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Immune Responses to Labial Infection of BALB/c Mice with Herpes Simplex Virus Type 1

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The kinetics of appearance of five humoral antibody responses (micro-neutralization assay [NT], complement fixation [CF], enzyme-linked immunosorbent assay [ELISA], radioimmunoassay [RIA], antibody-dependent cell-mediated cytotoxicity [ADCC]) were compared during labial infection of BALB/c mice with herpes simplex virus type 1 strain Patton. The ELISA/RIA antibody responses were present in most mice by day 5 after infection, at the beginning of the herpetic lip lesions; antibody effective in ADCC showed identical early kinetics. In contrast, NT/CF antibodies were not detected in most mice until day 10, at the time of resolution of the herpetic lip lesions. The humoral immune responses persisted for at least 6 months after infection. The NT and CF responses were closely correlated in time of appearance and titers ($r = 0.9$), as were the ELISA and RIA responses ($r = 0.99$). However, there was little correlation between NT/CF and ELISA/RIA responses ($r = 0.02$). The kinetics of the delayed type hypersensitivity response showed similar kinetics of appearance to the ELISA/RIA/ADCC humoral responses, and peaked similarly, but waned gradually over 2 months. The importance of antibody in protection against labial herpes simplex virus type 1 infection was demonstrated by the ability of passively transferred convalescent serum (that produced a minimum NT titer of 10 in recipient mice) to protect against development of herpetic lesions and death.

Herpes labialis is one of the most frequent manifestations of disease with herpes simplex virus type 1 (HSV-1). Considerable interest has recently been directed toward animal models of this infection which attempt to establish efficacy of antiviral treatments (15, 20, 21) and immunization procedures (7, 23). We have demonstrated that infection of the lip with HSV-1 reproducibly causes the typical herpetic mucocutaneous lesion that can be monitored clinically and virologically (7, 15). Similar to the human disease, there is limited mortality, and in the majority of mice the virus establishes latent infection in the trigeminal ganglion, which is readily accessible for determination of latent virus.

Most experimental studies of immune responses to HSV have focused on systemic infection. We have established, however, that the immune responses differ considerably between systemic and vaginal infection of mice with HSV-2, partly due to the different pathogenesis of the two infections (14). Studies in humans have focused on recurrent oral infection with HSV-1, and not on the immune responses surrounding establishment of latency after primary oral infection with HSV-1. The presence studies, therefore, were undertaken to define several humoral and cell-mediated immune responses that occur during primary lip infection with HSV-1. These provide a basis for defining changes in the infectious process and immune responses that occur in immunized animals (7) or in animals treated with antiviral drugs or immunomodulators (15).

MATERIALS AND METHODS

Animals. Male, 5- to 6-week-old BALB/c mice were obtained from Laboratory Supply, Indianapolis, Ind., or Simonsen Laboratories, San Diego, Calif., and acclimated for 7 days before use.

Cells. Human epidermoid carcinoma (HEp-2) cells and African green monkey kidney cells (Vero) were obtained from Flow Laboratories, Inc., Rockville, Md., and were grown in Eagle minimal essential medium with Earle balanced salt solution, supplemented with 2 mM glutamine and 10% heat-inactivated fetal calf serum (10% FCS/CGM).

Virus. A pool of plaque-purified HSV-1, strain Patton, was prepared as previously described (7, 15). Virus infectivity titer was determined by measuring plaque-forming units (PFU) using a plaque count assay on Vero cells as previously described (14).

