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Alteration of Host Defense Mechanisms by Murine Cytomegalovirus Infection

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An animal model of a sublethal infection, utilizing murine cytomegalovirus (MCMV), was developed to determine whether immunological factors could contribute to the establishment of a persistent viral infection. Adult female C3H mice inoculated intraperitoneally with 10^5 plaque-forming units of MCMV developed splenomegaly 5 to 12 days after infection. Virus replicated to peak titers (10^3 to 10^6 plaque-forming units per g of tissue) in liver, spleen, lung, kidney, and salivary gland tissue during the acute phase of the infection (3 to 12 days); it then decreased to undetectable levels in all tissues except salivary gland. Serum interferon was detected as early as 12 h after infection, peaked at 36 h (1,093 U/ml), and was undetectable by 4 days after infection. MCMV-infected animals were hyporeactive to interferon induction with Newcastle disease virus on days 5 to 9 of the infection. Splenic lymphocyte reactivity to phytohemagglutinin and lipopolysaccharide was normal early during the course of the infection, was suppressed during the acute phase of the infection, and had returned to normal by day 18. These data indicate that several parameters of host defense are transiently suppressed during the course of a MCMV infection. The capacity of cytomegaloviruses to alter host resistance may be one factor that contributes to the establishment of a persistent infection.

Observation of both humans and animals have indicated that the cytomegaloviruses (CMV) may persist in host tissues for long periods of time despite a demonstrable humoral immune response (16, 21). Persistence of this virus in human tissues has resulted in a variety of clinical problems. Its presence in donor tissues such as whole blood used in transfusions (2) or transplanted kidneys (9) has been associated with infection in recipients. Furthermore, the presence of the virus in cervical secretions of apparently healthy, pregnant women (18) may be associated with an increased risk of infection to the newborn infant during the neonatal period. This problem of chronic CMV infection has been investigated in several animal model systems. Henson et al. (8) were able to isolate murine cytomegalovirus (MCMV) from abdominal lymph node and spleen cell suspensions, prepared from chronically infected mice by cocultivation on mouse fibroblasts, in the absence of histological and electron microscopic evidence of infection. These findings were confirmed by Olding and his colleagues (15), who isolated virus from the spleens of mice infected in utero or at birth as long as 5 months after infection by cocultivating spleen cell suspensions on allogeneic mouse fibroblasts or by lipopolysaccharide (LPS)-induced blastogenesis. He concluded that latent virus was highly associated with the B-lymphocyte in mice. Utilizing the murine model, several investigators have reported that certain determinants of host resistance may be suppressed during the course of MCMV infection. Osborn and Medearis (17) demonstrated that the ability of MCMV-infected mice to produce interferon and antibody to Newcastle disease virus (NDV) was impaired. Furthermore, NDV, which does not normally replicate in mice, replicated to a limited extent in MCMV-infected mice. In a subsequent series of experiments, Osborn et al. (16) showed that the antibody response to sheep erythrocytes and the generation of hemolytic plaque-forming spleen cells against this antigen were suppressed by acute MCMV infection. Howard et al. (10) have reported that mitogen-induced blastogenesis and mixed-lymphocyte reactions are suppressed and skin graft survival is prolonged during MCMV infection. In general, evidence has accumulated indicating that CMVs interact with their respective host's immune system and that during infection certain parameters of host defense may be suppressed. Whether or not this alteration

of the host response is a factor in the development of a persistent infection remains to be established.

Although the immune function in MCMV-infected mice has been investigated, the results have not been correlated with the pathogenesis of the infection. Therefore, the purpose of this investigation was to define the relationship of virus replication in target organs to the effect of the virus on host defense. Toward this objective we developed an animal model of a sublethal MCMV infection in an inbred strain of mice. Studies carried out with this model include an assessment of: (i) the development of splenomegaly, (ii) the replication of virus in major target organs, (iii) the interferon response to MCMV, (iv) the effect of the infection on the ability of the host to produce interferon in response to exogenous viral challenge, and (v) splenic lymphocyte reactivity to LPS and phytohemagglutinin (PHA).

