INFECTION AND IMMUNITY, Aug. 1977, p. 378-381

Vol. 17, No. 2
Printed in U.S.A.

Role of Viremia in the Suppression of T-Cell Function During Murine Cytomegalovirus Infection

J. BOOSS[1]* AND E. F. WHEELOCK

Department of Microbiology, Thomas Jefferson University, Philadelphia, Pennsylvania 19107

Received for publication 8 February 1977

The suppression of T-cell deoxyribonucleic acid (DNA) synthesis by serum from mice acutely infected with murine cytomegalovirus (MCMV) was investigated. Spleen cells from uninfected mice were exposed to concanavalin A in the presence of serum taken from mice at various times after infection with MCMV. The capacity of the serum to suppress DNA synthesis first appeared at day 3 postinfection and was associated with free infectious virus. Addition of MCMV to serum from uninfected mice also suppressed DNA synthesis. Ultracentrifugation of serum from mice acutely infected with MCMV removed most of the virus and abrogated the inhibition of DNA synthesis. However, in two of four experiments, serum from mice in weeks 4 and 5 postinfection did not contain infectious MCMV but did suppress. Therefore, it appears that MCMV itself can suppress DNA synthesis of T cells; however, this may not be the exclusive mechanism of suppression exerted by serum from MCMV-infected mice.

Acute murine cytomegalovirus (MCMV) infection impairs T-cell function, as shown by delayed rejection of skin grafts (7) and decreased in vitro response to T-cell mitogens by spleen cells from infected mice (1, 2, 7, 12). The impairment of the response of spleen cells to the T-cell mitogen, concanavalin A (ConA), precedes clinical signs of infection in infected DBA/2 mice, suggesting that depression of T-cell function is crucial in the pathogenesis of MCMV-induced disease. It has also been noted that serum from DBA/2 mice acutely infected with MCMV suppressed the in vitro incorporation of [^{3}H]thymidine (TdR) by resting and ConA-stimulated spleen cells of uninfected mice (2). We now report that the suppressive activity of this serum first occurs in the presence of viremia, is not associated with a stable circulating lymphokine, and can be reproduced by addition of MCMV to serum from uninfected mice. In addition, serum from DBA/2 mice in week 4 or 5 after infection that does not contain detectable infectious MCMV can exert a suppressive effect.

MATERIALS AND METHODS

Virus. Mouse-passed MCMV stock, free of lactic dehydrogenase virus, was prepared as a 10% (wt/vol) salivary gland suspension and stored at −70°C with 10% dimethyl sulfoxide as previously described (1). Virus in 0.2 ml of RPMI 1640 medium was inoculated into experimental mice intraperitoneally.

Mice. Adult DBA/2 female mice, 7 weeks of age, were obtained from the Jackson Laboratories, Bar Harbor, Maine. They were used for experimental purposes within 3 weeks of delivery.

Virus titration. Mouse embryo tissue culture was prepared from CD-1 embryos in week 3 of gestation and was used in passages 1 and 2 for MCMV titration. Serum, resuspended pellets, and supernatants of ultracentrifuged serum were assayed for virus content as previously described (2). Briefly, serial dilutions of 0.1 ml were assayed in quadruplicate in 96-well plates, using tragacanth as the overlay (10). The plates were incubated in an atmosphere containing 5% CO_2 at 37°C for 4 days and then were fixed and stained with naphthalene black for counting.

ConA assay. The ConA assay was performed as previously described (2) except that the spleen cells were dispersed with a Dounce tissue homogenizer. Cell yield and percent viability were equivalent to those obtained with the former technique of passage through a fine-mesh screen. A total of 2×10^6 spleen cells from uninfected control mice in the presence of 2% mouse serum were used in all assays reported here. The effect of 2% serum from acutely infected mice or from mice that had recovered from acute MCMV infection was compared with the effect of 2% serum from uninfected control mice.

Manipulation of serum. (i) Addition of MCMV. Fresh mouse serum was collected from uninfected donors, pooled, and mixed with MCMV and used in the ConA assay.

(ii) Ultracentrifugation. Initially, freshly collected, pooled mouse serum was layered over a 25% sucrose cushion and ultracentifuged for 2 h at 100,000 × *g*. However, variable results were ob-

[1] Present address: Neurology Service and Virology Laboratory, Veterans Administration Hospital, West Haven, CT 06516,* and Departments of Neurology and Laboratory Medicine, Yale University School of Medicine, New Haven, CT 06510.

