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Characteristics of Infection of B and T Lymphocytes from Mice After Inoculation with Cytomegalovirus

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Viremia was produced by inoculating intravenously BALB/c mice with murine cytomegalovirus. Virus was detected in plasma and granulocytes only during the first 8 days after infection. Lymphocyte-associated viremia, detectable by cocultivation on syngeneic or allogeneic fibroblasts, persisted for at least 4 weeks. Eight to 10 days after infection, sonicated lymphocytes had no demonstrable free virus. When whole lymphocytes with no demonstrable free virus were enclosed in a Millipore chamber and placed on a fibroblastic feeder layer, T cells produced free virus but B cells did not. Compared to normal calf serum, specific hyperimmune mouse serum reduced B cell-associated infectious centers by 74% and T cell-associated infectious centers by only 38%. Normal mouse sera reduced by 36% and 30% infectious center production by B cells and T cells, respectively. Lymphocytes enriched with Fc receptor-positive cells produced significantly more infectious centers than receptor-negative cells.

After mice are infected with sublethal doses of murine cytomegalovirus (MCMV), the virus has been found to persist in leukocytes (4, 9, 21). The precise status of the virus in these cells is not well defined. It is not known whether such infection represents a persistent, remitting lytic infection or a truly latent infection. The distinction is important to make, although it should be recognized that in the absence of a rigorous virological definition of latency with respect to MCMV, an unambiguous distinction may be impossible. Operationally, we and others have defined latency in a negative sense, i.e., an infection in which no lytic virus can be found prior to activation (11, 17).

A model of latent infection was described by Olding et al. (21), who infected mice in utero or neonatally with cell culture-attenuated MCMV. After maturing, survivors showed no apparent lytic virus in their organs, but B lymphocytes of the spleen released virus when cocultivated on allogeneic, but not syngeneic, mouse fibroblasts. Cheung and Lang (4) transfused whole blood from infected mice that were free of apparent virus to allogeneic recipients, and virus was later recovered from the recipients' salivary glands. Both Mayo et al. (17) and Jordan et al. (11) obtained latent infection in mice by waiting up to 4 months after infection. These infections were "activated," as evidenced by production of free virus in salivary glands, after the mice were treated with various immunosuppressants. Mayo et al. (17) also found that spleen cells from such latently infected mice were activated if transferred to syngeneic recipients or immunosuppressed allogeneic recipients, or if they were cocultivated on either syngeneic or allogeneic fibroblasts.

The present work began with the assumption that study of infection of blood cells may reveal the nature of viremia by MCMV and possibly yield further information on viral latency. Such blood cells were obtained from infected animals rather than from in vitro infection because the latter may not encompass the full range of cell-virus relationships.

At present, the cell-virus relationships in early and later phases of infection are unclear. There is no information available on whether MCMV interacts differently with different populations of leukocytes. The statement has been made that B lymphocytes are latently infected, but this has not been confirmed, and the relationship of MCMV to T lymphocytes is unclear. This study does show differences in infection of B and T lymphocytes which may be relevant to both viral latency and the immunosuppression of the host after cytomegalovirus infection.

MATERIALS AND METHODS

Mice. Inbred BALB/c mice of either sex, 8 to 12 weeks of age, and pregnant outbred Swiss-Webster (CD-1) mice were obtained from Charles River Mouse Farm, New Wilmington, Mass. Outbred ICR mice were obtained from Hilltop Laboratories, Pittsburgh, Pa. Some inbred BALB/c mice (originally from Charles River) were bred in our laboratory.

Virus. The Smith strain of MCMV was donated by

J. Osborn, University of Wisconsin, Madison. It has been serially passed through CD-1 mouse salivary glands (23). Two virus stocks gave a titer of 6×10^7 to 8×10^7 plaque-forming units (PFU) per ml and 10^7 PFU/ml, respectively.

