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Resistance of Female Mice to Vaginal Infection Induced by *Herpesvirus hominis* Type 2: Effects of Immunization with *Mycobacterium bovis*, Intravenous Injection of Specific *Herpesvirus hominis* Type 2 Antiserum, and a Combination of These Procedures

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The susceptibility of 4- to 6-week-old female white Swiss mice to intravaginal inoculation with *Herpesvirus hominis* type 2 (HVH2) and the effect of prior intravenous immunization with *Mycobacterium bovis* (BCG) and/or treatment with specific HVH2 antiserum (SAS) were investigated. Mice inoculated intravaginally developed vaginitis, posterior paralysis, encephalitis, and death. Prior immunization with BCG either had no effect or appeared in some cases to enhance the course of the disease, whereas a single 0.5-ml intravenous injection of SAS provided significant protection. However, synergistic interaction of BCG immunization and treatment with SAS produced the greatest degree of protection in mice challenged intravaginally with HVH2.

Infections of the human female reproductive system by *Herpesvirus hominis* type 2 (HVH2) are being recognized with increasing frequency. In addition to the severe discomfort experienced by patients, the devastating effects of neonatal infections and the possible role of vaginal herpetic infections in cervical cancer have aroused further interest in these agents (15-18). Investigations in rabbits (13), humans (1), and mice (14, 19) have established the important role of cell-mediated immunity in the control of herpes infections. It has been observed that although the sera of patients with recurrent herpetic lesions frequently contain specific neutralizing antibody, these antibodies alone do not prevent recurrence of the disease.

The present studies were undertaken to determine the effects of nonspecific immunity induced by vaccination with *Mycobacterium bovis* (BCG), passive administration of specific HVH2 antiserum (SAS), and a combination of these procedures upon the course of HVH2-induced vaginitis in mice. Since vaginal herpes infections run an acute course in mice, BCG vaccine was administered prior to infection with virus. The data indicate that protection in mice against vaginal inoculation with HVH2 is dependent upon stimulation of the reticuloendothelial system as well as the presence of specific antibodies.

MATERIALS AND METHODS

Animals. Female white Swiss mice were from the colony maintained at the Rocky Mountain Laboratory at Hamilton, Mont.

Virus preparation. Strain 196 of HVH2 was obtained from W. E. Rawls, Baylor University College of Medicine, Houston, Tex. Virus pools were prepared by infecting confluent monolayers of Vero cells. After 24 h of incubation at 37 C, the cultures were subjected to three rapid freeze-thaw cycles in an ETOH-dry ice bath and then centrifuged at 2,500 $\times$ *g* for 30 min. The supernatant fluid was dispensed in ampoules, frozen, and stored at -70 C until needed. Virus pools were titrated in Vero cells and contained from 4×10^5 to 2×10^6 mean tissue culture infective doses ($TCID_{50}$)/ml. Virus suspensions were diluted in either Eagle minimal essential medium or Hanks basic salt solution prior to challenge.

BCG vaccine. Mice were immunized intravenously (i.v.) with 0.2 ml of Dubos broth containing 10^7 viable units of BCG. The strain of BCG used was originally obtained from the Pasteur Institute (Paris, France), and has been maintained in our laboratory by transfer every 6 months on Hohn medium slants (Difco) and storage at 4 C.

The BCG vaccine was prepared in Dubos broth, which was inoculated from a stock culture of Hohn medium and incubated for 7 to 9 days at 37 C. A 5-ml sample of this culture was transferred to fresh Dubos broth. Seven days later, the bacteria were harvested by centrifugation, washed twice with Dubos broth and suspended in a fresh sample of the same medium. The cell suspension was filtered through a coarse sintered-

glass filter and adjusted to a turbidity of 200 with a Klett-Summerson photoelectric colorimeter. Samples of 5 ml were frozen and stored at −70 C.

For vaccination, a vial of the BCG vaccine was diluted with an equal volume of Dubos broth. Serial 10-fold dilutions were made and plated on Dubos agar to determine viability. The colonies were counted after the plates were sealed in plastic bags to prevent desiccation and were incubated for 30 days at 37 C.

