INFECTION AND IMMUNITY, Apr. 1982, p. 89-95
0019-9567/82/040089-07$02.00/0

Vol. 36, No. 1

Murine Cytomegalovirus-Induced Immunosuppression

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Received 23 September 1981/Accepted 1 December 1981

The mechanism of murine cytomegalovirus-induced immunosuppression was investigated by examining the roles played by lymphocytes and adherent cells derived from spleens of infected SWR/J mice. As few as 100 infected cells per spleen were correlated with complete abrogation of mitogen responses at 4 and 5 days after infection. In a series of cell mixing experiments it was shown that the deficiency in infected spleens was due partly to the adherent cells, which apparently secreted an immunosuppressive factor, and partly to the infected lymphocytes, which upon exposure to this factor could no longer respond to concanavalin A presented to them by normal adherent cells.

Murine cytomegalovirus (MCMV) produces a number of interesting pathogenetic features in different strains of mice and offers the opportunity for studying parameters involved in cytomegalovirus infections and disease (for recent reviews see references 14 and 24). One interesting feature which has concerned us is the phenomenon of immunosuppression, a property shared with many other viruses (27, 29, 30). The MCMV-mediated immunosuppression has been documented by several groups of workers, who have employed a variety of immune responses to measure this phenomenon, e.g., responses to foreign antigens, interferon inducers, and mitogens; mixed leukocyte reactions; and skin graft survival (5, 6, 11-14, 17, 18, 20, 25, 28). The effect is transient, however, and infected mice eventually produce vigorous cell-mediated and humoral responses to MCMV (2, 3, 10, 22, 26).

A consequence of the transitory immunosuppression is the marked susceptibility of the infected mice to secondary microbial infections (9), apparently due to an impaired ability of the reticuloendothelial system to clear the organisms.

It has been pointed out elsewhere (14) that the immunosuppression cannot be explained by a direct toxic effect of the virus upon lymphocytes. An alternative explanation is that MCMV infects macrophages or monocytes and thereby indirectly affects lymphocyte function. This is in accord with our observations on spleen cells in vitro, in which the virus associates predominantly with macrophages (19). In addition, we have recently correlated the immunosuppression with the presence of infected adherent cells (possibly macrophages) in the spleen (21).

In the present study we attempted to gain a better understanding of the mechanism of this phenomenon by examining the relative roles played by macrophages and lymphocytes.

MATERIALS AND METHODS

Virus. The Smith strain of MCMV was used in this study. The virus was propagated in mouse embryo cultures at low multiplicity (0.01 plaque-forming units per cell) and was assayed by plaque formation (standard assay) as previously described (23). Infectious centers (IC) were measured on monolayers of mouse embryo cells as described previously (19).

Mice. SWR/J mice were obtained from Jackson Laboratories (Bar Harbor, Maine). Females 8 to 12 weeks old were inoculated intraperitoneally with the indicated amount of virus in 0.2 ml of phosphate-buffered saline or with the saline alone, and spleens were removed 3 to 5 days later.

Spleen cultures. Spleens from 3 or 4 mice were pooled and disaggregated, and cultures were established and maintained in RPMI 1640 containing 10% (vol/vol) fetal bovine serum as described elsewhere (15). Whenever desired, cultures were fractionated into adherent and nonadherent populations by incubation for 2 h at 37°C in tissue culture dishes or Linbro microtiter plates (19). Reconstituted cell mixtures were formed by adding nonadherent cells to the appropriate adherent cells in wells of Linbro microtiter plates. Unless otherwise indicated, total cell concentrations were 10^6 cells per 0.2 ml per well.

Mitogen assays. Spleen cultures were incubated with concanavalin A (ConA; Sigma Chemical Co.), lipopolysaccharide (LPS; Difco Laboratories), or an equivalent volume of medium for 40 h unless specified otherwise. Final concentrations for ConA and LPS were usually 2.5 and 40 μg/ml, respectively. [*methyl*-^{3}H]thymidine (40 Ci/mmol; New England Nuclear Corp.) was added at 2.5 μCi per well during the last 8 h of incubation, except as indicated otherwise. Cells were then deposited onto fiber glass filters, which were washed three times with phosphate-buffered saline and twice with ethanol, by means of a multiple

automated sample harvester (MASH II; Microbiological Associates). The dried filters were counted for radioactivity in a Beckman Isocap 300. In all mitogen assays the counts per minute quoted represent the means of quadruplicate cultures. In most cases the variation among replicates was within ±10% of the mean. Error bars have been omitted from graphs for the sake of clarity.