Cell cultures and media. Secondary cell cultures of 16- to 18-day-old mouse embryos (METC) were prepared from CD-1, ICR, and BALB/c mice by the methods previously described (17). The METC was apportioned into either 6- or 24-well plates (Flow Laboratory, Bethesda, Md., catalog no. FB6TC and 16247C). Medium 199 supplemented with penicillin, streptomycin, 0.2% $NaHCO_3$, and 10% heat-inactivated newborn calf serum (growth medium) was used for initiation of cultures. For maintenance, 1% rather than 10% calf serum was used (maintenance medium). Eagle minimum essential medium supplemented with 0.08% $NaHCO_3$ and 5% heat-inactivated fetal calf serum (MEM) was used for column purification of lymphocytes. All cultures, unless stated otherwise, were maintained at 37°C in a water-saturated atmosphere of 5% CO_2 in air. Purchased media were from GIBCO, Grand Island, N.Y.

The overlay medium for plaque titration of MCMV was tragacanth gum (Nutritional Biochemical Co., Cleveland, Ohio), at a final concentration of 0.8%, in medium 199 plus antibiotics, 0.2% $NaHCO_3$, and 5% newborn calf serum.

Infection assays. (i) Plaque assay. The plaque assay method for MCMV described by Mirchamsy and Rapp (19) was used with modification. Serial 10-fold dilutions of virus were made, and 0.05 ml of each dilution was inoculated into each well of METC in a 24-well plate. After adsorption for 90 min, 2.5 ml of overlay medium was added. The plate was incubated for 7 days. The overlay was then decanted, the plate was stained with about 1.0 ml of a staining solution, and plaques were scored under a dissecting microscope. The staining solution consisted of gentian violet (0.5%), NaCl (0.9%), Formalin (5%), absolute ethyl alcohol (52.6%), and distilled water (41%).

Plasma for virus assay was obtained by centrifuging heparinized cardiac blood at $600 \times g$ for 20 min at 5°C. Sonicated leukocyte lysates were prepared by subjecting chilled cell suspensions to ultrasonic vibrations for 50 s by a Branson Sonifier Cell Disruptor (model W185D) set at 50 W. Less than 0.5% intact cells remained by microscopic examination. Control experiments showed that sonication produced no decrease in titer of a known virus suspension. The amount of heparin used for collecting blood also did not inactivate free MCMV.

(ii) Cocultivation system. Leukocytes obtained by various procedures were adjusted to desired concentrations and inoculated into each well of a 24-well plate containing METC. Inoculated cultures were maintained for 6 weeks. After the first week, the medium was changed twice a week. Cell sheets were patched with freshly prepared mouse embryo cells when needed. Each culture well was examined at least once every other day, and the development of characteristic cytopathic effects (CPE) was recorded. Unless otherwise stated, BALB/c cells were cocultivated on allogeneic (CD-1) fibroblasts.

(iii) Chamber culture system. Chambers were prepared by cementing two membrane filters (type HA, 0.45-μm pore size, catalog no. HAWP01300; Millipore Corp., Bedford, Mass.) on each side of a Millipore chamber ring (PR00001401, Millipore) with MF cement (XX7000000, Millipore). All chambers thus prepared were tested for leakage before use by blowing air into the chamber under water. Blood lymphocytes obtained from animals after infection were injected into the chamber via a hole on the side, which was then sealed. Each chamber was then immobilized in a well of a six-well plate with MF cement. Secondary mouse embryo cells, approximately 2×10^5 cells in 2.5 ml of growth medium, were added to each well so that the chambers were immersed, and the plate was incubated for 1 week. Thereafter, the medium was changed twice a week, and the cell sheet was patched with fresh mouse embryo cells when needed. The culture was examined daily for virus CPE.

(iv) IC assay. A designated number of fibroblasts or lymphocytes infected with MCMV in 0.1 ml was inoculated into a well containing METC of a 24-well plate and incubated. For fibroblasts, the adsorption time was 60 min, and for lymphocytes it was 14 to 16 h. After adsorption, 1 ml of tragacanth medium was overlaid on each well, and the plate was incubated for 5 more days for fibroblasts and 7 days for lymphocytes. The plates were then stained, and the number of plaques were scored as infectious centers (IC).