(Portions of this work were presented at the Eleventh National Meeting of the Reticuloendothelial Society, December 1974, in Seattle, Wash.)

MATERIALS AND METHODS

Animals. Female C3H mice (8 to 10 weeks of age) obtained from the vivarial colony maintained at the University of Utah were used in all experiments. The original breeding stock was obtained from Jackson Laboratories, Bar Harbor, Me.

Media. Eagle minimum essential medium consisted of Earle balanced salt solution supplemented with vitamins and amino acids (Grand Island Biological Co., Grand Island, N.Y.), glutamine (300 μg/ml), penicillin (100 U/ml), streptomycin (50 μg/ml), $NaHCO_3$ (2 mg/ml), and 10% heat-inactivated fetal bovine serum (Grand Island Biological Co.).

RPMI 1640 with glutamine (300 μg/ml; Grand Island Biological Co.) was supplemented with penicillin (100 U/ml), streptomycin (50 μg/ml), and $NaHCO_3$ (2 mg/ml). The pH of the medium was adjusted to 7.2 with 1 N HCl just before membrane filter sterilization. Where noted, 2% heat-inactivated, filtered human plasma was added to this medium.

Cell cultures. Primary mouse embryo fibroblast cultures were prepared as described previously (13). A continuous line of mouse L-929 cells obtained from the American Type Culture Collection, Rockville, Md., was used in the interferon assay.

Virus. The Smith strain of MCMV was obtained from June Osborn, University of Wisconsin, Madison. In her laboratory this pool had been cloned twice, passaged six times in mouse embryo fibroblast cell culture, and then passed three times in lactic dehydrogenase virus-free, Swiss-Webster, weanling mice obtained from the National Laboratory Animal Co., Creve Coeur, Mo. In our laboratory the virus was passed twice in 3- to 4-week-old C3H female mice by a procedure described previously (12). Pools prepared in this manner were found to be free from serum lactic dehydrogenase enzyme-elevating activity as assayed by the procedure of Wroblewski and LaDue (27). The virus pool was also free from culturable mycoplasma and titered 10^7 plaque-forming units (PFU)/ml when assayed on secondary mouse embryo fibroblast cells. The Herts strain of NDV was originally obtained from Samuel Baron, National Institutes of Health, Bethesda, Md. Stock NDV pools were prepared by propagating the virus in the allantoic membrane of embryonated chicken eggs. Allantoic fluids were harvested and assayed for virus on primary chicken embryo cells. Pools prepared in this manner regularly titered 10^9 PFU/ml. Vesicular stomatitis virus, Indiana strain, was obtained from the American Type Culture Collection. This virus was propagated in confluent chicken embryo cell monolayers, and supernatants were collected as stock virus. Pools of this virus routinely titered 10^8 PFU/ml when assayed on mouse L-929 cells.

Viral assay. MCMV was assayed, as described previously (12), by employing a modification of a technique described by Wentworth and French (26).

Preparation of murine tissues for viral assay. Tissues from infected animals were prepared as described previously (12). Briefly, liver, spleen, lung, kidney, and salivary gland tissues from groups of three infected mice were excised at various times after infection, pooled by group, and weighed, and 10% (wt/vol) homogenates were prepared in minimal essential medium. Serial 10-fold dilutions of these homogenates were assayed for virus on secondary mouse embryo fibroblast cells. Homogenized whole blood was assayed in a similar manner. Since low dilutions of most tissue preparations were found to be toxic to mouse embryo fibroblast cells, virus titers less than $10^{2.4}$ PFU/g of tissue for solid organs and $10^{1.4}$ PFU/ml for whole blood were not detectable by this procedure. Spleen tissue homogenates were not as toxic, and a sensitivity of $10^{1.2}$ PFU/g was achieved.