Inoculations. Prior to inoculation, virus suspensions were diluted to the desired concentration with minimal essential medium or Hanks basic salt solution. Mice were challenged by the vaginal route by the method of Nahmias et al. (A. J. Nahmias, Z. M. Naib, A. K. Highsmith, and W. E. Josey. Pediat. Res. 1:3). Briefly, sterile absorbant cotton pellets 3 to 4 mm in diameter were saturated with 0.05 ml of the virus suspension and immediately inserted into the vagina with small Teflon-coated forceps. All mice were toe-clipped to facilitate individual identification and were examined daily for signs of vaginitis, encephalitis, and death. Mean death times were analyzed by the Mann-Whitney U test.

Antiserum. Antiserum used for passive transfer experiments was obtained from New Zealand and Dutch Belted rabbits which had been challenged with HVH2 by corneal scarification and had developed encephalitis but subsequently recovered (13). These animals were bled 4 to 6 weeks after infection, and the sera were harvested and heat-inactivated for 50 min at 56 C. Specific neutralization titers were determined by standard in vitro methods with Vero cells. Sera with titers of 1:128 or greater were pooled. Mice received 0.5 ml of the pooled SAS i.v. 4 h prior to vaginal challenge with HVH2.

Sequence of therapy. The sequence of therapy used in all BCG experiments was as follows. Female mice (21 to 28 days old) were immunized i.v. with BCG vaccine and treated i.v. 7 to 10 days later with SAS. Four hours after treatment with SAS, the mice were challenged with virus.

RESULTS

Infectivity of HVH2 administered by the vaginal route to sexually mature female mice. Groups of 6 to 8 female mice approximately 28 days old were infected by the vaginal route. The highest dose of virus administered contained 7.5×10^4 $TCID_{50}$ per 0.05 ml and caused characteristic disease and death in 88% of the mice tested. Challenge with 7.5×10^3 and 7.5×10^2 $TCID_{50}$ of virus produced death in 86 and 67% of the mice, respectively. An inoculum of 7.5×10^1 $TCID_{50}$ was insufficient to cause disease, and none of the mice given this amount of virus showed evidence of infection (Fig. 1).

Vaginal inflammation accompanied by a mucopurulent exudate appeared by the third day after inoculation. Posterior paralysis and encephalitis followed. Death usually occurred by the 21st day. Most of the animals died within 5 to 11 days after administration of virus.

Age-dependent resistance to vaginal infection. The susceptibility of mice to herpesvirus administered by extraneural routes decreases with increase in age. In consequence, many workers have used suckling mice for in vivo studies (9–11). The present studies could not be accomplished in suckling mice. To determine the maximal age at which mice could be infected by the vaginal route, groups of mice 26, 46, and 120 days old were injected with varying dilutions of HVH2. Susceptibility decreased with the age of mice, and only mice about 4 weeks old or less were useful for studies of active resistance to herpesvirus infections established by vaginal instillation of virus (Fig. 2).

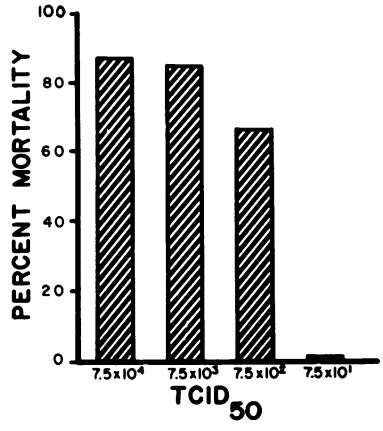

FIG. 1. *Percent mortality among groups of 28-day-old female white Swiss mice infected intravaginally with decreasing doses of HVH2.*

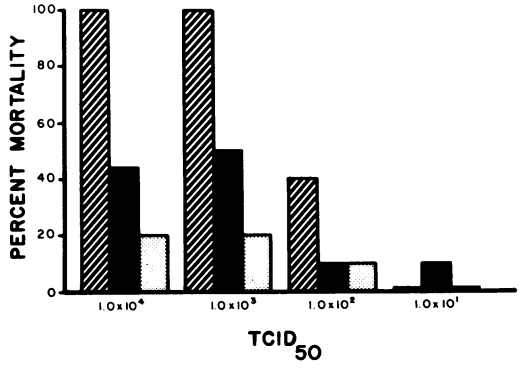

FIG. 2. *Effect of age upon the mortality of female white Swiss mice infected intravaginally with decreasing doses of HVH2. Legend:* ▨, *25-day-old mice;* ■, *46-day-old mice; and* ▩, *120-day-old mice.*

Effects of nonspecific immunity induced by BCG vaccination, passive administration of SAS, and a combination of these procedures upon the course of vaginal HVH2 infections. The feasibility of infecting adult female mice vaginally with HVH2 (as indicated above) and the data of other workers showing the importance of both cell-mediated immunity (9, 13, 19, 20) and specific antibody (4, 14) in controlling HVH infections were used as the basis of the following experiment.