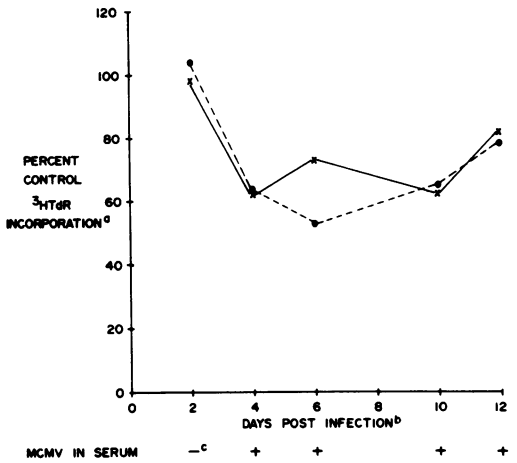

FIG. 1. *Suppression of [³H]TdR incorporation into uninfected spleen cells by pooled serum from mice acutely infected with MCMV. Footnotes: (a) 100% = [³H]TdR incorporation by 2 × 10⁶ uninfected spleen cells in the presence (——) or absence (- - -) of ConA. Five replicate samples of a pool of four to nine mice were assayed at each point. (b) 6.9 × 10⁴ PFU in 0.2 ml intraperitoneally. (c) Virus isolated from all unmanipulated serum samples except on day 2 when virus was only isolated from the resuspended pellet after ultracentrifugation (100,000 × g for 2 h).*

tained when this serum was used in the ConA assay. In particular, the effect of the supernatant of the normal mouse serum was frequently altered in comparison to the effect of uncentrifuged serum. Subsequently, the serum was ultracentrifuged without the sucrose cushion in 1.3- by 5.1-cm cellulose nitrate tubes with a Beckman SW50.1 rotor at 37.5 × 10^3 rpm for 2 h. The upper portion of the serum sample was then removed for experimental purposes. On two of three occasions, the effect of centrifuged, uninfected normal mouse serum on ConA stimulation was unchanged. In all experiments in which serum was ultracentrifuged, the supernatant as well as the pellet of the experimental groups was assayed for virus content. The pellet was resuspended to the original volume in RPMI 1640 for assay.

RESULTS

Suppressive effect of serum from mice acutely infected with MCMV on the in vitro response of spleen cells to ConA. The kinetics of suppression of the incorporation of [³H]TdR into normal uninfected spleen cells at rest and exposed to ConA by pooled serum from adult DBA/2 female mice acutely infected with 6.9 × 10^4 plaque-forming units (PFU) of MCMV is shown in Fig. 1. Suppressive effects were observed in serum 4 days postinfection and throughout the subsequent 8-day period of this study. Virus was detected in the serum on each of these days. On day 2 postinfection, when no serum suppressive effect was present, virus could only be isolated after ultracentrifugation and resuspension of the pellet. This may represent a virus dose dependence of the suppression. Clinical illness (huddling, ruffled fur, and decreased activity) was apparent in 100% and deaths occurred in 27% of the mice. In a confirmatory experiment, virus and the suppressive effect were found in the serum by day 3 postinfection.

Suppressive effect of MCMV added to normal mouse serum. Since serum from acutely infected mice that suppressed the response to ConA contained virus, the effect of addition of MCMV to serum from uninfected mice was studied. Addition of 680 PFU of MCMV to each culture resulted in reduced incorporation of [³H]TdR into the ConA-exposed normal spleen cells (Table 1).

The virus preparation used above was a clarified supernatant of centrifuged, disrupted, infected salivary glands and therefore could have contained soluble factors that were toxic for the spleen cell culture. The experiment was therefore performed using virus that had been partially purified by pelleting from a clarified suspension through a 25% sucrose cushion at 100,000 × *g*. Suppression of the stimulated [³H]TdR incorporation was produced by this partially purified virus preparation.

Ultracentrifugation of serum from mice infected 4 days previously with MCMV. In an attempt to isolate a serum mediator of T-cell suppression, virus was pelleted from pooled serum from infected mice by ultracentrifugation under conditions in which a soluble mediator such as interferon would not be sedimented (3). The serum was centrifuged at 100,000 × *g* for 2 h, and the upper portion of the spun serum was removed for use without disturbing the pellet. In two of three experiments, the response of spleen cells to ConA in the presence of ultracentrifuged serum from infected mice was in-

TABLE 1. *Effect of addition of MCMV to normal serum on ConA stimulation of normal spleen cells*

PFU of MCMV added to normal serum[a]	[³H]TdR incorporation into normal spleen cells (cpm ± SD, ×10⁻³)[b]		Stimulation index[c]
	Spontaneous	ConA exposed	
0	15.8 ± 1.7	222.3 ± 6.3[d]	14.1
680	11.2 ± 0.7	157.8 ± 21.1[d]	14.1

[a] Pooled serum from four uninfected mice.