Neutralization tests. A test serum or fluid was serially diluted twofold in maintenance medium. Stock virus was diluted so as to contain approximately 100 PFU/0.1 ml. A 0.4-ml sample of the virus preparation was added to 0.4 ml of each serum dilution and incubated at room temperature for 60 min. Four replicates of each dilution were inoculated for plaque assay. The reciprocal of the highest dilution that neutralized 50% plaques was taken as the titer of the material.

To test whether spleen cell sonic extracts from mice had any inactivating or neutralizing activity, 10^7 spleen cells in 1.0 ml were mixed with 1.0 ml of virus suspension containing about 1,000 PFU and sonicated. The lysate-virus mixture was incubated at room temperature for 60 min and titrated. The spleen cells were prepared from normal, uninfected animals and from BALB/c mice 1 week after inoculation with 10^5 PFU of MCMV. The virus titer was not affected by spleen cell sonic extracts from either normal or infected mice.

Immunological reagents. (i) Hyperimmune serum. Thirty-five CD-1 mice were infected initially with 2×10^5 PFU in 0.2 ml intraperitoneally. Five weeks postinfection, the animals were challenged with weekly doses of 1.5×10^6 PFU for 7 weeks. Two weeks after the last dose, serum was obtained through cardiac puncture. The neutralizing titer of this serum was 512.

(ii) Guinea pig complement. Guinea pig complement was obtained from Cappell Laboratories, Inc., (Cochranville, Pa.; lot no. 9738). A 1:4 dilution of complement did not inactivate MCMV.

(iii) Normal sera. Normal mouse and rabbit sera were obtained in this laboratory and found to have no anti-MCMV activity in vitro. Newborn calf and fetal calf serum were purchased from GIBCO, Grand Island, N.Y.

(iv) Mitogens. Phytohemagglutinin-P (lot no. K-1954, Wellcome Reagents Ltd., Burroughs Wellcome Co., Research Triangle Park, N.C.) was at a final

concentration of 2 μg/ml in maintenance medium. Lipopolysaccharide (LPS) (*Escherichia coli* O127:B8, lot no. 568787; Difco Laboratories, Detroit, Mich.) was added at a final concentration of 10 μg/ml.

Lymphocyte preparation. The procedures for obtaining enriched T and B lymphocytes was adapted from Julius et al. (12) and Handwerger and Schwartz (8) with modifications. Equal volumes of pooled heparinized cardiac blood and Seligann balanced salt solution were mixed and overlaid carefully on a preformed Ficoll-Hypaque gradient (71:29, vol/vol). After centrifuging at 440 × *g* for 30 min at 20°C, the interphase, enriched with mononuclear leukocytes, and the sediment, rich in granulocytes and erythrocytes, were collected separately and washed twice in MEM. The mononuclear leukocytes were resuspended in warm MEM, and the concentration of leukocytes was adjusted to 2×10^5 to 5×10^5 cells per ml. The cell suspension was quickly passed through a glass-wool column, and the eluted cells were collected and concentrated by one wash. Lymphocyte preparations were contaminated with 1.8% monocytes, as assessed by the latex particle ingestion test (15).

Nylon-wool columns consisted of 1.5 g of washed nylon wool packed into a 10-ml syringe barrel, which had been washed first with 30 ml of Hanks balanced salt solution and then with 30 ml of warm MEM. The column was incubated at 37°C for 1 h before use. After addition of the cell suspension, the column was incubated for another hour. The nonadherent cells were eluted with 35 ml of MEM. This population contained largely T cells, as shown by the fact that 87% cells had surface θ antigen by immunofluorescence, 2.4% cells had surface immunoglobulin G (IgG) by immunofluorescence, and 74% cells were killed by anti-theta serum plus complement as measured by trypan blue exclusion. The adherent B cells were released by pressing the nylon wool. This fraction was 67% positive for surface IgG, and only 2% of cells were lysed by anti-theta serum plus complement.

Preparation of Fc receptor-positive [FcR (+)] cell-enriched lymphoid cell populations. The method described by Berman and Weigle (2) with modification was used. To prepare mouse antiserum against sheep erythrocytes (SRBC), 12- to 16-week-old BALB/c mice were injected weekly with 10^9 washed SRBC in 0.2 ml of phosphate-buffered saline (PBS) for 4 weeks. One week after the last injection, cardiac blood was collected, and serum was obtained. The hemagglutination titer was 1:1,000.