RESULTS

We have shown previously that in MCMV-infected SWR/J mice the peak of immunosuppression occurs at 4 to 5 days after inoculation (21). Therefore in this study we have concentrated our attention on this time period to understand the mechanism of the immunosuppression.

Kinetics of mitogen response. The response of 4-day-infected and uninfected spleen cells to ConA and LPS was measured by [^{3}H]thymidine incorporation either in the form of 4-h pulses or as a continuous exposure. The results are plotted as stimulation indices in Fig. 1 for ConA and in Fig. 2 for LPS. Evidently the kinetics are similar as measured by pulse-labeling (solid lines) and continuous labeling (broken lines) and are similar for the two mitogens, except that stimulation values are invariably lower for LPS. In all cases the infected cells were virtually unresponsive, and prolonged exposure to mitogens did not overcome this defect.

The spleens of 4-day-infected mice were identical to control spleens in appearance and contained normal numbers of macrophages, T-cells, and B-cells (21). The number of infected spleen cells in the experiment described above (measured as IC) was only 136 per 10^6 viable cells. In addition the loss of cell viability during the cultivation of these infected spleen cells was no greater than in the uninfected spleen population. Consequently the extent of immunosuppression observed could not be explained by death of the lymphocytes and must be due, therefore, to an indirect effect of the virus.

Number of IC required for immunosuppression. If a correlation exists between virus infection and immunosuppression, then the degree of suppression observed should be related to the dose of virus inoculated. Such a relationship is demonstrated in Fig. 3, in which the log of the number of infected cells (solid symbols) and the stimulation index (by ConA, open symbols) are plotted against virus dose. There is clearly an inverse relationship between the number of infected cells and the ability of the cells to respond to ConA. A similar relationship holds for LPS-treated cells. It can be calculated from the results shown in Fig. 3 that a 50% suppression of

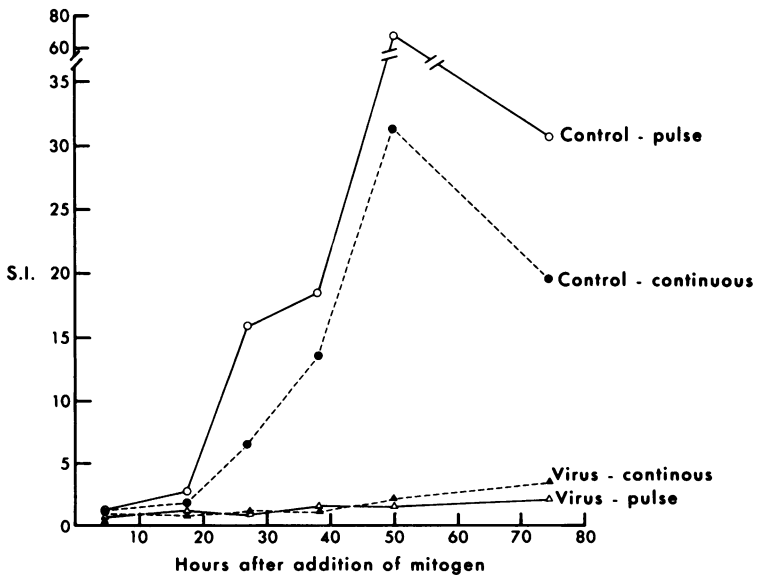

FIG. 1. Response to ConA of spleen cells at four days postinoculation. Spleens were removed from control (uninfected) and infected mice at 4 days after intraperitoneal inoculation with, respectively, saline or 10^6 PFU of MCMV per mouse. The spleen cultures, at 5×10^6 cells/ml, were incubated with or without 2.5 μg of ConA per ml plus 2.5 μCi of [^{3}H]thymidine per well. In the case of pulse-labeling, replicate cultures received isotope at 4 h before harvest. Continuously labeled cultures received isotope, together with 10^{-5} M nonradioactive thymidine, from time zero until harvest. S.I., Stimulation index: counts per minute in the presence of ConA/counts per minute in the absence of ConA.