Labial infection of BALB/c mice with HSV-1. Labial infection of BALB/c mice with HSV-1 was performed as previously described (15; W. J. Payne, Jr., B. K. Murray, and P. H. Coleman, Abstr. Annu. Meet. Am. Soc. Microbiol. 1977, S311, p. 331), using methoxyflurane (Pitman-Moore, Inc., Washington Crossing, N.J.) inhalation anesthesia, a soft wire bristle brush attached to a dental drill to slightly abrade the

lip, and then swabbing the abrasion site with a cotton-tipped applicator dipped in a virus preparation containing 2×10^7 to 3×10^7 PFU/ml. Lesions were examined on days 4 to 18 and were scored on a scale of 0 to 3, where 1 represented erythema and swelling, 2 represented a lesion less than 1 mm in diameter, and 3 represented a lesion greater than 2 mm in diameter. Thirty days after infection, mice were assayed for latency using a modification of the method of Wohlenberg et al. (15, 34). Briefly, ipsilateral and contralateral trigeminal ganglia were obtained, cultured in 2% FCS/CGM for 72 h at 37°C to reactivate latent virus, and sonicated to disrupt the ganglia and release virus, and the presence of reactivated HSV was detected by inoculation of the sonic extract on Vero cells, incubation, and observance of typical cytopathic effect.

Antibody titration by ELISA. Antibody titers of mouse sera were measured with an enzyme-linked immunosorbent assay (ELISA) modified from the method of Ruittenberg et al. (29). Commercially prepared rabbit antiserum to mouse immunoglobulin G heavy and light immunoglobulin chains, conjugated to horseradish peroxidase (Cappel Laboratories Inc., Cochanville, Pa.), was used. The infected cell extract HSV antigen was diluted in carbonate coating buffer (0.1 M Na_2CO_3, pH 9.6), and 0.3 ml was added to each well of 96-well Cooke (Dynatech Laboratories, Inc., Alexandria, Va.) microtiter flat-bottom plates. After incubation for 24 h at 4°C in a humid chamber to allow antigen to coat the plates, the plates were washed three times in phosphate-buffered saline (0.02 M, pH 7.4) with 0.05% Tween 20 (PBS-T) and stored at 4°C until used.

Test sera were diluted in PBS-T, and 0.3 ml was added to each well and incubated for 3 h at room temperature or overnight at 4°C to allow reaction of antigen with antibody. The plates were again washed three times with PBS-T to remove nonreacted antibody. Then 0.3 ml of the peroxidase-conjugated antiserum diluted 1:200 was added to each well and the plates were incubated as before to allow antigen-antibody reaction. The plates were again washed in PBS-T, and 0.3 ml of substrate containing 0.005 M 5-amino salicylic acid (Aldrich Chemical Co., Milwaukee, Wis.) and 0.004% H_2O_2 (CMC Co., Nashville, Tenn.) was added to each well. After 30 min at room temperature, the reaction of substrate and enzyme was blocked by adding 0.05 ml of 3 M NaOH. Replicate wells were pooled, and the optical density was read at 450 nm. The antigen and conjugate concentrations were independently standardized to give a reading of 1.0 adsorbance with a known positive serum before running the test. Each assay included a known positive mouse serum, normal mouse serum, positive rabbit serum, wells receiving buffer rather than serum, and wells receiving only substrate. For each serum, a standard curve was established by plotting sequential serial dilutions against optical density, and the dilution corresponding to 0.2 optical density was interpolated and taken as the endpoint.

RIA. For radioimmunoassay (RIA), antigen-coated plates were prepared from HSV-1-infected cell lysates as described for the ELISA antibody assay. After washing with PBS-T, the test serum dilutions were added in replicate to the appropriate wells and incubated as above. After three washes with PBS-T, the wells were overlaid with 0.3 ml of ^{125}I-labeled rabbit anti-mouse immunoglobulin G heavy and light chains. The antiserum was iodinated by the chloramine-T method (6) and was kindly provided by J. Tew, Virginia Commonwealth University, Richmond. After overnight incubation at 4°C, the plates were washed three times with PBS-T and five additional times with tap water. Wells were cut from the plate with a band saw, and each well was counted individually in a Beckman gamma counter. Values were taken as the average counts per minute of the two replicate wells after subtraction of the background counts per minute. The endpoint was taken as the last point on the linear response portion of the titration curve.

