Mechanism of Acquired Resistance to Herpes Simplex Virus Infection as Studied in Nude Mice

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SUMMARY

The role of antibody and cell-mediated immunity in the resistance of adult mice to intracutaneous infection with herpes simplex virus type 1 (HSV-1) was studied in nu/nu and nu/+ mice. In nu/+ mice, local skin lesions began to appear at the site of inoculation on the 4th day after intracutaneous challenge with the virulent Hayashida strain of HSV-1. Zosteriform skin lesions were observed in some animals. Almost complete regression of the lesions had occurred by the 16th day p.i. In contrast, all of the nu/nu mice that developed local skin lesions died after development of severe zosteriform skin lesions. After repeated intraperitoneal inoculations with the avirulent SKa strain of HSV-1, nu/nu mice did not produce detectable amounts of neutralizing antibody and succumbed to infection, indicating no development of resistance.

Passively transferred neutralizing antibody prevented nu/nu mice from developing zosteriform skin lesions by the Hayashida strain of HSV-1, as long as the minimum concentration of serum antibody was maintained and prolonged their survival time. Adoptive transfer of 1·0 × 10⁷ immune nu/+ spleen cells to nu/nu mice provided almost complete recovery from infection with production of sporadic low levels of anti-HSV antibody. The protective action of the immune spleen cells was lost after pre-treatment with anti-θ serum and fresh guinea pig serum prior to transfer of the cells. These data indicate that T cell-mediated cellular immunity plays a major role in recovery from intracutaneous HSV infection in mice, while antibody-mediated protection due to passive administration of HSV antibody is effective only in limiting the spread of virus.

INTRODUCTION

Resistance of animals to virus infections is due to several specific and non-specific immune responses. Although macrophages may play an important role against herpes simplex virus (HSV) infection at an early stage of infection (Zisman et al. 1970; Mogensen, 1977), the significance of T cell-mediated immunity in control and recovery from HSV infection has been confirmed in studies with neonatally thymectomized mice (Mori et al. 1967) or antithymocyte serum (ATS) treated mice (Oakes, 1975) and by adoptive transfer experiments with immune spleen cells (Ennis & Wells, 1974; Oakes, 1975; Rager-Zisman & Allison, 1976). Immune T cells in HSV infected animals mediate many kinds of immunological responses such as: antibody production (Burns et al. 1975), cytotoxic T cell induction (Pfizenmaier et al. 1977), elicitation of delayed type hypersensitivity reactions (Lausch et al. 1966), and production of lymphokines including interferon (Lodmell & Notkins, 1974).

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Therefore, the mechanism of T cell-mediated immunity \textit{in vivo} in HSV infection is complex and poorly understood.

In previous studies from our laboratory, we have used neonatally thymectomized mice (Mori \textit{et al.} 1967). The congenitally athymic nude (nu/nu) mouse, which is markedly deficient in T cells, is unique for the study of thymus dependency of acquired resistance in virus infections. When normal mice are challenged intracutaneously with the virulent strain of HSV type 1 (HSV-1), they display local skin lesions as early as the 4th day after inoculation, with subsequent development of zosteriform skin lesions (Sydiskis & Schultz, 1965). This clinical course serves as an excellent model for the study of HSV infection. Skin is the portal of entry for HSV and the development of zosteriform skin lesions indicates virus spread from the ganglion to the skin.

In the present study, we examined the ability of passively transferred antibody or immune spleen cells to protect HSV infected nude mice in an attempt to separate antibody-mediated protection from T cell-mediated cellular protection.

\textbf{METHODS}

\textbf{Mice.} The nu/nu or nu/+ mice with a Balb/c genetic background were purchased from the Central Laboratories of Experimental Animals, Ltd., Tokyo, and all experiments were conducted with 6- to 10-week-old animals. During this study, all mice were kept in a conventional environment and fed with sterilized food and water. Colony bred CFI mice, supplied by Kyushu University Animal Centre, were used for the preparation of immune sera.

\textbf{Virus.} HSV type 1 (HSV-1), strain Hayashida, was isolated from the vesicular skin lesion of an adult with \textit{Herpes labialis} and had been passaged five times in Vero cells. Strain SKa, an avirulent strain of HSV-1, was kindly supplied by Dr K. Yoshino of the Institute of Medical Science, University of Tokyo. The strain previously passaged in Vero cells was used for immunization. Virus titres were determined using a plaque forming assay in Vero cells and expressed as p.f.u./ml.