To prepare sensitized SRBC, 2 ml of 5% washed SRBC was mixed with 2 ml of 1:2,000 anti-SRBC antiserum. The cell-serum mixture was incubated at 37°C for 1 h. After two washes with PBS, sensitized SRBC were refrigerated at 5°C until used.

To rosette FcR (+) lymphocytes, lymphocytes enriched by passage through a glass-wool column were incubated at a ratio of 1:100 with sensitized SRBC for 5 min at 37°C and subsequently spun at 140 × *g* for 20 min. The pellet was kept at 5°C for 30 min, gently resuspended, and layered on a Ficoll-Hypaque gradient. The gradient was spun at 440 × *g* for 25 min. The lymphocytes at the interphase and the sediment were collected and washed twice in PBS. The cells were resuspended in 0.5 ml of MEM and submitted to rapid osmotic lysis in 2.0 ml of water to lyse the SRBC. Immediately, hypertonic PBS was added to restore the tonicity, and the suspension was washed twice in PBS before the lymphocytes were counted.

Statistical methods. Differences between the fractions of cultures which were positive for virus after various treatments were tested by χ^2. Correction for continuity was made where appropriate. Differences between the means of various serum treatments on formation of infectious centers were tested by analysis of variance techniques employing standard procedures to deal with unequal subclass numbers and appropriate adjustments for multiple comparisons (7).

RESULTS

Course of viremia. Six- to 8-week-old BALB/c mice were given 10^5 PFU of MCMV intravenously. Heparinized cardiac blood was obtained from 10 mice at each interval after infection (Fig. 1). Each 0.1-ml sample of whole blood was diluted by adding 0.9 ml of maintenance medium. After centrifugation, the diluted plasma was collected for plaque assay. After two additional washes of the sediment, the whole-blood cells were adjusted to a concentration of 10^6 cells per ml. A 0.1-ml sample containing 10^5 cells was cocultivated with allogeneic (CD-1) METC for 4 to 6 weeks. The development of CPE was recorded, and the proportion of mice with either plasma or cell-associated virus was computed. Virus was found in the plasma only on days 6 and 8. However, the cell-associated viremia lasted for at least 2 weeks.

Infection of various blood cells. In another experiment, at various intervals after infection, cardiac blood was obtained from 10 mice (Fig. 2). Diluted plasma prepared as described above was collected for virus titration. Washed blood cells were pooled from the 10 mice and fractionated into T-lymphocyte, B-lymphocyte, and erythrocyte-granulocyte-rich fractions as described in Materials and Methods. For each cell fraction, 6 to 12 replicates of 10^5 leukocytes were cocultivated, and CPE was scored. Virus in plasma was detected only on days 3, 6, and 8. Virus was recovered from the erythrocyte-granulocyte-rich fraction during the first 8 days of infection; thereafter, virus was no longer associated with this fraction. On the other hand, virus was detected by cocultivation in the lymphocyte fractions for at least 3 weeks. Lymphocyte-associated viremia was more frequently associated with T lymphocytes early in the infection.

Free virus in lymphocyte-associated infection. Plasma and different cell fractions were again collected from pooled blood of 10 to 30 mice at the intervals indicated (Table 1). A portion of the cells was cocultivated, and a portion was sonicated for determination of free virus

in the cell lysates. On days 3 and 6 virus was found to be associated with all the cell fractions, and plasma virus was also detected. On day 6 a low titer of free virus was detected in the cell lysates of sonicated lymphocytes. No lytic virus was detected from sonicated lymphocytes at any other time. By cocultivation, virus was demonstrated to be associated with both T and B lymphocytes for 19 to 28 days.

