific antibody, presumably by neutralizing virus as it traveled extracellularly between adjacent cells (19). Antibody may also prevent virus spread in vivo by similar mechanisms. However, the data in our study do not support the hypothesis that passive antibody inhibited virus spread solely by neutralization of extracellular virus. In animals which were immunosuppressed with sublethal doses of irradiation, passive antibody had no effect on the final outcome of infection (Fig. 3). If antibody was primarily protecting the host by neutralization of extracellular virus, immunosuppression would not have interfered with these reactions. The inability of passive antibody to protect irradiated immunosuppressed recipients indicates that a radiation-sensitive component in the host is required in addition to specific antibody for protection against subcutaneous HSV-2 infections.

A possible explanation for the data shown in Fig. 3 is that protection against HSV-2 in nonirradiated mice requires the combined action of early passively transferred antibody plus physiologically synthesized antibody which normally can be detected by day 6 postinfection (Fig. 2). In the irradiated animals, antibody synthesis was compromised, resulting in death of the host. However, Rager-Zisman and Allison have achieved significant protection of cyclophosphamide-immunosuppressed mice by passive transfer of nonimmune spleen cells and specific antibody (20). These workers suggested that their data support the concept that antibody-dependent cell cytotoxicity is involved in immunity to herpesviruses in vivo. However, the nature of the radiation-sensitive component and the mechanisms by which it cooperated with passively administered antibody to inhibit virus spread in our experiments are not known.

In passive transfer studies, antibody given later than day 2 postinfection did not significantly protect recipients from HSV-2 (Table 4). These observations indicate that antibody must be present soon after exposure to have any effect on the course of infection. Because neutralizing antibody was not detectable for 6 days after primary infection (Fig. 2), it is doubtful whether humoral immunity is effective in controlling primary HSV-2 infection. Thus, the potential of specific antibody to localize HSV-2 at initial sites of exposure may become important in subsequent infections when antibody can be produced by the host soon after contact with virus. Neutralizing antibody may also be important in keeping recurrent HSV infections localized at subcutaneous sites after spread of the virus to these areas from sensory ganglia. A noncompromised host response also appears to be required in these reactions because HSV often spreads from localized areas to other sites in individuals receiving immunosuppressive drugs (1, 15).

Other herpesviruses are also affected by specific antibody during replication in the host. Varicella-zoster virus, another herpesvirus having many biological properties in common with HSV-2, is susceptible to specific antibody when given within 72 h after exposure (3, 9). Therefore, herpesviruses may be susceptible to the humoral immune mechanisms of the host under the proper circumstances. The most important of these may be the requirement for an intact

immune response to provide not only specific antibody but additional immunological mechanisms to inhibit virus spread.

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