Antibody titration by CF. The protocol of the Centers for Disease Control, Atlanta, Ga., as standardized for diagnostic laboratories (32), was followed strictly for antibody titration by complement fixation (CF). Serial dilutions of antisera were mixed with an optimal antigen dilution (predetermined, usually 1:8 or 1:16) and complement, followed by incubation and addition of sensitized sheep erythrocytes. Controls included those for antigen anti-complementary activity, antibody anti-complementary activity, complement back titrations, and cell controls.

Antibody titration by NT. For the complement-dependent micro-neutralization assay (NT), test sera were inactivated at 56°C for 30 min. The initial serum dilution was 1:10 in 10% FCS/CGM. Twofold serial dilutions of the serum were made in 96-well flat-bottom plates using a 0.025-ml microdiluter with 10% FCS/CGM diluent. Infectious virus (0.025 ml) containing 100 50% tissue culture infective doses and a final complement dilution of 1:15 was added to each well and the mixture was incubated for 45 min at 36°C. After incubation, 0.05 ml of Vero cells (4.0×10^5 cells per ml) was added to each well and incubation was continued. After 48 to 72 h, when the Vero cells formed a confluent monolayer and 50% cytopathic effect was observed in wells containing the negative control serum, the plates were examined microscopically. The endpoint was taken as the last dilution which showed less than 10% cytopathic effect.

DTH assay. The delayed type hypersensitivity (DTH) response was measured in the ear pinna of the mouse similar to our previous assay in the footpad for HSV-2 (14). Control or HSV-1 antigen (0.02 ml) was inoculated in the outer margin of the pinna of infected, inoculated, or control mice using a 27-gauge needle. Antigen was prepared by Formalin inactivation (1:400) of HSV-1-infected HEp-2 cell lysate. At various times after HSV-1 infection, antigen was injected and, 24 h later, 0.2 ml of ^{125}I-labeled human serum albumin (specific activity, 9.5 μCi/mg; Mallinckrodt, St. Louis, Mo.) was injected intraperitoneally. Four hours later, the mice were killed by cervical dislocation, and the ears were amputated at the base. Each ear was counted for radioactivity in a Beckman gamma counter. For each mouse a stimulation ratio (SR) was calculated by dividing the counts per minute in the ear receiving HSV-1 antigen by the counts per minute in the opposite ear. Two sets of controls were included: normal mice receiving HSV-1 antigen and infected, convalescent mice receiving uninfected cell antigens.

The SRs of these control mice were compared by analysis of variance, and no significant difference among controls was observed. Thus, the combined average SR in control animals was used for statistical comparisons. To define a response in an individual mouse as either positive or negative, a tolerance interval was established. Variations from controls were calculated at the 0.01 confidence level so that the tolerance interval would contain 99% of the control values, which means that there was only a 1 in 100 chance of falsely assigning a positive value. The mean SR of the control mice was 1.27, and the upper tolerance limit was 2.33 (0.01 confidence limits). Mice with a greater SR were defined as positive, whereas those mice whose SR fell within the tolerance interval were defined as negative.

ADCC. Mouse sera (1:20 dilution) were assayed for the presence of antibody effective in antibody-dependent cell-mediated cytotoxicity (ADCC), using HSV-1-infected Chang liver cells incubated with C57BL/6 mice peritoneal effector cells at a ratio of 60:1 for 18 h as previously detailed (9).

RESULTS

Pathogenesis of HSV-1 labial infection. After labial challenge with HSV-1, the primary infection followed a predictable pattern in BALB/c mice. By day 3, mice presented an erythematous swelling in the infected area of the lip, followed at days 4 to 5 by the appearance of a raised, yellowish vesicle which was most severe on days 8 to 10 and then began to resolve. Mice also usually experienced mild to severe conjunctivitis in the eye adjacent to the lesion and loss of facial hair. Some mortality (10 to 35%) was apparent, with herpetic encephalitis and death occurring between days 6 and 12. Surviving mice were killed at day 30, when trigeminal ganglia were assayed for latent virus. Virtually every mouse surviving primary infection was latently infected with HSV-1; titrations revealed from 10^3 to 10^4 PFU per ganglion.