Kinetics of the appearance of serum neutralizing antibody. The appearance of neutralizing antibody may profoundly influence the nature of viremia. Serum was obtained from groups of five mice at intervals after infection for determination of its neutralizing titer in the presence and absence of complement (Fig. 3). Neutralizing antibody was detected as early as 3 days after infection. It did not, however, reach a peak until 3 weeks after infection. Appearance of antibody occurred earlier in these BALB/c mice than in Swiss-Webster mice, in which it was not detected until 19 to 26 days after infection (1, 16). Complement enhances the neutralizing activities in the early phase of infection (1, 13). The appearance of antibody, however, did not prevent the persistence of infected cells.

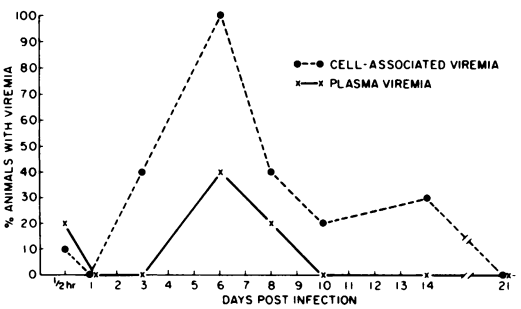

FIG. 1. *The course of viremia due to MCMV. Each point represents the percentage of 10 animals with MCMV detected in either plasma* (×) *or blood cells* (●).

Effect of cocultivation and mitogens on lymphocyte-associated MCMV. Blood cells were obtained from mice when lytic viremia was demonstrable, i.e., 1 to 6 days after infection, and later when it was not. We investigated whether lymphocyte-associated infection differed by CPE production upon cocultivation with either allogeneic or syngeneic fibroblasts (Table 2). No significant difference was found ($P > 0.05$).

To test the enhancing effect of mitogens (Table 3), cells were collected 7 and 14 days after infection and cocultivated on syngeneic fibroblasts in the continuous presence of either phytohemagglutinin or LPS. A "no treatment" group served as the control. As shown in Table 3, phytohemagglutinin treatment did not affect the frequency of detection of lymphocyte-associated infection. LPS significantly enhanced in-

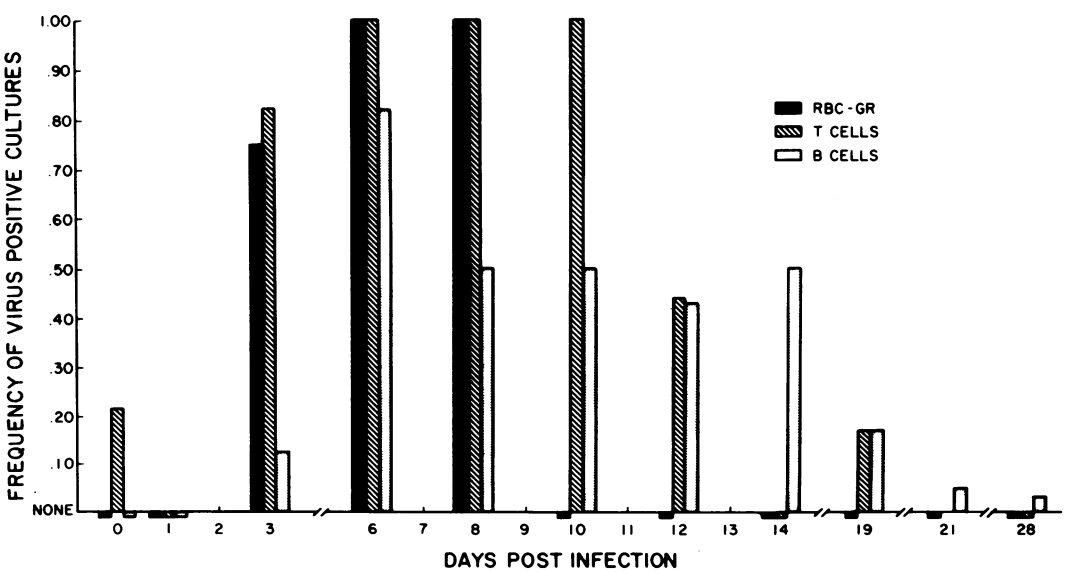

FIG. 2. *The frequency of viremia associated with different blood cells. Each point represents the proportion of replicate cultures demonstrating CPE after inoculation of 10^5 leukocytes per replicate. Virus was assayed in corresponding pooled plasma and found only on days 3, 6, and 8 as follows: 6, 4.5, and 8.3 PFU per 0.01 ml, respectively. Abbreviations: RBC-GR, erythrocyte-granulocyte-rich fraction; T, purified T lymphocytes; B, purified B lymphocytes.*