[b] Five replicate samples. SD, Standard deviations.

[c] Spontaneous counts per minute divided into ConA-exposed counts per minute.

[d] $t = 5.86$; $P < 0.001$ by Student's t test.

TABLE 2. *Effect of ultracentrifugation of serum of infected mice on the ConA response of normal spleen cells*

Serum[a]	[3H]TdR incorporation into normal spleen cells (cpm ± SD, ×10⁻³)[b]		Stimulation[c] index	PFU/ml
	Spontaneous	ConA exposed		
NMS	6.6 ± 1.1	93.3 ± 5.5	14.2	
NMS supernatant	5.1 ± 0.5	97.1 ± 15.7	18.9	
IMS	5.0 ± 0.5	17.7 ± 2.2[d]	3.5	1.1×10^3
IMS supernatant	4.6 ± 0.7	119.2 ± 17.4[d]	26.0	0.25×10^0

[a] Pools of serum from eight uninfected and fourteen infected mice. NMS, Normal mouse serum; IMS, infected mouse serum collected 4 days postinfection with 5.6×10^5 PFU of MCMV; supernatant, top portion of serum after ultracentrifugation at 100,000 × *g* for 2 h.

[b] Five replicate samples. SD, Standard deviation.

[c] Spontaneous counts per minute divided into ConA-exposed counts per minute.

[d] $t = 11.58$; $P < 0.001$ by Student's *t* test.

creased. In those experiments, there was no effect of ultracentrifugation on the control uninfected serum on ConA stimulation. One experiment is shown in Table 2. Over 99% of the infectious MCMV was removed, and a suppressor of T-cell function could not be demonstrated in the supernatant of the centrifuged serum.

Serum collected from mice in weeks 4 and 5 after infection. In an attempt to demonstrate the suppressive effect of serum on T cells in the absence of infectious virus, mice that had recovered from an infection with MCMV were studied. In two of four experiments, pooled serum of mice in weeks 4 and 5 postinfection did not contain infectious virus but did have suppressive activity in comparison to pooled serum of uninfected mice of the same shipment (Table 3).

DISCUSSION

Spleen cells from mice infected with MCMV are as viable as uninfected controls but have a decreased response to ConA (1). We recently reported that serum from mice acutely infected with MCMV suppresses the incorporation of [3H]TdR into resting and ConA-exposed normal spleen cells (2). The experiments reported here demonstrated that the suppressive effect of the serum is not found until infectious virus is present and may require a certain minimal amount of virus. In addition, MCMV added to normal serum suppresses the response of normal spleen cells to ConA. Therefore, the viremia itself could be responsible for suppression of [3H]TdR incorporation into normal spleen cells. Selgrade et al. have reported that high but not low doses of virulent MCMV added to spleen cells from uninfected BALB/c mice suppressed the response to ConA (12).

Further evidence that the suppressive effect of serum from acutely infected mice was due to virus comes from experiments in which ultracentrifugation of this serum at 100,000 × *g* significantly reduced the virus content and also abrogated the suppression of [3H]TdR incorporation. Therefore, it is unlikely that suppression results from a stable circulating mediator such as interferon or other lymphokine (13). Although our experiments have not examined the possible in vitro induction of a suppressive factor, Selgrade et al. found no evidence for the in vitro production of such a factor by spleen cells (12).

Suppressive activity in the absence of free infectious virus was demonstrated in the serum of mice in weeks 4 and 5 after an MCMV infection. The mechanism of this suppression remains to be examined. Competition by increased levels of circulating TdR is a possibility, although it is not supported by the somewhat elevated spontaneous [3H]TdR incorporation in the presence of serum from infected mice in the experiment shown (Table 3). Since the salivary glands remain productively infected after the appearance of antibody (9), one possible mechanism is the action of circulating vi-

TABLE 3. *Effect of serum from mice in week 4 after MCMV infection on ConA stimulation of normal spleen cells*

Mice from which serum[a] was taken	[3H]TdR incorporation into normal spleen cells (cpm ± SD, ×10⁻³)[b]		Stimulation index[c]	PFU/ml of serum
	Spontaneous	ConA exposed		
Uninfected	5.5 ± 0.9	180.2 ± 22.8[d]	32.5	
Infected[e]	7.2 ± 1.7	78.7 ± 4.0[d]	10.9	0

[a] Pooled serum from four uninfected and six infected mice.

