Herpesvirus hominis Type 2 Infections in Rabbits: Effect of Prior Immunization with Attenuated Mycobacterium bovis (BCG) Cells

C. L. LARSON, R. N. USHIJIMA, R. KARIM, M. B. BAKER, AND R. E. BAKER

Department of Microbiology and Stella Duncan Memorial Institute, University of Montana, Missoula, Montana 59801, and Department of Biology, Northern State College, Aberdeen, South Dakota 57401

Received for publication 24 April 1972

Angora, New Zealand, and Dutch-belted rabbits were tested for their susceptibility to graded doses of Herpesvirus hominis type 2 administered by vaginal instillation, intracorneal injection, and scarification of the cornea. Central nervous system involvement and death occurred after infection by the various routes employed. Prior immunization of Dutch-belted and New Zealand rabbits with viable Mycobacterium bovis (BCG) cells injected intravenously provided protection against subsequent infections with type 2 virus.

Nonspecific resistance to a variety of human and animal diseases (including tumors) has been demonstrated after administration of attenuated live Mycobacterium bovis BCG cells (2). Nonspecific resistance is associated with cell-mediated immunity and with specific delayed hypersensitivity against the organism employed to induce this resistant state (14). It is concerned with agents which are facultative intracellular parasites (23). Cell-mediated immunity depends upon sensitization of a population of lymphocytes, resulting in activation of macrophages, which play an important nonspecific role in controlling agents other than the one used to induce sensitization (13).

Herpesvirus hominis (HVH) type 2 infection is regarded as a venereal disease in humans and induces genital lesions (16) in both sexes. Aside from the discomfort experienced by these patients, current concern is over the association of this disease with cervical cancer (18-20). Without doubt, effective preventive and therapeutic measures to control herpetic infections are needed, and the use of several compounds such as idoxuridine (7) or double-stranded ribonucleic acid (17) or of photo-inactivation (15) is being investigated. In view of the studies reported by Hirsch et al. (8), showing that HVH type 1 infection in mice is dictated by the ability of macrophages to control the virus, it seemed appropriate to determine whether immunization with BCG cells would affect the course of HVH type 2 infection in experimental animals.

The present report is concerned with HVH type 2 infection in normal rabbits and rabbits previously immunized with BCG cells. It was found that prior immunization with BCG cells considerably reduced the mortality rate in rabbits challenged with HVH type 2 administered orally or vaginally.

MATERIALS AND METHODS

HVH type 2, strain 196, was obtained from W. E. Rawls, Baylor University College of Medicine, Houston, Tex. A pool of virus was prepared by infecting primary cultures of rabbit kidney cells and maintaining the tissue culture at 37°C for 48 hr, at which time the cells showed marked cytopathic effects. The cultures were subjected to three rapid freeze-thaw cycles and were centrifuged at approximately 2,500 × g for 30 min. The supernatant fluid was harvested, distributed and sealed in ampoules, and frozen at −70°C. The preparation contained 8 × 106 tissue culture infective doses (TCID) per ml.

Angora, New Zealand, and Dutch-belted strains of rabbits were used. Infection with virus was initiated by instillation of 0.25 ml of suspension into the vagina, by injection of 0.05 ml intracorneally, or by scarification of the cornea with a 20 gauge needle followed by dropping 0.05 ml of the preparation over the scratched surface. Three horizontal and three vertical scratches were made on the cornea.

Rabbits were immunized by intravenous injection of 0.2 ml of Dubos liquid medium containing 4 × 104 viable units of M. bovis (BCG). The organisms were grown and harvested as previously described (10). The culture was originally received from the Pasteur Institute, Paris, France, and has been maintained in this laboratory for 8 years. Animals were challenged with virus 4 weeks after immunization with BCG.
RESULTS

The infectivity of the pool of HVH was analyzed by determining the amount of virus capable of producing encephalitis and death when administered by scarification of the cornea or intracorneal injection. The eyes of five groups of Angora rabbits each were infected by the methods described above. Separate groups of animals were infected by corneal scarification with suspensions containing $4 \times 10^5$, $4 \times 10^4$, $4 \times 10^3$, and $4 \times 10^2$ TCID of virus, and by corneal injection with suspensions containing $4 \times 10^5$, $4 \times 10^4$, $4 \times 10^3$, and $4$ TCID of virus. The LD$_{50}$ of virus was found to be $4 \times 10^{4.8}$ after corneal scarification and $4 \times 10^{1.2}$ after corneal injection. HVH type 2 was infective by either route, but, to produce comparable results, infection by corneal scarification required about 1,000 times more virus than infection by corneal injection.

A comparison was made of the relative susceptibility of Angora and New Zealand rabbits to infection with HVH type 2 introduced by corneal scarification. Both strains of rabbits were uniformly susceptible to infection with $4 \times 10^4$ or $4 \times 10^3$ TCID, and all developed fatal infections. None of five Angora rabbits died after introduction of $4 \times 10^3$ TCID, although two developed encephalitis. Three of five New Zealand rabbits developed encephalitis after infection with this dose of virus, but only one of the three died.

In an experiment to determine the infectivity of HVH type 2 introduced vaginally, it was found that none of three New Zealand rabbits exposed to $10^4$ TCID developed vaginitis, but three of four animals exposed to $10^4$ TCID developed vaginitis, and two of these subsequently died. Among two groups of four New Zealand rabbits infected with either $10^4$ or $10^3$ TCID, all developed severe vaginitis and eventually succumbed after displaying paralysis of the hind limbs followed by generalized encephalitis.