Interferon induction and assay. The interferon response to MCMV was determined in mice that had been infected intraperitoneally with 10^5 PFU of virus. Blood was obtained by cardiac puncture from groups of five infected mice at 12-h intervals up to 3 days postinfection and daily thereafter, pooled by group and allowed to clot, and the serum was then separated. Sera were diluted 1:20 in minimal essential medium and adjusted to pH 2 for 3 days to inactivate MCMV. The interferon titer for L-929 cells was determined by the plaque reduction method with vesicular stomatitis virus as the challenge virus, as has been described (23). The ability of MCMV-infected mice to produce interferon was measured by inoculating groups of three MCMV-infected and three uninfected control mice intraperitoneally with 10^8 PFU of NDV at various times after infection. Blood was collected from these animals 4 h postinoculation and processed in a similar manner, except that serum samples were pH treated for 5 days to inactivate residual NDV. An internal laboratory interferon standard was included with each assay. Periodically, the sensitivity of our assay system was standardized against the international reference mouse preparation obtained from the National Institutes of Health. In our system this reference interferon standard had a titer of approxi-

mately 5,000 U compared with the designated titer of 6,500 U. Each unit of interferon depicted in our figures represents 1.3 international reference units.

Preparation of spleen cell suspensions. Spleens from animals killed with chloroform were removed aseptically and placed in warm (37°C) RPMI 1640. Whole organs were teased into fine fragments with a sterile, 20-gauge, hypodermic needle, and the suspension was then filtered through sterile, 80-mesh, stainless-steel gauze (Granger Wire and Iron Works, Murray, Utah). The spleen cells were sedimented by centrifugation at 100 × *g* for 5 min. The cell pellet was then suspended by gentle pipetting in warm (37°C) tris(hydroxymethyl)aminomethane-buffered NH_4Cl (1) to lyse erythrocytes and then centrifuged immediately for 5 min at 100 × *g*. The remaining cells were suspended, in RPMI 1640 with 2% human plasma, to a final concentration of 2×10^6 viable cells per ml; their viability was determined by trypan blue dye exclusion. Portions of 2 ml each were dispensed into round-bottomed, disposable, glass test tubes (16 by 100 mm; Van Water and Rogers, Salt Lake City, Utah) that had been cleaned, rinsed, and dry-heat sterilized at 250°C for 45 min.

Mitogen-induced blastogenesis. Spleens from groups of three infected and three uninfected animals were pooled by group, and cell suspensions were prepared at various times after infection with MCMV. Cultures were prepared in triplicate, and to each tube either 0.1 ml of a 1:100 dilution of PHA (PHA-P, Difco Laboratories, Detroit, Mich.) or 50 μg of LPS from *Escherichia coli* O111:B4 (LPS-W, Difco) was added. Triplicate cultures without mitogen served as controls. Samples were incubated for 48 h at 37°C in an atmosphere of 5% CO_2 and air. At this time, 1 μCi of [^{3}H]thymidine (specific activity, 20 Ci/mmol; New England Nuclear Corp., Boston, Mass.) was added to each tube. After a further incubation period of 24 h, the tubes were gently blended in a Vortex mixer and centrifuged at 100 × *g* for 5 min. The supernatant was decanted, and 5 ml of ice-cold 5% trichloracetic acid was added to each tube. The tubes were allowed to stand for 30 min at 4°C, after which they were spun at 1,000 × *g* for 5 min. The supernatant was discarded, and the residual pellet was suspended in 10 ml of Aquasol (New England Nuclear Corp.). The amount of radioactivity in each sample was determined by liquid scintillation spectrometry in a Nuclear-Chicago Mark II liquid scintillation spectrometer. Disintegrations per minute of counting were determined by the channels ratio method, utilizing an external standard. In general, the counting efficiency for all samples was between 25 and 30%.

RESULTS

Pathogenesis of the infection. In an initial series of experiments, several parameters of the MCMV infection were examined. Mice infected intraperitoneally with a sublethal inoculum (10^5 PFU/mouse) of MCMV began to exhibit fur ruffling, hunching, slight weight loss, and lethargy by day 5. These symptoms persisted for an additional 5 to 7 days and then gradually resolved. During the course of the infection, infected animals developed marked splenomegaly. In Fig. 1 the spleen weight, expressed as grams per kilogram of total body weight, is presented for MCMV-infected and uninfected control mice. In each of three experiments, spleens from three mice were pooled and weighed. Splenomegaly was first evident by day 5, was maximal on day 7, and had resolved by day 18.