\textbf{Production of skin lesions.} Skin lesions were produced in mice according to the method of Sydiskis & Schultz (1965). The hair was manually removed from the midflank area measuring approx. 1 inch diam. The depilated nu/+ or nu/nu mice were challenged intracutaneously at the midflank with $2.5 \times 10^4$ p.f.u./0.05 ml of the virulent Hayashida strain of HSV-1. The development and severity of skin lesions were scored each day and given the following numerical designations: none, 0; vesicle, 2; local erosion, 3; ulceration of the local lesion, 4; mild zosteriform ulceration, 6; moderate zosteriform ulceration, 8; severe zosteriform ulceration, 10; and death (Fig. 1).

\textbf{Immunization schedule.} Adult nu/nu, nu/+ or CFI mice were immunized intraperitoneally with $10^8$ p.f.u. of the SKa strain of HSV. The virus was inoculated nine times in 0.2 ml vol. with intervals of 3 to 4 days between inoculations. For transfer experiments, sera or spleen cells were taken 6 to 7 days after the last immunization.

\textbf{Neutralization test.} Mice were bled from the retroorbital plexus and sera were separated and inactivated at 56 °C for 30 min. Vero cells or RK13 cells were grown to confluency on Microtest II plastic trays (Falcon Plastics, California). The cells were grown in Eagle's minimal essential medium (MEM) supplemented with 10% heat-inactivated calf serum (CS). The medium was removed and the cell monolayer was washed once with phosphate-buffered saline and 0.025 ml MEM containing 2% CS was added. Twofold dilutions of the test serum were made in transfer plates (Cooke Laboratories, Virginia, U.S.A.), with the aid of a microdiluter, by serially transferring 0.025 ml of test serum to wells containing 0.025 ml of medium. One hundred p.f.u. of the Hayashida strain of HSV-1 in 0.025 ml and 0.025 ml of a 1:10 dilution of guinea pig serum were added to each well and incubated at
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(a) score 2  (b) score 6  (c) score 10

Fig. 1. Scales for scoring skin lesions in mice that were infected intracutaneously with the virulent Hayashida strain of HSV-1 at the midflank: (a), (b), (c), nu/nu mice; (d), (e), (f), nu/+ mice. The development and severity of skin lesions were scored on a daily basis and given the numerical designations: none, 0; vesicle, 2; local erosion, 3; ulceration of the local lesion, 4; mild zosteriform ulceration, 6; moderate zosteriform ulceration, 8; severe zosteriform ulceration, 10.

37 °C in 5% CO₂ atmosphere for 3 to 4 days. The microplaques produced in each well were counted with a light microscope. The antibody titres were expressed as the highest serum dilution causing 80% plaque reduction.

Antiserum preparation. Herpes simplex antiserum was prepared in CFI mice by multiple intraperitoneal (i.p.) inoculations with the SKa strain of HSV-1 containing 10⁶ p.f.u./0.2 ml. The serum titred 1:64 to 1:128 using the standard virus plaque neutralization assay described above.

Spleen cell suspensions. The spleens were removed aseptically either from immunized or non-immunized mice, cut into pieces, pressed between glass slides and passed through a nylon sieve into ice-cold MEM containing 10% calf serum. Spleen cell suspensions were centrifuged at 1000 rev/min for 5 min and the supernatant fluids were removed. After treatment with 0.83% NH₄Cl to deplete erythrocytes for 2 min, spleen cells were washed three times in MEM and the cells were counted using the trypan blue dye (0.5%) exclusion test. The viability varied between 80 and 90%.
Treatment of cells with anti-0 serum. The anti-0 serum was obtained from Bionetics Lab., Kensington, Md., U.S.A. (Lot No. 231-76-6). One ml of spleen cell suspension containing $10^8$ cells was mixed with 1 ml of a 1:10 dilution of anti-0 serum and incubated at 4 °C for 15 min. Two ml of a 1:10 dilution of fresh guinea pig serum was added and incubated at 37 °C for 30 min. The cells were washed twice with 10% CS-MEM, suspended in 10% CS-MEM, counted and tested for viability. Under these conditions, 99% of the C3H thymocytes were killed, but only approx. 3% of the immune nu/+ spleen cells died as determined by the trypan blue dye exclusion test. Twenty-four hours before infection 10T viable cells were transferred to nu/nu mice via the tail vein.