TABLE 1. *Distribution of cell-associated viremia and plasma viremia*

Days after infection	CBC[a]		RBC-GR[b]		T cells		B cells		Plasma (PFU per 0.01 ml)
	Whole cell[c]	Sonicated[d]	Whole cell	Sonicated	Whole cell	Sonicated	Whole cell	Sonicated	
1	0/8	0	0/4	0	0/4	0	0/4	0	0
3	3/3	2.1	4/4	8.5	3/3	0	2/8	0	6.0
6	6/8	5.3	4/4	17.8	8/8	0.3	7/8	0.3	4.5
12	0/4	0	0/4	0	4/9	0	3/7	0	0
19	0/4	0	0/4	0	1/6	0	1/6	0	0
28	0/4	0	0/4	0	0/8	0	1/8	0	0

[a] CBC, Complete blood cells, before first fractionation as described in the text.

[b] RBC-GR, Erythrocyte-granulocyte fraction obtained from Ficoll-Hypaque gradient; T and B lymphocytes were obtained after passage through glass- and nylon-wool columns.

[c] Fraction of virus-positive cultures (numerator) after cultivating 10^5 cells on replicates (denominator) of allogeneic fibroblasts.

[d] Number of PFU per 10^5 cell equivalents. Four replicates were inoculated. 0 signifies <0.25 PFU per 10^5 cells.

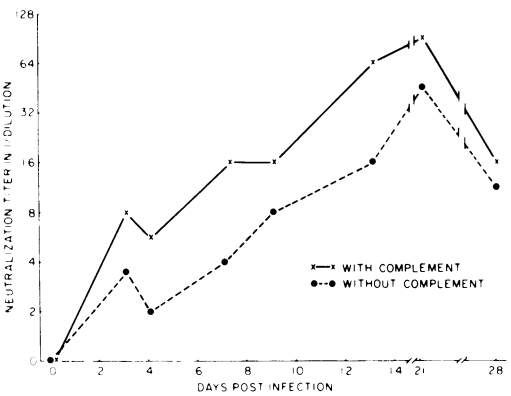

FIG. 3. *The kinetics of development of serum neutralizing antibody after MCMV infection in BALB/c mice. Groups of mice were each infected with 10^5 PFU in 0.2 ml at day 0. Serum was obtained at intervals by pooling the serum from five mice, and its neutralization titer was determined. Titer of each serum preparation was obtained with* (×) *or without* (●) *added (1:8) diluted guinea pig complement.*

TABLE 2. *Effect of cocultivation on allogeneic and syngeneic fibroblasts on lymphocyte-associated MCMV*[a]

Cell type	Allogeneic fibroblasts		Syngeneic fibroblasts	
	Fraction positive[b]	Frequency	Fraction positive[b]	Frequency
CBC	10/25	0.40	12/21	0.57
RBC-GR	8/12	0.67	13/20	0.65
T	11/15	0.73	7/14	0.50
B	9/20	0.45	9/18	0.50

[a] Pooled blood was obtained 1 to 6 days after infection of BALB/c mice, and 10^5 designated cells were inoculated on a well containing CD-1 or BALB/c fibroblast monolayer. Abbreviations as in Table 1.

[b] Wells with viral CPE/total number of wells inoculated.

fection of B lymphocytes from 14-day-infected mice. There is, therefore, a stimulating effect of LPS on B-cell infection in accordance with the results of Olding et al. (21). The effect of LPS on B lymphocytes obtained on day 7 was not significant. This may have been due to the lower number of cells employed, or LPS may enhance latent, but not lytic, infection.

Is free virus produced during cocultivation? In the foregoing, we found that virus infection was associated with both B and T cells even when no free virus was found in sonicated cells at the time of cocultivation. One simple explanation would be that the cycle of infection or the production of complete virions was accomplished later in each cell type in the presence of a feeder layer. Therefore, we attempted to determine whether either lymphocyte is able to produce complete lytic virions.