We next examined the MCMV replication in major target organs at various times after infection. Data from a representative experiment are plotted in Fig. 2. Virus was detectable at low levels in the liver on day 5, replicated to peak titers by day 7, and was cleared to undetectable levels between 18 and 30 days after infection. In the kidneys, MCMV was present on day 5, with peak titers on day 7; in salivary gland and lung tissues, it was first detected on day 7. Virus replicated to high titers by day 9 in salivary gland tissue and remained at these levels throughout the 30-day course of the experiment. Virus was cleared from lung tissue by 12 days postinfection. In one of four similar experiments, a low-level viremia (less than 10^2 PFU/ml) was present on days 5 through 12 (data not shown). In a separate experiment, spleen tissue homogenates were assayed for the presence of virus at various times after infection. These data are also shown in Fig. 2. Virus was detectable at low levels as early as 24 h after infection, replicated to peak titers by day 12, and was cleared to undetectable levels 30 days postinfection.

Interferon response of MCMV-infected mice. To correlate the pathogenesis of the MCMV infection with the capacity of infected mice to produce interferon, we initially determined the serum interferon response to the virus itself. The data from three experiments are sum-

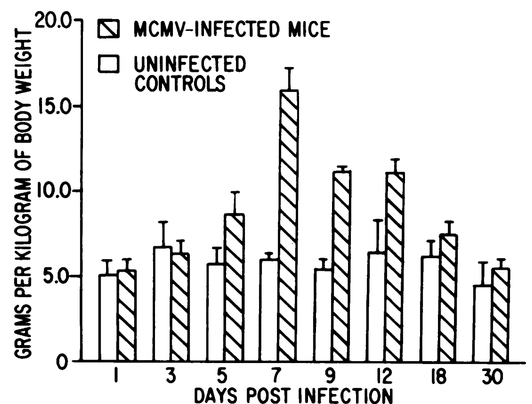

FIG. 1. *Spleen weight in C3H mice during MCMV infection and in uninfected control mice. The mean ± 1 standard deviation for three experiments is shown.*

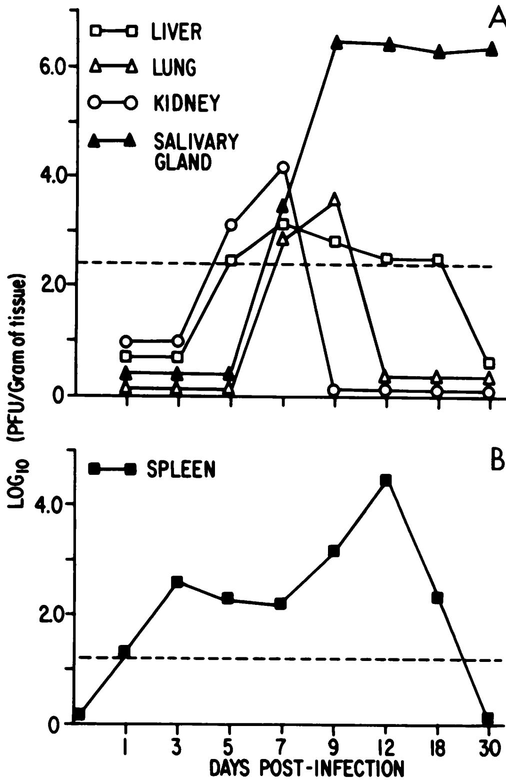

FIG. 2. *(A) Virus titers in various tissues during MCMV infection of C3H mice. The horizontal, dotted line indicates that virus titers of less than $10^{2.4}$ PFU/g of tissue were not detectable in this system. (B) Virus titers in spleen tissue during MCMV infection of C3H mice. The horizontal, dotted line indicates that virus titers of less than $10^{1.2}$ PFU/g of tissue were not detectable in this system.*

marized in Fig. 3. The interferon response to MCMV occurred early, with low levels of activity at 12 h after infection and a peak titer of 1,093 ± 280 U/ml by 36 h. No interferon activity was detected from 4 to 30 days after infection.