Comparison of assays to measure antibody to HSV-1. Four different assays (NT, CF, RIA, ELISA) were used to measure antibody in 23 different sera obtained from individual mice at various times during infection. By the NT test, 8 of the 23 sera were judged negative (<10). By CF, 3 of those 8 negative sera were positive, and only one serum negative by CF was positive by NT. However, whereas the CF test was slightly more sensitive in detecting serologically converted mice, there was not a significant difference between the mean titers by the two assays (NT titer of 18 ± 5 standard error and CF titer of 25 ± 6). The ELISA assay detected 21 positive sera of the 23; 6 of these had previously been negative by NT and 4 also had been negative by CF. The RIA assay detected HSV antibody in 22 of the 23 sera, including 1 serum negative by ELISA. The mean RIA titer (3,300 ± 695) was slightly higher than the ELISA mean titer (1,020 ± 236). When the ratios of each serum were compared, the RIA was 4.03 ± 0.34 times more sensitive than the ELISA. The ELISA assay, on the other hand, was 75 ± 20 times more sensitive than the NT assay.

Examination of the correlation between individual titers provided additional evidence suggesting that the NT/CF and the ELISA/RIA assays measure a different part of the antibody population as well as possibly reflect differences in biological activities. There was little correlation ($r = 0.02$) between the ELISA and NT titers obtained on 85 different sera. Comparing 60 sera by CF and ELISA also revealed no correlation ($r = 0.01$). In contrast, comparison of NT with CF on 61 sera revealed a highly significant correlation ($r = 0.9$, $P < 0.001$). A strong correlation was also observed between the RIA and ELISA titers ($r = 0.99$, $P < 0.001$) of 19 sera obtained from mice between 5 and 22 days after labial HSV-1 infection.

Kinetics of the humoral immune response to labial HSV-1 infection. Individual mice were assayed for antibody responses during the course of labial HSV-1 infection. The initial NT and CF responses occurred between days 5 and 10, reached an apparent plateau by day 17, and remained stable for at least 6 months (Fig. 1). The ELISA antibody response revealed a steeper rise in titer between days 5 and 10, indicating a more rapid appearance of ELISA antibody, plateaued at day 10, and remained constant thereafter. The kinetics of the RIA antibody response were very similar to the ELISA antibody response. When the rate of seroconversions was compared at day 5, only 1 of 10 mice was positive by NT and only 4 of 10 were positive by CF. Moreover, the titer of the sera from the positive mice was 10, indicating the responses were just at the level of detectability. In contrast, the ELISA assay detected 7 of 9 of the same mice positive, whereas the RIA detected 8 of 9. Also, ELISA and RIA titers at day 5 were significantly greater than the NT or CF titers.

Ten individual mice were serially, retro-orbitally bled over 30 days, and the sera were assayed by the humoral assays (Fig. 2). In examining the serological response of Mouse-1, a slower response (compared to the population kinetics, Fig. 1) occurred in each of the four assays, with the CF titer selectively depressed. The other three mice represented seemed more typical of the population kinetics response, although individual variations were apparent. In this particular experiment, all of the mice tested were negative for NT antibody at day 5, and two of the mice did not reach peak NT titers until