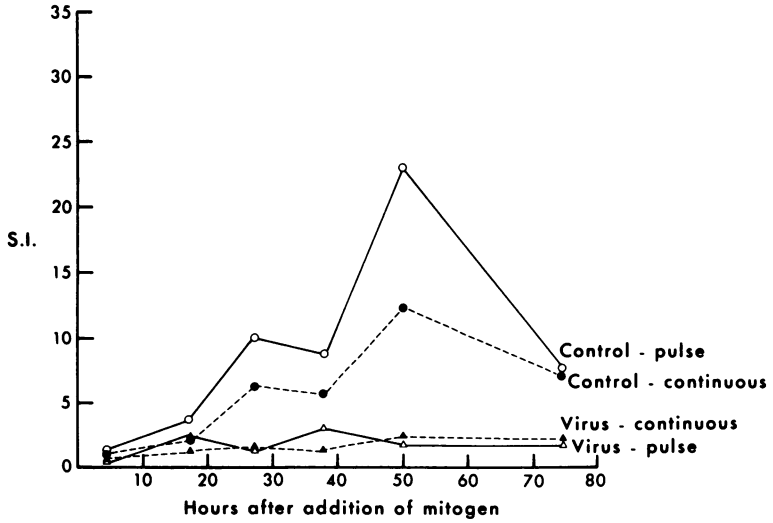

FIG. 2. Response to LPS of spleen cells at 4 days postinoculation. Experimental protocol identical to that given in Fig. 1, except that ConA was replaced by 40 μg of LPS per ml.

the response to ConA could be produced by as few as 13 IC per 10^6 cells, or approximately 200 IC per spleen. However this was only true at 3 days postinoculation, since at 4 and 5 days postinoculation in the same group of mice, complete abrogation of the response to ConA and LPS was obtained with the lowest virus dose (1.5×10^5 PFU), which gave rise to approximately 100 IC per spleen. Thus the degree of immunosuppression is proportional to both the number of infected spleen cells and to the duration of the infection up to 5 days postinoculation.

Type of cell responsible for immunosuppression. Since the majority of the infected spleen cells appear to be macrophages (20), then if these cells were responsible for causing the immunosuppression, their removal should allow restoration of the spleen responses to mitogens. Preliminary experiments indicated that this was the case.

This experimental approach was utilized for a more detailed analysis of the roles of adherent and nonadherent cells. In Fig. 4 are the data showing the relative responses to ConA and to LPS of spleen cells from uninfected mice and from mice at 4 days after one of three different virus inocula. Responses were measured on unfractionated cells, which gave the usual depressed values, and on the nonadherent populations left after removal of plastic-adherent cells. In all cases, including cultures not exposed to any mitogen, removal of the bulk of adherent cells allowed the nonadherent cells to respond well to ConA and LPS. Evidently the nonadherent cell populations of infected spleens contained sufficient residual macrophages or monocytes to permit a healthy response to take place. In additional experiments it was shown that the original state of stimulation or suppression could be partly restored by readdition of the appropriate adherent cells.

Thus it appears that a component of the adherent cell population, when infected with MCMV, is inhibitory to mitogen responses. However at very high virus doses (1.5×10^7

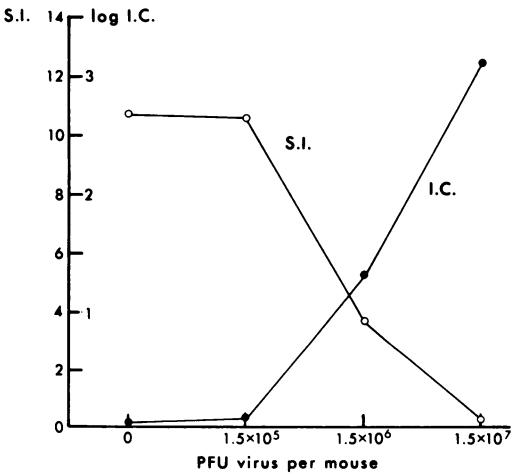

FIG. 3. Correlation between virus dose, infectious centers, and suppression of ConA response. Groups of mice were inoculated with the indicated doses of MCMV. At 3 days postinoculation spleens were removed and assayed for IC and stimulation index (S.I., as defined in the legend to Fig. 1).