RESULTS

Failure of nu/nu mice to develop acquired resistance

Nu/nu or nu/+ mice were infected intracutaneously at the midflank with $2.5 \times 10^4$ p.f.u./0.05 ml of the virulent Hayashida strain of HSV-1. In nu/+ mice, local skin lesions began to appear as early as the 4th day p.i. with zosteriform skin lesions developing in some. However, almost complete regression of the lesions had occurred by the 16th day after infection (Fig. 2a). In contrast, all of the nu/nu mice that developed local skin lesions died by the 18th day p.i. after development of severe zosteriform skin lesions (Fig. 2b).

After immunization with the avirulent SKa strain of HSV-1, only two of eight nu/+ mice developed small skin lesions which localized at the challenged site (Fig. 2c), but immunized nu/nu mice developed severe zosteriform skin lesions and died along with the non-immunized nu/nu control mice (Fig. 2d).
**Fig. 3.** (a), (b) and (c) The development of skin lesions of mice challenged with HSV-1 after transfer of hyperimmune mouse serum. (a) Control nu/nu mice; (b) 1 ml of hyperimmune CF1 mouse serum with neutralizing antibody titre of 1:64 was inoculated intraperitoneally into nu/nu mice 6 h before challenge and on days 2 and 6 after challenge. Immune serum was transferred to maintain antibody titre (arrows). (c) Hyperimmune mouse serum with neutralizing antibody titre of 1:128 was administered to nu/nu mice 6 h before challenge and on days 2, 5, 8 and 11. Immune serum was transferred to maintain antibody titre (arrows). Numbers on the top of lines indicate time of death (days).

**Failure of nu/nu mice to produce neutralizing antibody after repeated immunization**

In order to examine the ability of mice to produce neutralizing antibody against HSV, nu/nu and nu/+ mice were inoculated nine times i.p. with $1 \times 10^6$ p.f.u./0.2 ml of the
Fig. 4. (a) and (b) Passive transfer of protective immunity with immune spleen cells. Six nu/nu mice received $10^7$ (a) non-immune or (b) immune nu/+ spleen cells 6 h before challenge. The mice were challenged with $2.5 \times 10^4$ p.f.u./0.05 ml of the Hayashida strain of HSV-1 on day 0.

avirulent SKa strain of HSV-1. None of the nu/nu or nu/+ mice had detectable neutralizing antibody prior to immunization. After nine i.p. immunizations, all of the eight nu/+ mice produced antibody which titred 1:32 to 1:128, whereas only one of the seven nu/nu mice produced detectable antibody (1:8). In the remaining six nu/nu mice, neutralizing antibody was undetectable even after nine immunizations. These data indicate the thymus-dependence of neutralizing antibody production to HSV in mice.

Transfers of hyperimmune mouse serum

Nu/nu mice immunized with the SKa strain of HSV-1 could neither produce neutralizing antibody nor acquire resistance to HSV infection. To evaluate the significance of circulating antibody in controlling intracutaneous (i.c.) HSV infection, the influence of antibody was studied by transferring sera from hyperimmunized CF1 mice.

All of control nu/nu mice died by the 16th day after i.c. challenge with the virulent Hayashida strain of HSV-1 (Fig. 3a). In the serum-transferred group, 1 ml of hyperimmune serum with neutralizing antibody titre of 1:64 was inoculated i.c. into nu/nu mice 6 h before challenge. On days 2 and 6 after challenge, equal vol. of immune sera were transferred to maintain antibody titre. As shown in Fig. 3(b), transferred antibody did not inhibit the production of local skin lesions, but in four of the five mice zosteriform skin lesions did not develop until approx. 8 days after the last serum administration. The mice were bled on days 3, 9 and 16 for serum neutralizing antibody determinations and the titres were found.
to be well maintained until day 9 (average titre 1:16). The prolonged survival in serum treated mice was significant at $P < 0.05$ using Fisher's test.

When serum with neutralizing antibody titre of 1:128 was administered 6 h before challenge and on days 2, 5, 8 and 11, the development of skin lesions was delayed as well as the survival time (Fig. 3c). Two of six mice did not develop skin lesions and did not die. Administration of high titred serum for 11 days increased the survival time as compared to low titred serum for 6 days duration. This was significant at $P < 0.05$. These data indicate that the development of zosteriform skin lesions can be delayed by immune serum administration.

Transfer of resistance by spleen cells

Immune spleen cells were obtained from nu/+ mice 7 days after immunization with the avirulent SKa strain of HSV-1. Five nu/nu mice received intravenous injections of $10^7$ viable spleen cells per mouse 6 h before challenge. As shown in Fig. 4(a), five of six nu/nu mice which received non-immune spleen cells developed skin lesions and died along with untreated nu/nu mice (data not shown), whereas four of the five nu/nu mice which received
immune spleen cells survived (survival $P < 0.05$): three recovered from local skin lesions and one from zosteriform skin lesions; one animal was refractory to treatment (Fig. 4b). Three of six nu/nu mice which received immune spleen cells did not produce a detectable amount of neutralizing antibody (lower than 1:8), although they acquired resistance to the virus.