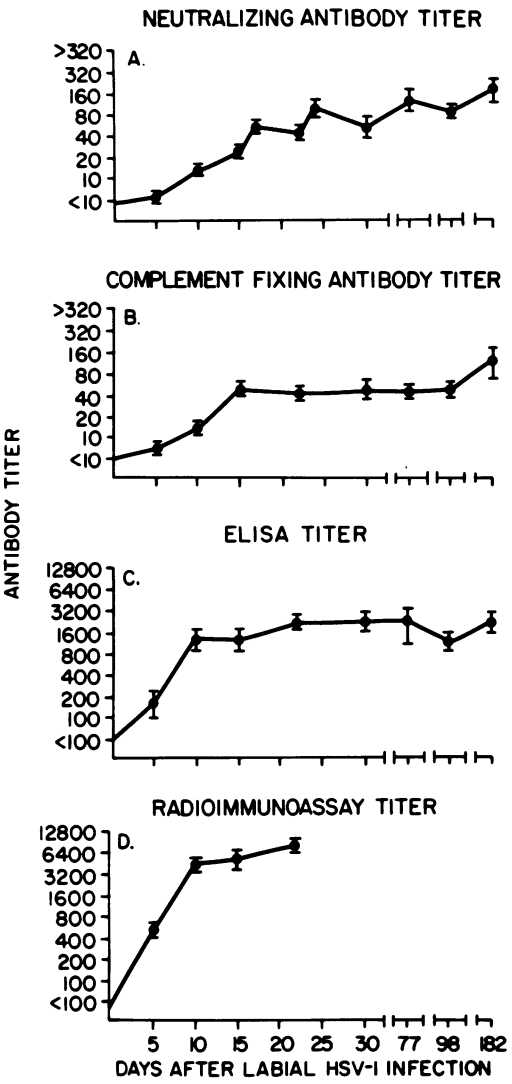

FIG. 1. *Humoral immune response in mice after labial infection with HSV-1. Mice were assayed for the presence of anti-HSV-1 antibody by four separate assays. Each point represents the mean of at least 7 mice with the standard error represented by the bars. Values of 10 were considered to be zero for statistical purposes.*

day 22. ELISA and RIA antibodies were present in all mice at day 5, but the maximum ELISA response was delayed until day 15 in two mice.

The kinetics of appearance of antibody detected by ELISA and ADCC were found to be virtually identical (Table 1). The two assays detected about the same number of positive immune responses. Moreover, the ELISA titer and ADCC percent cytotoxicity correlated well. Humoral immune responses, as measured by both assays, were present in most mice by day 5 after labial infection.

Kinetics of DTH response during labial infection with HSV-1. Mice were infected with HSV-1 labially, and their DTH response was followed over a period of several weeks. At day 5 after labial HSV-1 infection, two of five mice showed a positive DTH response and by day 10 all mice were positive (Fig. 3). At days 20 through 40, about 60 to 70% of the mice showed positive responses, whereas by day 75 only 2 of 6 mice were positive. The mean SR for the positive mice revealed that the maximal response (5.1) occurred at day 12, with the DTH values remaining relatively constant otherwise between days 5 and 40. Thus, a maximal DTH response appeared to occur at about days 10 to 15, persisted for several weeks, and waned after the second month after labial challenge.

Protection elicited by passive transfer of immune sera. Mice were exsanguinated by cardiac bleed 30 days after labial HSV-1 challenge, and sera were pooled and titrated (NT titer = 80). The resulting NT antibody titer in the recipient mice 24 h after intravenous inoculation of this serum was 10. These mice were then challenged labially with HSV-1, and showed significant protection against the primary herpetic lesion (Fig. 4). Mice receiving the serum were also protected against death (0/10 dead, as compared to 9/30 dead in the controls). Latency was reduced marginally from 15/15 in controls to 7/10 in the mice receiving the antibody. Mice that received less serum showed passive antibody titers of <10, but also showed protection from death and some decrease of clinical lesion scores.