Female mice (24 to 28 days old) were vaccinated i.v. with 10^7 viable BCG bacilli. Unvaccinated mice of the same age were retained as controls. Six to eight days later, 0.5 ml of SAS was given i.v. to half of the mice in each group. After an additional 2 to 4 h, the animals were challenged per vagina with an inoculum of 10^4 to 10^5 $TCID_{50}$ of HVH2.

The results from four similar experiments were collectively tabulated and appear in Fig. 3. Each experimental group represents 38 to 42 mice.

It was evident that i.v. administration of BCG vaccine alone had no effect upon the course of vaginal HVH2 infections in the mouse. A single i.v. injection of SAS into normal mice (group 2, Fig. 3), however, significantly extended their mean time of death. Serum treatment of mice immunized with BCG (group 3, Fig. 3) resulted in an extended mean death time

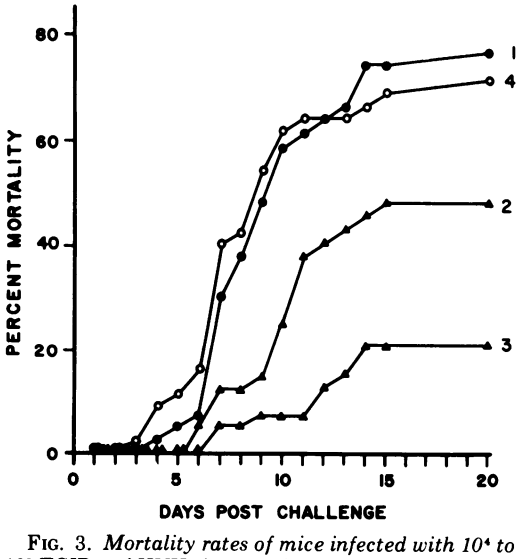

FIG. 3. *Mortality rates of mice infected with 10^4 to 10^5 $TCID_{50}$ of HVH2 intravaginally. Tabulated results from four experiments show the effects of (1) immunization with M. bovis (BCG); (2) i.v. injection of HVH2 antiserum (SAS); (3) a combination of (1) and (2); and (4) no treatment.*

that was statistically significant when compared with any of the other groups of mice tested.

Antibody response of mice to injection of SAS. In experiments with BCG-immune, serum-treated mice, two periods of time were observed when these mice succumbed to herpes infection. The first period was 7 to 9 days postchallenge. The few deaths noted at this time occurred among those mice that developed vaginitis 3 to 4 days after challenge. This phase was followed by a lag period of 2 to 3 days during which no deaths occurred. The second period during which numerous deaths occurred from 11 to 15 days postchallenge. The animals that died during this latter period had no symptoms of infection until the 9th or 10th day after vaginal challenge.

Since the serum used was obtained from rabbits, the deaths occurring late in the course of the experiments were considered to be associated with depletion of rabbit serum due to the immune response of the host. To test this possibility, four groups of five female mice were injected i.v. with 0.5 ml of SAS. The animals in each group were bled at intervals from 2 to 16 days later, and the sera of mice from each group were pooled at each interval. Precipitin tests were performed by using SAS as antigen. Precipitins were noted in two of four pools by day 8 and in all pools by day 13.

The time at which antibody against rabbit serum appeared in the circulation correlates with the time at which protection against vaginal challenge with HVH2 began to wane in mice immunized with BCG and treated with SAS.

BCG enhancement of HVH2 neurovirulence. In a few experiments involving vaginal challenge of mice with HVH2, BCG immunization appeared to enhance the virulence of the virus (Fig. 4). Although this observed difference in the BCG-immune versus normal mice appeared to be marked, analysis of the data showed no significant difference between the results obtained in the two groups of mice. Vaginitis was frequently apparent in the BCG-immunized mice 48 h after challenge, compared with 72 and 96 h for the normal control. Furthermore, the course of the disease from first appearance of clinical signs to fatal outcome was often more rapid among individual mice sensitized with BCG than among normal mice.