Effects of treatment of immune spleen cells with anti-θ serum and fresh guinea pig complement

In this experiment nu/nu mice were inoculated with $10^7$ viable immune spleen cells 24 h before challenge. As shown in Fig. 5(a) five nu/nu mice which received untreated immune spleen cells developed minimal or no skin lesions and recovered completely. Four of five recipient mice did not produce a detectable amount of antibody on the 10th day p.i. Two of them died accidentally on the 18th day p.i. Treatment of immune spleen cells with anti-θ serum and complement significantly reduced the ability of these cells to protect the recipient nu/nu mice from developing fatal HSV-1 infection ($P < 0.05$; Fig. 5b). This finding suggests that immune T cells play a significant role in recovery from HSV skin infection.

DISCUSSION

The role of the thymus in host resistance and antibody production to HSV infection has already been well documented (Mori et al. 1967; Ennis & Wells, 1974; Burns et al. 1975; Oakes, 1975; Rager-Zisman & Allison, 1976). In this paper, by using T cell deficient athymic nude mice, we have confirmed T cell dependency of resistance to HSV infection. Since production of antibody is T cell-dependent (Burns et al. 1975), it is difficult in vivo to separate antibody-mediated protection from that afforded by T cells alone. In this study, we examined antibody-mediated protection and T cell-mediated cellular protection independently, by transferring neutralizing antibody or immune spleen cells into nude mice.

It has been shown that neutralizing antibody did not prevent cell to cell spread of HSV in vitro (Ennis, 1973; Lodmell et al. 1973), or restore resistance in X-irradiated mice (Oakes, 1975) or alter the course of infection in mice immunosuppressed with cyclophosphamide (Rager-Zisman & Allison, 1976). Recent studies have emphasized the in vitro significance of antibody-dependent cell-mediated cytotoxicity (ADCC), in which non-immune non-T effector cells in combination with antibody lyse virus infected cells (Shore et al. 1974; Shimizu et al. 1977). Mice immunosuppressed with cyclophosphamide were significantly protected when antibody and normal spleen cells were administered simultaneously. The protective ability of this cell-dependent antibody-mediated protection in vivo was abrogated, when normal spleen cells were pre-treated with silica prior to transfer of the cells (Rager-Zisman & Allison, 1976), suggesting non-immune macrophages played a major role in this type of protection. Therefore, the in vivo experiments with antibody and normal spleen cells strongly suggested that protective ability of antibody-mediated protection in HSV infected mice was due to the mechanism of ADCC. Nevertheless, the recipient mice in their experiments may have obtained some T cells from the normal spleen cells. These experiments could not show the nature of the contribution of T cells in the protection observed. In our experiments, neutralizing antibody prepared in mice was transferred to T cell-deficient nude mice. The protective ability of passively transferred antibody was demonstrated in this study by inhibition of the development of zosteriform skin lesions and by the prolongation of the survival time after intracutaneous infection with HSV. Since nude mice are rich in effector cells involved in ADCC (Lovchik & Hong, 1977), it is tempting to speculate that this mechanism may have been operative in our serum transfer experiments. Our results show that antibody-mediated protection in vivo is effective in limiting the spread of virus, but not in protecting mice from fatal HSV infection.

However, transfer of immune nu/+ spleen cells in our experiments significantly protected
nude mice from fatal HSV infection. Our results are consistent with the observations in other studies involving transfer experiments with immune spleen cells. In the recipient mice in such experiments, others have shown the production of detectable amounts of neutralizing antibody (Ennis & Wells, 1974; Oakes, 1975; Rager-Zisman & Allison, 1976). In our experiments, some nude mice which received immune nu/+ spleen cells did acquire resistance to HSV, although they did not produce detectable neutralizing antibody. Furthermore, protective action of the immune spleen cells was significantly reduced after pre-treatment with anti-0 serum and fresh guinea pig serum prior to transfer of the cells. These data strongly suggested that immune T cells were able to protect nude mice against fatal HSV skin infection without antibody-mediated protection.

In conclusion, we have successfully shown antibody-mediated protection and T cell-mediated protection in nude mice. Our data indicate that T cell-mediated cellular protection plays a major role in recovery from HSV skin infection in mice, whereas antibody-mediated protection is effective only in limiting the spread of virus.

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