As part of these studies, we also examined the ability of MCMV-infected mice to produce interferon in response to an exogenous viral challenge with NDV, a potent inducer of interferon in mice. Groups of three infected and three uninfected control mice were inoculated intraperitoneally with 10^8 PFU of NDV at different times after MCMV infection. In our laboratory, 10^8 PFU of NDV induces 2,000 to 3,000 U of interferon per ml in normal C3H mice. The results from two such experiments, expressed as a percentage of the interferon titer induced in uninfected control mice inoculated with NDV at the same time, are shown in Fig. 4. MCMV-infected animals were able to produce interferon in response to NDV early during the course of the infection but became profoundly hyporeactive on days 5 to 9, during the acute phase of the infection. On days 12 to 30, MCMV-infected animals appeared to recover their capacity to produce interferon.

Response of splenic lymphocytes to mitogen. The ability of splenic lymphocytes obtained from groups of three infected mice to respond to PHA or LPS at different times during the MCMV infection was compared with the response obtained with splenic lymphocytes from three uninfected control mice. Data from one of three similar experiments for PHA are shown in Fig. 5. In this experiment, PHA responsiveness was normal to near normal on days 1 to 3, reduced to low levels on days 5 to 12, and recovered by day 18. The effect of the MCMV infection on splenic lymphocyte reactiv-

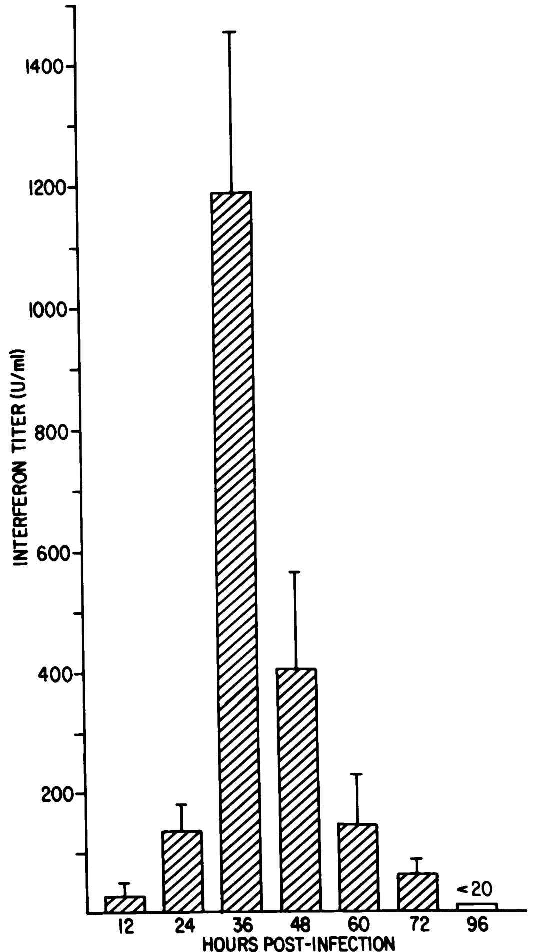

FIG. 3. *Interferon response to MCMV in C3H adult mice. The mean titer ± 1 standard deviation for three experiments is shown.*

ity to LPS is depicted in Fig. 6. The LPS response was normal early during the course of the infection on days 1 to 5, was reduced on days 7 to 12, and had returned to normal by day 30. It appeared from these studies that splenic lymphocyte responsiveness during the course of the MCMV infection to both a T- and a B-lymphocyte mitogen was suppressed during the acute, replicative phase of the infection and that, as the animals recovered, mitogen responsiveness was restored concomitantly.

DISCUSSION

The nature of immunity to CMV infections and the mechanisms responsible for viral persistence and/or reactivation of a latent infection remain incompletely defined. The present studies were designed to examine the host response and correlate the pathogenesis of MCMV infection with the alteration of several parameters of host defense during the course of a model infection in C3H mice.