To increase the antibody titer in the recipient mice, a hyperimmune serum (NT titer = 400) prepared in rabbits against HSV-1 strain Patton was used for passive immunization, resulting in an NT titer of 80 in the recipient mice. This treatment also offered marked protection against the primary lesion and protected mice against death. There was significant protection against latency (an incidence of 4/11 as compared with 10/10 in the controls, $P < 0.01$), and partial protection to those animals that became latent, evidenced by the reduction from $>10^3$ PFU in the controls to 10^2 PFU per ganglion. Thus, mice can be partially protected from latency, which may be an important factor in the development of recurrences of other sequelae.

Protection elicited by adoptive transfer of immune lymphocytes. Mice were labially challenged with HSV-1, survivors were taken at days 12 to 15 after infection, the mice were killed, and their spleens and right cervical lymph nodes were removed, minced, and forced through a 200-mesh, stainless-steel screen. The

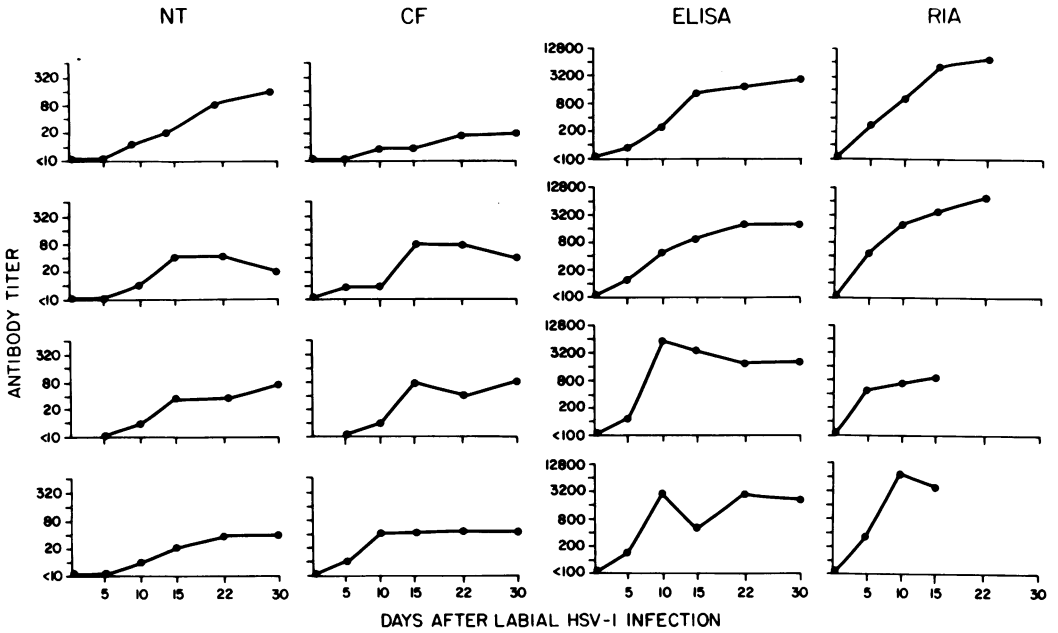

FIG. 2. *Comparison of antibody response against labial HSV-1 challenge in four representative, individual mice. Each mouse was assayed by four serological assays over a period of 30 days.*

TABLE 1. *Kinetics of ELISA and ADCC antibody responses during labial HSV-1 infection*

Day after infection	ELISA[a] (titer)	ADCC[b] (% cytotoxicity at 1:20 serum dilution)
3	<10	0
	<10	0.6
	<10	1.6
	24	4.2
	60	3.4
	1/5[c]	0/5[c]
5	50	5.7
	80	6.8
	90	9.3
	150	11.3
	210	13.2
	5/5[c]	5/5[c]
10	125	29.1
	350	28.8
	400	21.3
	875	24.3
	4/4[c]	4/4[c]

[a] ELISA values greater than 50 are considered positive.

[b] ADCC cytotoxicity values greater than 5% are considered positive.