[b] Five replicate samples. SD, Standard deviation.

[c] Spontaneous counts per minute divided into ConA-exposed counts per minute.

[d] $t = 8.77$; $P < 0.001$ by Student's *t* test.

[e] 6.9×10^4 PFU of MCMV 24 days before sacrifice.

rus-antibody complexes (5). Rubella virus-antibody complexes are capable of reducing the lymphocyte response to phytohemagglutinin (8). Further, circulating virus-antibody complexes might induce interferon (4), which in turn could inhibit cell division (6). Finally, it is possible that virus-antibody complexes become dissociated in vitro, releasing MCMV.

There are several molecular mechanisms by which a nonlytic MCMV infection could directly impair T-cell function. Attachment of complete virus, defective virus, virus membrane fragments, or neutralized virus could interfere with ConA receptors. MCMV infection might also alter the intrinsic membrane structure. Further, virus components or virus-coded products could inhibit host cell deoxyribonucleic acid synthesis. Moon et al. have recently found that MCMV can suppress host fibroblast deoxyribonucleic acid synthesis even if the virus is inactivated and no virus replication occurs (11). Our findings support a direct role of MCMV in the suppression of T-cell function during the viremic stage of infection.

ACKNOWLEDGMENTS

We thank Saidee Thompson for expert technical assistance, V. Mercadante for manuscript preparation, and Emanuel Lerner for statistical assistance.

This work was supported by Public Health Service research grant R01 AI 12853-01 from the National Institute of Allergy and Infectious Diseases.

John Booss was the recipient of a Public Health Service Special Research Fellowship from the National Institute of Neurological and Communicative Disorders and Stroke.

LITERATURE CITED

1. **Booss, J., and E. F. Wheelock.** 1975. Correlation of survival from murine cytomegalovirus infection with spleen cell responsivenes to Concanavallin A. Proc. Soc. Exp. Biol. Med. 149:443-446.
2. **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.
3. **Fantes, K. H.** 1973. Purification and physico-chemical properties of interferons, p. 171-200. *In* N. B. Finter (ed.), Interferons and interferon inducers. North Holland Publishing Co., American Elsevier Publishing Co., Inc., New York.
4. **Fujibayashi, T., J. J. Hooks, and A. L. Notkins.** 1975. Production of interferon by immune lymphocytes exposed to herpes simplex virus-antibody complexes. J. Immunol. 115:1191-1193.
5. **Gorczynski, R., S. Kontiainen, N. A. Mitchison, and R. E. Tigelaar.** 1974. Antigen-antibody complexes as blocking factors on the T lymphocyte surface, p. 143-154. *In* G. M. Edelman (ed.), Cellular selection and regulation in the immune response. Raven Press, New York.
6. **Gresser, I., M.-T. Bandu, M. Tovey, G. Bodo, K. Pauker, and W. Stewart.** 1973. Interferon and cell division. VII. Inhibitory effect of highly purified interferon preparations on the multiplication of leukemia L1210 cells. Proc. Soc. Exp. Biol. Med. 142:7-10.
7. **Howard, R. J., J. Miller, and J. S. Najarian.** 1974. Cytomegalovirus induced immune suppression. II. Cell mediated immunity. Clin. Exp. Immunol. 18:119-126.
8. **Lee, J. C., and M. M. Sigel.** 1974. A differential effect of IgM and IgG antibodies on the blastogenic response of lymphocytes to Rubella virus. Cell. Immunol. 13:22-31.
9. **Medearis, D. N., Jr.** 1964. Mouse cytomegalovirus infection. II. Observations during prolonged infections. Am. J. Hyg. 80:103-112.
10. **Mirchamsky, H., and F. Rapp.** 1968. A new overlay for plaquing animal viruses. Proc. Soc. Exp. Biol. Med. 129:13-17.
11. **Moon, H. M., V. J. Sapienza, R. I. Carp., and K. S. Kim.** 1976. DNA synthesis in mouse embryo fibroblast cells infected with murine cytomegalovirus. Virology 75:376-383.
12. **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.
13. **Waksman, B. H., and Y. Namba.** 1976. On soluble mediators of immunologic regulation. Cell. Immunol. 21:161-176.