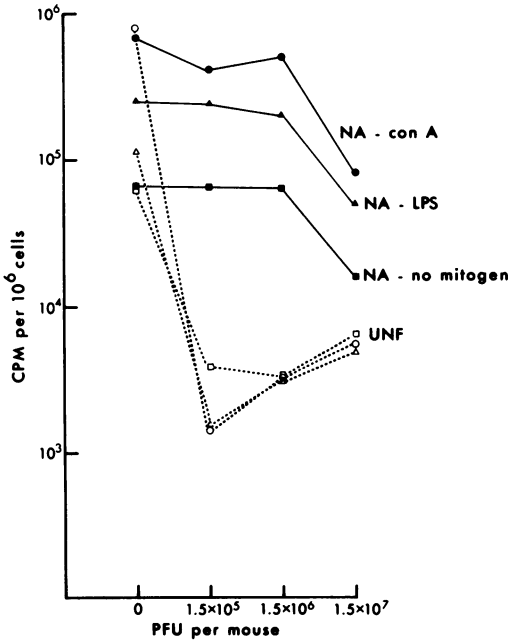

FIG. 4. Mitogen responses in spleen cells before and after removal of adherent cells. Spleens were removed from different groups of MCMV-infected and uninfected mice at 4 days postinoculation. Responses to ConA, LPS, and no mitogen were measured by [^{3}H]thymidine uptake between 24 and 36 h after mitogen addition. UNF (unfractionated), Spleen cells before removal of adherent cells; NA, nonadherent cells, i.e., cells which did not adhere to plastic tissue culture dishes in 2 h at 37°C; (●, ○), ConA added; (▲, △), LPS added; (■, □), no mitogen added.

PFU per mouse) the nonadherent population is still markedly inhibited (Fig. 4). This may be due to the larger number of infected macrophages and the consequent larger number of them remaining in the nonadherent population, or it may reflect an additional effect on the lymphocytes themselves.

To distinguish between effects of the virus on macrophages and on lymphocytes, cell mixing experiments were performed. For this purpose spleens from uninfected and infected mice at various times after infection were fractionated into adherent and nonadherent cell populations, and the four possible permutations were reconstituted for measurement of responses to ConA and LPS. The results were calculated and expressed as a ratio of counts per minute of virus-infected cells/counts per minute of uninfected cells relative to the value for the reconstituted mixture of uninfected adherent cells and uninfected nonadherent cells (Fig. 5). The greatest degree of suppression was always observed in the doubly infected cell mixture, i.e., infected adherent cells and infected nonadherent cells. The presence of only one of the infected cell populations was much less inhibitory, although suppression was always observed in the 3- to 9-day infected mixtures.

After 10 days of infection, responses to both mitogens were restored to normal levels, and there were no significant differences between the various reconstituted mixtures.

Binding and presentation of ConA. Since the

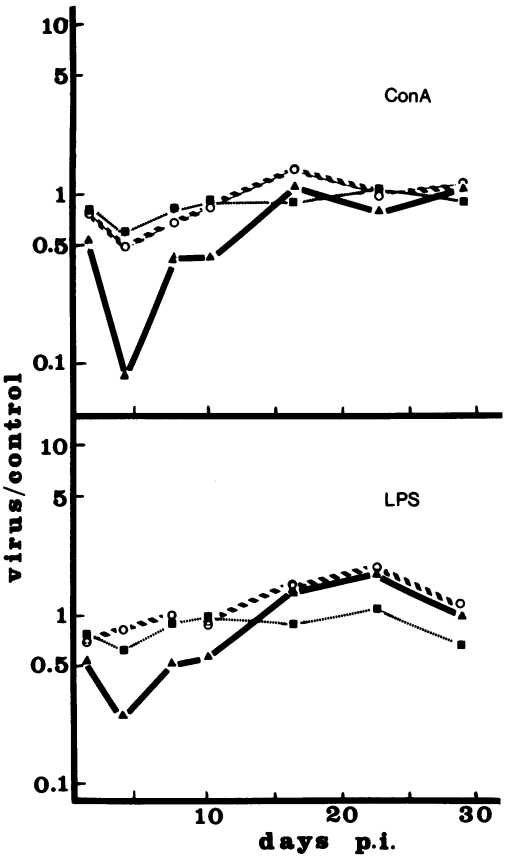

FIG. 5. Mitogen responses of reconstituted spleen cultures. A group of mice were inoculated with 10^6 PFU of MCMV per mouse or mock infected with phosphate-buffered saline. At various times after infection three infected and three uninfected spleens were removed. Both groups of spleens were fractionated into adherent and nonadherent populations of cells, which were then reconstituted to give the four possible permutations. Mitogen responses to ConA and LPS were measured in the usual way, and the results were calculated as counts per minute relative to the counts per minute in the culture containing uninfected adherent cells and uninfected nonadherent cells. The latter mixture is referred to as the control in the ordinate axis. Symbols: ▲, infected adherent cells plus infected nonadherent cells; ■, uninfected adherent cells plus infected nonadherent cells; ○, infected adherent cells plus uninfected nonadherent cells.