Since interferon is thought to play a role in host resistance to viral infections (4-6), two interesting observations are that C3H mice produced a detectable serum interferon response beginning at 12 h and extending through 72 h and that infected animals were able to respond to challenge with NDV during the early stages of the infection but that, later during the stage of peak viral replication, this host defense system was suppressed. Several hypotheses may be suggested to explain this phenomenon. First,

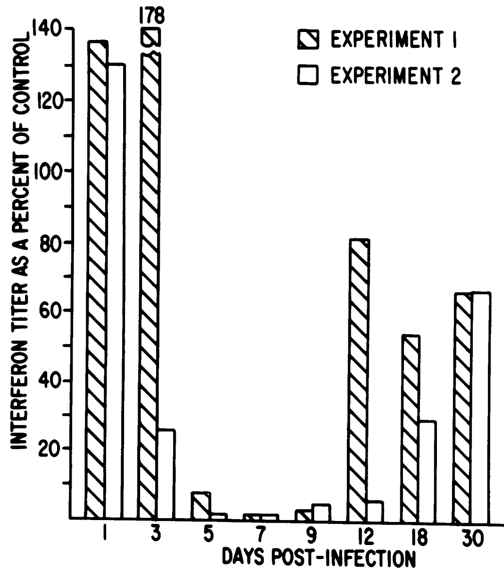

FIG. 4. *Interferon response to NDV in adult C3H mice during MCMV infection.*

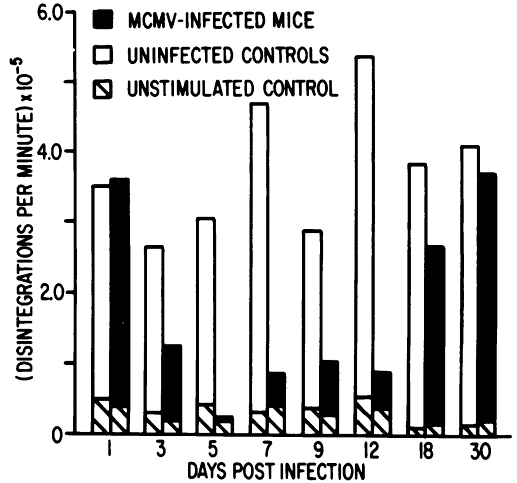

FIG. 5. *Splenic lymphocyte response to PHA in MCMV-infected and control, uninfected animals.*

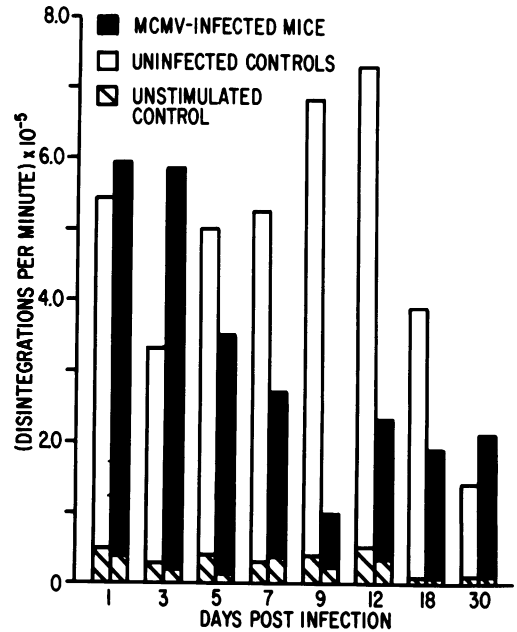

FIG. 6. *Splenic lymphocyte response to LPS in MCMV-infected and control, uninfected animals.*

certain cells critical to interferon production may be infected by the virus or otherwise impaired by the viral infection in their ability to produce interferon. This would be consistent with the observation that maximal suppression of interferon production occurs when tissue MCMV titers are the highest. It is interesting that Henson et al. (7) were able to show that mice given 450 rads of whole-body X-irradiation and then infected with MCMV had interferon

activity in liver tissue for as long as 6 days after infection, whereas nonirradiated, infected animals had none in liver tissue 6 days after infection. This occurred despite the fact that virus replicated to higher titers in the liver of irradiated animals than in nonirradiated animals. One interpretation of these data is that a radiosensitive cell population capable of suppressing the interferon response during MCMV infection was destroyed by X-irradiation. If this occurred, an alternate explanation of the hyporeactivity to interferon induction during the MCMV infection might be that cells are generated during the course of the infection that either themselves or through the production of factor(s) suppress the interferon response. Evidence for such a factor found in the serum of mice during an infection with encephalomyocarditis virus (22, 24), as well as other viruses, including MCMV (25), has been previously reported by this laboratory.