[c] Positive/total.

resulting cell suspension was washed, suspended in Hanks balanced salt solution with 20 U of heparin per ml at a leukocyte density of 10^8 cells

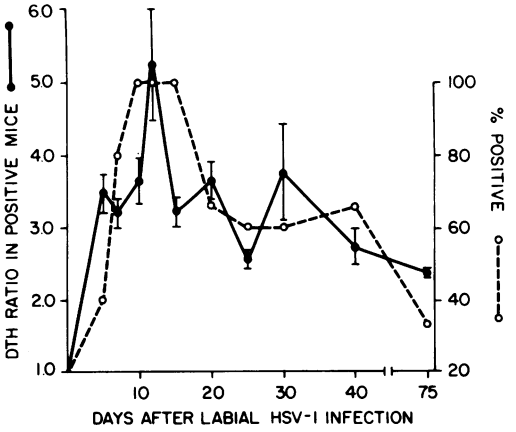

FIG. 3. *DTH response in mice after labial challenge with HSV-1. The values are for between 5 and 12 animals at each time point.*

per 0.5 ml, and 0.5 ml was injected into the lateral tail veins of mice. Some mice were examined for a DTH response at 24 h, whereas the rest were challenged labially with HSV-1. The controls included mice that received no inoculation as well as those receiving lymphocytes from normal mice. Twenty-four hours after virus challenge, two mice were examined for the presence of anti-HSV antibody with the ELISA assay; no detectable antibody was present. The passive transfer of spleen and lymph node cells produced a DTH response in three of four mice, with a

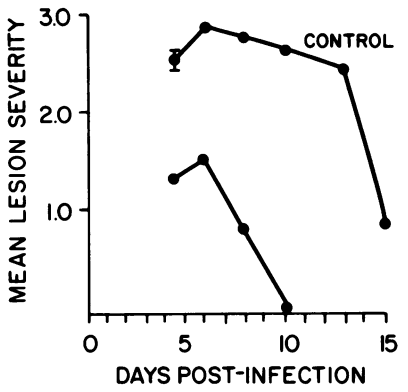

FIG. 4. *Mean lesion severity and duration after labial HSV-1 challenge in mice passively immunized with mouse antiherpesvirus antiserum. The bars represent one standard error on the mean lesion scores of control mice.*

mean stimulation ratio in mice with positive DTH responses of 2.75. However, there was no significant protection against the primary lesion, in the clinical course of the herpetic lesions, or in mortality. The control group showed 30% mortality (6/20 dead), whereas the treated group showed 21% mortality (3/14 dead). Latency was marginally reduced in the groups receiving immune lymphocytes (latency incidence of 7/10 compared with 10/10 in controls).

DISCUSSION

The present results demonstrate that functional antibody effective in ADCC against HSV-infected cells showed early kinetics identical with the primary ELISA antibody test. These data provide support for the role of ADCC antibody early in the localized infection elicited by labial HSV-1 infection. Recent reports have demonstrated that antibody effective in ADCC also appears early after systemic infection of mice with HSV-1 (8) or cytomegalovirus (24).

Moreover, the present data indicate that the kinetics of appearance of NT antibody appear relatively similar to localized or systemic (labial, intraperitoneal, intravenous, and subcutaneous) HSV infections in mice (14, 18, 25). The earlier kinetics of appearance of the ELISA/RIA as compared with NT/CF antibody responses indicate that, in part, different populations of antibody are probably being measured. The host may be responding to virus-induced antigens that appear earlier or in higher concentrations than the glycoproteins associated with neutralization. It has been established that the antibody effective in ADCC is immunoglobulin G type antibody (13), and that it is effective against early antigens present after infection of cells (28).