DISCUSSION

Herpesvirus infections in man may produce, in addition to the usual epithelial lesions, such severe illnesses as encephalitis, generalized in-

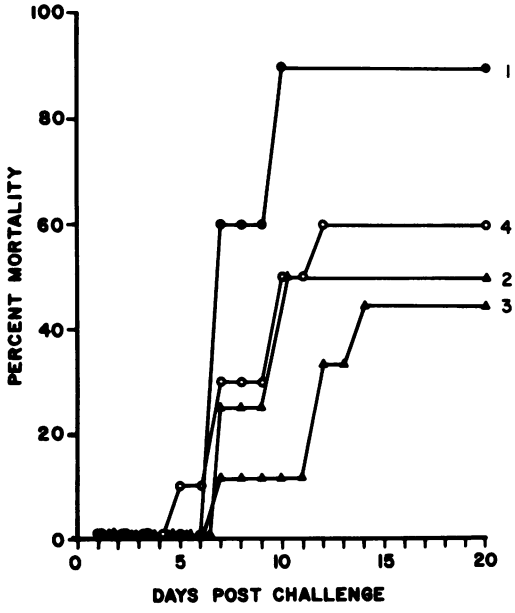

FIG. 4. *Mortality rates of mice infected with 1.0 × 10^4 $TCID_{50}$ of HVH2 intravaginally. In this experiment, the effects of (1) immunization with BCG; (2) i.v. injection of SAS; (3) a combination of (1) and (2); and (4) no treatment are compared.*

fection of the newborn, and recurrent infections. In addition, such infections may ultimately be found to be the cause of cervical cancer (15–18). In spite of the increased interest in these manifestations, treatment of herpes infections has not been satisfactory.

Iodoxuridine and adenosine arabinoside, and possibly cytosine arabinoside, appear to be effective in the treatment of severe HVH infections in man (12). These drugs may, however, produce toxic side effects. Application of 0.1% neutral red or proflavine dye to HVH skin lesions and subsequent exposure to incandescent light has also resulted in healing of lesions and a decrease in recurrent infections (7). However, Rapp et al. (6) question the advisability of this procedure since the infectivity but not the oncogenic potential of the virus may be destroyed.

Although capable of neutralizing HVH in vitro, specific antiglobulin does not appear to be effective in controlling clinical infections since much of the virus spreads directly from cell to cell through cytoplasmic bridges (5). Thus, HVH infections can persist and recur even in the presence of high titers of neutralizing antibody. Immune lymphocytes (4) as well as antiviral antibody plus complement can interact with HVH-induced cell surface antigens and cause destruction of the infected cells. However, this immunological mechanism does not prevent the spread of HVH infections, presumably since infective virus is produced before the cell develops virus-induced membrane antigens (14).

In the majority of cases, HVH infections are confined to a single site and the lesions heal without treatment. This restriction and recovery appears to result from a two-phase immunological response, cellular followed by humoral intervention. Lodmell et al. (14) demonstrated such a two-phase response in vitro by exposing virus-infected cells to leukocytes stimulated with sodium caseinate. The treatment suppressed the infection but did not destroy all infected cells. On the other hand, when low concentrations of antiviral antibody and complement were added to the system, infection was eliminated. These investigators conclude that the normal immunological response to HVH eliminates the infection through the combined attack of stimulated leukocytes and antiviral antibody. The leukocytes exert a toxic effect upon some of the virus-infected cells, break intracellular bridges, and inhibit viral reproduction. Viral-infected cells not killed by the leukocytes and extracellular virus are destroyed by antiviral antibody and complement before intracellular bridges are reestablished.

Specific stimulation of cell-mediated immunity with BCG has no effect upon the course of the disease. Immunization with BCG appears in some instances to increase the severity of the virus infection, but analysis of pooled data from several experiments shows no statistical difference between the results obtained in BCG-immune and normal mice. Treatment of mice with a single i.v. injection of SAS 4 h prior to challenge significantly increases their mean survival time. Mice immunized with BCG and treated with SAS display the greatest resistance to infection.

There is abundant evidence that BCG produces activated macrophages (2, 3) and that such cells are capable of nonspecifically destroying cells possessing foreign membrane antigens (8). The activation of macrophages by immunization with BCG, together with administration of specific antibody, yields in vivo results similar to those obtained by Lodmell et al. (14) in their in vitro studies. It is of interest that Anderson et al. (1) have shown that immunization with BCG controls recurrent genital herpes infections in human beings whose serum contains specific neutralizing antibodies.

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