The depression of mitogenesis to PHA, a T-lymphocyte mitogen (11), and LPS, a B-lymphocyte mitogen (3), may be explained by several different mechanisms. First, the virus could infect lymphocytes and inhibit deoxyribonucleic acid synthesis. Some evidence for MCMV directly impairing lymphocyte responsiveness to various mitogens has been reported. Selgrade et al. (20) showed that the addition of MCMV to spleen cell suspensions from normal BALB/c mice could suppress the lymphocyte response to a number of mitogens. The splenic lymphocyte response to LPS could also be suppressed with a relatively low inoculum of either a tissue culture-attenuated or a virulent strain of MCMV, whereas 10 times more virus of the virulent strain was required to inhibit the response to concanavalin A, a T-cell mitogen. These data suggested to the authors that B-cell mitogenesis was more susceptible to the virus than T-cell mitogenesis. This concept was supported by the work of Olding et al. (15), who demonstrated that MCMV could be isolated from lymphocytes in culture and could be reactivated from a latently infected murine spleen cell suspension by culturing in the presence of LPS or by cocultivating partially purified B-cell suspensions on allogeneic fibroblast cells. The interaction between MCMV and the B-lymphocyte, therefore, could be similar to that of a related herpesvirus, Epstein Barr virus of humans (19). On the other hand, evidence for specific infection of either T-lymphocytes or macrophages with MCMV has yet to be established. Although the suppression of splenic lymphocyte reactivity to LPS might be a result of the direct infection of B-lymphocytes and subsequent interference with deoxyribonucleic acid synthesis, this is not likely to be the cause of a decreased response to T-cell mitogens such as PHA. Data from our laboratory (unpublished) indicate that the splenomegaly associated with this infection corresponds to an increase in cell number in infected spleens and that the viability of spleen cell suspensions (greater than 85%) obtained during the acute phase of the infection does not differ from that of the control. Given the relatively low titers of virus (10^3 to 10^4 PFU/g of tissue) that can be recovered from splenic tissue homogenates in the presence of an increased number of viable cells, it seems unlikely that this infection results in enough cell damage to account for the suppression of mitogen responses. An alternative explanation for these observations is that the subpopulations of specifically immune lymphocytes that might be generated in response to the viral infection are not as responsive to mitogenic agents. Finally, it is also possible that suppressor cells are generated during the course of the infection that are capable of suppressing mitogen responsiveness of splenic lymphocytes. Such suppressor cell activity has been demonstrated in viral tumor systems such as the Moloney sarcoma of mice (14). Since MCMV-infected mice recover from the infection, presumably by mounting an effective immune response to the viral pathogen, it is possible that the hyporeactivity to interferon induction and the diminished response of splenic lymphocytes to various mitogens observed during the acute course of the infection may be normal components of the regulation and control of the primary immune response to the viral infection rather than the results of direct cell damage by the virus.

Although it has been suggested that alteration of host resistance during CMV infections may be a factor contributing to the establishment of a persistent infection, our data indicate that nonspecific determinants of host resistance, e.g., mitogenesis and the interferon response, are suppressed only during the acute phase of the infection. Further work is needed to define the kinetics of generation of the specific immune response of the host to this virus and to determine whether suppression of host defense mechanisms may play a role in the establishment of the persistently infected state.

ACKNOWLEDGMENTS

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ADDENDUM

Since submission of this manuscript, Booss and Wheelock (J. Infect. Dis. **135**:478–481, 1977) reported

that serum from mice lethally infected with MCMV could suppress the response of lymphocytes from uninfected mice to concanavalin A, a T-cell mitogen.

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