Differences between NT and RIA antibodies have been noted in a recent study of human sera associated with recurrent herpes labialis (27). Antibody taken between recurrent episodes had a lower proportion of NT to RIA antibody, whereas antibody assayed during recurrent oral lesions had a higher proportion of NT to RIA antibody (higher efficiency NT antibody). In our primary labial HSV infection, higher efficiency NT antibody did not appear until late in recovery of the animal. In both studies, the RIA assay was designed to detect the total antibody response rather than NT antibody only. A modification, for ELISA/RIA similar in specificity to NT but with greater sensitivity, could be made by preparing the antigen-coated plates with the glycoprotein fraction of HSV.

The DTH response, similar to the ELISA/RIA/ADCC humoral immune responses, occurred early during labial HSV-1 infection. The DTH response was present in about one-third of the mice by day 5, and reached a maximum both in number of mice responding and in level of response by day 12. However, the DTH response declined gradually over 2 months while the antibody titers persisted. The persisting antibody may be the result of virus being harbored in, and periodically reactivated from, a latent state, leading to continued antigenic stimulus for antibody.

Additional insight into the role of antibody was gained by passively transferring hyperimmune serum to normal mice. Mice receiving an amount of mouse antiserum that yielded an NT titer of only 10 in the recipient had significant protection from death, marked reduction in clinical lesions, and some reduction in incidence of latency. Transfer of higher-titered antisera produced significant protection against latency. Passive antibody has previously been shown capable of reducing the number of ganglionic cells infected with HSV-1 (33) and of restricting latent infection (12). Passive antibody, however, is not protective in all HSV-1 infections. The protective effect varies considerably depending upon the route of virus infection and subsequent pathogenesis, whether the animal is immunocompromised, and whether the infection is severe or inapparent. In general, for significant protection, the recipient animal must be immunocompetent (3, 8, 12, 17, 19, 25, 31). Data of Davis et al. (3) and Rager-Zisman and Allison (25) suggest that, in addition to a humoral factor, a cell that is sensitive to irradiation or cyclophosphamide treatment must be present; such a cell could be effective in the ADCC reaction (9).

The role of cell-mediated immunity was investigated by the transfer of immune spleen and

lymph node cells. Normal mice received immune lymphocytes from early convalescent animals (days 12 to 15), resulting in a transfer of positive DTH response in three of five mice. Mice receiving both spleen and lymph node cells showed slight protection against latency, but no protection from death or clinical symptoms. In some other experimental HSV infections, immune cells taken from hyperimmune animals have transferred marked protection (3, 5, 18, 25). Whether the present results are related to the time of harvesting immune cells, inoculation into immunocompetent rather than immunosuppressed animals, time of challenge after lymphocyte transfer, or differences in viral pathogenesis is not clear. In any event, very low levels of passively transferred convalescent antibody appeared more effective than convalescent immune cells in the labial HSV-1 infection model.

Other factors in the immune response to HSV also must play a role in protection, including activated macrophages (2, 16), NK cells (24, 26), interferon, and other lymphokines (11). Peritoneal macrophages activated for antiviral activity were not found during the labial HSV-1 infection (unpublished data); however, this does not preclude activation of macrophages in the local areas around labial infection.

In addition to the direct relationship between the immune response and the primary herpetic lesion, there is probably a relationship between the level of certain immune parameters and the maintenance of latency and control of recurrent infection. We have shown that immunization can reduce the number of latent animals and degree of latency in the HSV labial infection model (7; Thomas, Murray, and Morahan, manuscript in preparation). Price et al. (22, 23) have suggested that preexisting antibody may enhance the propensity for latency of HSV under certain conditions, but this was not observed in our studies. Our data do not offer information on the role of antibody or other immune parameters in maintaining the latent state or regulating recurrence. Stevens and Cook (30) have suggested that antibody may be able to maintain the virus in a latent state, whereas others (1, 10) have suggested that immunoglobulin G may protect latently infected cells from immune destruction by blocking attachment sites involved in ADCC or complement-mediated lysis. To address these two hypotheses, it will be necessary to monitor the reactivation of latent virus, a task which has proved elusive in the mouse model of HSV infection.

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