T-cell response to ConA requires that the macrophages bind and "present" the mitogen to the lymphocytes, then a defect in this property of infected macrophages could explain the refractoriness of the response. This possibility was tested by incubating spleen cells, from 4-day-infected and uninfected mice, with various concentrations of ConA. Unbound ConA and nonadherent cells were removed by thorough washing. Cell mixtures were then made by adding infected or uninfected nonadherent cells which had not been exposed to ConA. Tritiated thymidine uptake was measured as usual. In addition parallel cultures were made in which the spleen cells had been incubated in 0.1 M α-methyl mannoside with ConA. As expected these latter cultures were not stimulated by ConA, except for the uninfected cells in 100 μg of ConA per ml.

The results are summarized in Fig. 6. The uninfected adherent cells were able to bind and present sufficient ConA for mitogen response, after the 2-h incubation with 2.5 μg/ml, although maximal response required an initial concentration of 50 μg/ml. The infected adherent cells were evidently deficient in binding or presentation (or both) at low concentrations of ConA (2.5 to 10.0 μg/ml), but this defect was easily overcome by increasing the ConA concentration. In contrast infected nonadherent cells were unable to respond to the ConA presented to them by either infected or uninfected adherent cells.

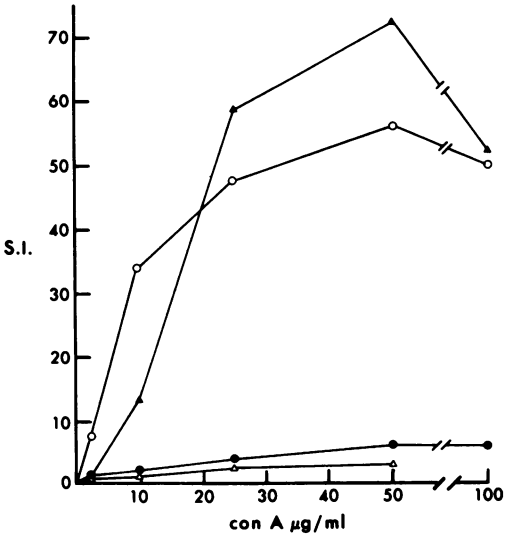

FIG. 6. Attempts to stimulate lymphocytes by ConA bound to infected and uninfected adherent cells. Spleens were removed from infected and uninfected mice at 4 days after inoculation with 1.5×10^6 PFU of MCMV per mouse and were fractionated into adherent and nonadherent cell populations. Replicate cultures of adherent cells were incubated with the indicated concentration of ConA for 2 h at 37°C and were then washed thoroughly with medium to remove unbound ConA. The nonadherent cells were then added to give the four possible permutations. Parallel cultures without ConA were treated identically. Tritiated thymidine was added at 40 h, and the cells were harvested at 52 h after ConA treatment. S.I., stimulation index, defined as in the legend to Fig. 1. Symbols: ○, uninfected ConA-bound adherent cells plus uninfected nonadherent cells; ▲, infected ConA-bound adherent cells plus uninfected nonadherent cells; ●, uninfected ConA-bound adherent cells plus infected nonadherent cells; △, infected ConA-bound adherent cells plus infected nonadherent cells.

DISCUSSION

MCMV, like many other viruses, causes a period of immunosuppression in mice. It appears, from the published data on various systems of study, that different viruses can bring about suppression in different ways, although in no case has a detailed account of the mechanism been proposed.

In theory a virus could produce immunosuppression either by a direct effect upon the lymphocytes themselves or by an indirect effect upon another cell type capable of influencing the properties of lymphocytes. The first type of mechanism probably does not apply to MCMV, since this virus has very little tendency to infect lymphocytes in vivo or in vitro, at least in SWR/J mice (15, 19). Conceivably such a mechanism could operate in other strains of mice which are more susceptible to the virus. Mere contact of MCMV with lymphocytes is insufficient since the suppression is known to require infectious virus (20).

An indirect effect could be due to (i) a change in numbers or function of a cell involved in lymphocyte responses, such as macrophages, or (ii) augmentation of suppressor cell function. The latter would require a nonspecific effect on different types of suppressor cells, in view of the widespread nature of the immune responses affected by MCMV. At present there is no evidence for this.