In an experiment performed to determine the effect of BCG immunization on the infectious process in New Zealand rabbits, the results indicated that the route of challenge with virus influenced the eventual course of the disease. One group of 12 animals was immunized with BCG and another 12 were retained as unimmunized controls. Four weeks later, the rabbits in both groups were infected with $10^4$ TCID of HVH, introduced either intracorneally or by scarification (Table 1). All animals developed encephalitis within 6 to 8 days after challenge. Of the six unimmunized animals infected by scarification, all died 2 to 17 days after onset of encephalitis. All of the immunized rabbits infected by intracorneal injection of virus died 3 to 17 days after development of neurological disturbances. Among the unimmunized rabbits infected by scarification, only two of six succumbed. One died 6 days and the other 16 days after onset of encephalitis. However, among the immunized animals infected by intracorneal injection of virus, death occurred in four of six animals, 1 to 2 days after encephalitis developed. Immunization with BCG cells increased resistance in rabbits challenged by the scarification procedure with a small amount of virus used for the infective dose, but appeared to hasten death among those challenged intracorneally. The animals given virus intracorneally received about 1,000 more infective doses of virus than those infected by scarification.

A summary of experiments performed in immunized and unimmunized New Zealand and Dutch-belted rabbits infected by scarification of the cornea with $10^4$ TCID of HVH type 2 is shown in Table 1. Thirty rabbits immunized with BCG cells were infected with virus. Encephalitis developed in $80\%$ (24 of 30) of these animals; however, only $27\%$ of all animals succumbed. Thus, only $33\%$ of immunized animals developing encephalitis progressed to a fatal outcome. Of the 30 unimmunized rabbits, 28 (93\%) developed encephalitis and 25 (83\%) died. The mortality rate was $89\%$ among those developing encephalitis.

Since HVH type 2 is essentially a venereal infection in humans and is a common cause of vaginitis, the influence of immunization with BCG cells upon viral vaginitis was studied in Dutch-belted and New Zealand rabbits. Groups of 14 Dutch-belted and 11 New Zealand rabbits were immunized with BCG cells; groups of 12 of the former and 10 of the latter strain of rabbits were maintained as unimmunized controls. After an interval of 4 weeks, the animals were divided as shown in Table 3 and infected by either corneal scarification or vaginal douching with $10^4$ TCID of HVH type 2. Dutch-belted rabbits immunized with BCG cells were resistant to ocular but not to...
vaginal infection. About 50% of New Zealand rabbits immunized with BCG cells resisted either vaginal or corneal infection. It was observed that, in addition to lesions in the vagina, many rabbits infected by the vaginal route developed lesions in the perineal region. It was also noted that paralysis of the hind limbs and loss of sphincter control preceded onset of encephalitis in animals challenged by the vaginal route.

**DISCUSSION**

The results of these experiments show that resistance of rabbits to HVH type 2 infection after intravenous injection of BCG cells is of considerable magnitude. The incidence of encephalitis in rabbits immunized with BCG cells and infected with virus by corneal scarification was not greatly decreased compared with that in unimmunized animals, but 33% of immunized rabbits developing encephalitis died whereas 83% of the unimmunized rabbits with encephalitis eventually succumbed. The reason for this difference is not immediately apparent, but local development of antibodies in the central nervous system may account for recovery of the BCG-immune animals. Bell et al. (1) described the development of neutralizing antibodies in the central nervous system of mice infected with rabies virus.

The resistance induced by BCG cells appears to be greater against HVH type 2 infections produced by corneal scarification than against infections produced by injection into the cornea or by vaginal instillation of virus. Both Dutch-belted and New Zealand rabbits developed resistance to virus introduced by scarification, but only New Zealand rabbits appeared to develop resistance to vaginal infection. Resistance to virus injected directly into the cornea did not develop in BCG-immune New Zealand rabbits. The amount of virus required to produce encephalitis and death in New Zealand rabbits was about the same whether administered by corneal injection or by instillation into the vagina. However, about 1,000-fold greater amounts of virus were required to produce the same effects when virus was introduced by corneal scarification. The amount of HVH type 2 required to produce death in rabbits infected by corneal scarification was at least 10-fold less than that required for HVH type 1 (15).

The use of BCG as an agent to prevent death in rabbits infected with HVH type 2 appears to be an example of nonspecific resistance. Recently, McGregor et al. (11) and Koster et al. (9) demonstrated that such resistance is due to sensitization of short-lived small lymphocytes which remain in the circulatory system and which contribute to increased activity of macrophages. Zisman et al. (24) have shown that, in mice, resistance to infection with HVH type 1 is related to the age of the animals and this, in turn, is related to the ability of the macrophages of adult mice to dispose of this virus.

Some workers (3, 12, 22) have adopted the position that resistance to facultative intracellular parasites is entirely nonspecific, but others (4, 6, 21) have indicated that the immunity afforded to the specific organism is greater than that afforded to nonspecific parasites. Recently, we have shown (5) that, in murine tularemia, cell-mediated immunity is clearly specific.

The present experiments demonstrate the effectiveness of nonspecific resistance developed in rabbits after intravenous injection of viable BCG cells. In view of the known chronicity of herpesvirus infections and their postulated relationship to cancer of the cervix, it may be of value to utilize BCG as a therapeutic agent in the treatment of chronic herpetic infections.

**ACKNOWLEDGMENTS**

This study was supported by Public Health Service grants 5 KO6 AI16502-10, 5 RO1 AI05370-10, and 1 ROI CA 12795-01.

**LITERATURE CITED**