Cells in the monocyte-macrophage series constitute good candidates for MCMV targets, since these cells are relatively easily infected by the virus. However, it has not been possible to identify with certainty the infected spleen cells as macrophages, since the proportion of infected cells is always low, and it remains possible that the relevant infected cell is a nonmacrophage type of cell with similar adherence properties. Further experiments need to be done in this area of study. In this context it is interesting that recent work by Bixler and Booss (4) has implicated an adherent cell in the MCMV-induced suppression of antibody-forming cells, and the human cytomegalovirus may suppress immune responses by similar mechanisms (7).

The suppression could result from contact between the infected cell and neighboring lymphocytes in such a way that the effector cell is inhibitory rather than stimulatory. Alternatively the infected cell could produce a factor which inhibits the normal responsiveness of lymphocytes.

The results described in this study support the latter explanation. Thus the data indicate that the suppression cannot be overcome by prolonged exposure of the spleen cells to mitogen or by exposure to higher concentrations of mitogen. But simple removal of the majority of the infected adherent cells restores partly the response to mitogens, and readdition of these cells results again in a suppression of the response. Several other observations argue in favor of the hypothesis that an immunosuppressive factor is involved, rather than cell-to-cell contact. First, the presence of very few infectious centers (100 per spleen) can bring about complete suppression of mitogen responses in vivo. It seems unlikely that contact between this small number of infected cells and the excessive number of lymphocytes affected is possible. At this time, however, we cannot rule out the possible contribution from nonproductively infected cells. Second, the suppression is difficult to demonstrate in cultures with fewer than 5×10^6 cells per ml. Third, the incomplete restoration of suppression in the cell mixing experiments (Fig. 4 and 5) which utilized washed cell populations is more compatible with the concept of a factor secreted into the medium. Fourth, the lymphocytes from 4-day-infected spleens are clearly defective in the response to mitogens even in the presence of uninfected adherent cells. This suggests that they have become refractory to mitogens because of their exposure to a factor in the spleens. Furthermore, recent experiments in our laboratory have demonstrated the presence of a suppressive factor in the supernatants of spleen cultures, infected in vitro and in vivo (Whyte and Hudson, unpublished results). Further characterization of this factor is in progress.

Other reports have provided evidence for suppressive factors during virus infections (8, 16). It will be of interest to determine whether such a factor is specifically induced by a given virus, or whether the suppression is simply due to the nonspecific secretion of immunosuppressive substances by macrophages which are activated by the virus (1).

ACKNOWLEDGMENT

Financial support was obtained from the Medical Research Council, Ottawa.

LITERATURE CITED

1. **Allison, A. C.** 1978. Mechanisms by which activated macrophages inhibit lymphocyte responses. Immunol. Rev. **40**:3–27.
2. **Araullo-Cruz, T. P., M. Ho, and J. A. Armstrong.** 1978. Protective effect of early serum from mice after cytomegalovirus infection. Infect. Immun. **21**:840–842.
3. **Bixler, G. S., and J. Booss.** 1980. Establishment of immunologic memory concurrent with suppression of the primary immune response during acute cytomegalovirus infection of mice. J. Immunol. **125**:893–896.
4. **Bixler, G. S., and J. Booss.** 1981. Adherent spleen cells from mice acutely infected with cytomegalovirus suppress the primary antibody response in vitro. J. Immunol. **127**:1294–1299.
5. **Booss, J., and E. F. Wheelock.** 1975. Correlation of survival from murine cytomegalovirus infection with spleen cell responsiveness to concanavalin A. Proc. Soc. Exp. Biol. Med. **149**:443–446.
6. **Booss, J., and E. F. Wheelock.** 1977. Progressive inhibition of T-cell function preceding clinical signs of cytomegalovirus infection in mice. J. Infect. Dis. **135**:478–481.
7. **Carney, W. P., and M. S. Hirsch.** 1981. Mechanisms of immunosuppression in cytomegalovirus mononucleosis. II. Virus-monocyte interactions. J. Infect. Dis. **144**:47–54.
8. **Chaturvedi, U. C., M. I. Shukla, and A. Mathur.** 1981. Thymus-dependent lymphocytes of dengue virus-infected mice spleens mediate suppression through prostaglandin. Immunology **42**:1–6.
9. **Hamilton, J., and J. C. Overall.** 1978. Synergistic infection with murine cytomegalovirus and *Pseudomonas aeruginosa* in mice. J. Infect. Dis. **13**:775–782.
10. **Ho, M.** 1980. Role of specific cytotoxic lymphocytes in cellular immunity against murine cytomegalovirus. Infect. Immun. **27**:767–776.
11. **Howard, R. J., D. M. Mattsson, M. V. Seidel, and H. H. Balfour, Jr.** 1978. Cell-mediated immunity to murine cytomegalovirus. J. Infect. Dis. **138**:597–604.
12. **Howard, R. J., J. Miller, and J. S. Najarian.** 1974. Cytomegalovirus-induced immune suppression. II. Cell-mediated immunity. Clin. Exp. Immunol. **18**:119–126.
13. **Howard, R. J., and J. S. Najarian.** 1974. Cytomegalovirus-induced immune suppression. I. Humoral immunity. Clin. Exp. Immunol. **18**:109–118.
14. **Hudson, J. B.** 1979. The murine cytomegalovirus as a model for the study of viral pathogenesis and persistent infections. Arch. Virol. **62**:1–29.
15. **Hudson, J. B., L. Loh, V. Misra, B. Judd, and J. Suzuki.** 1978. Multiple interactions between murine cytomegalovirus and lymphoid cells in vitro. J. Gen. Virol. **38**:149–159.
16. **Israel, E., B. Beiss, and M. A. Weinberg.** 1980. Viral abrogation of lymphocyte mitogenesis: induction of a soluble factor inhibitory to cellular proliferation. Immunology **40**:77–85.
17. **Kelsey, D. K., G. A. Olsen, J. C. Overall, Jr., and L. A. Glasgow.** 1977. Alteration of host defense mechanisms by murine cytomegalovirus infection. Infect. Immun. **18**:754–760.
18. **Kelsey, D. K., J. C. Overall, Jr., and L. A. Glasgow.** 1978. Correlation of the suppression of mitogen responsiveness and the mixed lymphocyte reaction with the proliferative response to viral antigen of splenic lymphocytes from cytomegalovirus-infected mice. J. Immunol. **121**:464–470.
19. **Loh, L., and J. B. Hudson.** 1979. Interaction of murine cytomegalovirus with separated populations of spleen cells. Infect. Immun. **26**:853–860.
20. **Loh, L., and J. B. Hudson.** 1980. Immunosuppressive effect of murine cytomegalovirus. Infect. Immun. **27**:54–60.
21. **Loh, L., and J. B. Hudson.** 1981. Murine cytomegalovirus infection in the spleen and its relationship to immunosuppression. Infect. Immun. **32**:1067–1072.
22. **Manischewitz, J. E., and G. V. Quinnan.** 1980. Antivirus antibody-dependent cell mediated cytotoxicity during immune cytomegalovirus infection. Infect. Immun. **29**:1050–1054.
23. **Mosmann, T. R., and J. B. Hudson.** 1973. Some properties of the genome of murine cytomegalovirus. Virology

54:135–149.
24. **Osborn, J.** 1982. CMV-herpesvirus of mice. *In* The mouse in biomedical research (in press). Academic Press, Inc., New York.
25. **Osborn, J. E., A. A. Blazkovec, and D. L. Walker.** 1968. Immunosuppression during acute murine cytomegalovirus infection. J. Immunol. **100**:835–844.
26. **Quinnan, G. V., J. E. Manischewitz, and F. A. Ennis.** 1978. Cytotoxic T-lymphocyte response to murine cytomegalovirus infection. Nature (London) **273**:541–543.
27. **Rouse, B. T., and L. A. Babiuk.** 1979. Mechanisms of viral immunopathology. Adv. Vet. Sci. Comp. Med. **23**:103–136.
28. **Selgrade, M. K., A. Ahmed, K. W. Sell, M. E. Gershwin, and A. D. Steinberg.** 1976. Effect of murine cytomegalovirus on the in vitro responses of T and B cells to mitogens. J. Immunol. **116**:1459–1465.
29. **Virelizier, J. L.** 1975. Mechanisms of immunodepression induced by viruses: possible role of infected macrophages. Biomedicine **22**:255–261.
30. **Woodruff, J. F., and J. J. Woodruff.** 1975. The effect of viral infections on the function of the immune system, p. 393–418. *In* A. L. Notkins (ed.), Viral immunology and immunopathology. Academic Press, Inc., New York.