Experimentally, 5×10^5 total lymphocytes derived from either day 10- or day 13-infected animals were enclosed in a 0.45-μm Millipore chamber and cocultivated on allogeneic and syngeneic fibroblasts. Closed chambers prevented migration of cells but not of virus or medium. As controls, lymphocytes were cocultivated without chambers ("no chamber") or in open chambers (Table 4).

As expected, there was no significant difference in the frequency of virus-positive cultures when chamber cultures were cocultivated with either syngeneic or allogeneic fibroblasts. Using lymphocytes from mice infected for 10 days, the fraction of positive chamber cultures on ICR and BALB/c fibroblast feeder layers was similar to the open chamber cultures. This was also the case with lymphocytes collected 13 days after

TABLE 3. *Effect of mitogens on lymphocyte-associated MCMV*

Cells obtained on day:	Cell type	Treatment	No. of cultures		Frequency of positive cultures (%)
			Positive	Total	
7[a]	T	None	11	24	45.8
		PHA[b]	7	27	25.9
		LPS	9	24	37.5
	B	None	3	24	12.5
		PHA	3	24	12.5
		LPS	5	24	20.8
14[c]	T	None	2	41	4.9
		PHA	7	42	16.7
		LPS	5	48	10.4
	B	None	1	42	2.4
		PHA	0	42	0.0
		LPS	8	40	20.0[d]

[a] 5×10^3 cells per culture were used.
[b] PHA, Phytohemagglutinin.
[c] 10^5 cells per culture were used.
[d] $P < 0.05$, LPS compared to no treatment; mitogen effect was not significant for other treatment groups.

TABLE 4. *Demonstration of the production of complete MCMV in chamber culture of lymphocytes*

BALB/c lymphocytes in:	Feeder layer (METC)	Fraction of MCMV-positive cultures	
		Day 10[a] (%)	Day 13[a] (%)
Chamber	ICR[b]	4/10 (40)	1/18 (6)
Chamber	BALB/c	7/10 (70)	3/20 (15)
Open chamber	ICR	2/6 (33)	1/6 (17)
No chamber	ICR[c]	24/24 (100)	11/36 (31)

[a] Each chamber (or culture) contained 5×10^5 total (T+B) lymphocytes derived from either day 10- or day 13-postinfected animals. Cell lysates were negative by direct virus titration.
[b] $P > 0.25$, compared with chamber cultured on syngeneic (BALB/c) fibroblasts; $P > 0.25$, compared with open chamber cultured on allogeneic (ICR) fibroblasts.
[c] Compared pooled results with open and closed chambers for day 10 were $P < 0.001$, and for day 13 were $P < 0.05$.

TABLE 5. *Need for cell-cell contact for B and T lymphocytes to replicate infectious virus*[a]

Lymphocytes[b]	IC per 10^5 cells	Fraction of MCMV-positive cultures	
		Chamber cultures	"No. chamber" control
T	51	6/15[c]	6/6
B	30	0/20	6/6

[a] 10^5 lymphocytes were cocultivated with secondary syngeneic (BALB/c) METC for 4 to 6 weeks or until the development of characteristic CPE.
[b] Purified lymphocytes were obtained from day 6-infected mice. No lytic virus was detectable in cell lysates.
[c] $P < 0.01$, compared with B cells.

infection. On the other hand, cultures were significantly more positive in the absence of chambers. Apparently, the chambers reduced lymphocyte infectivity, probably by restriction of lymphocyte-fibroblast contact. But clearly, lymphocytes in closed chambers in the absence of such contact still produced infectious virions.

To determine which lymphocyte type was responsible for virus production, separated B and T cells obtained from animals 6 days after infection were placed in chambers (Table 5). The number of infectious centers in 10^5 of each population was comparable. Forty percent of the T-cell chamber cultures resulted in infection of the feeder layer, compared to none of the B-cell chamber cultures. T cells were able to produce free virus in the absence of a feeder layer. These results suggest that B cells could not release infectious virus in the chambers, and could initiate infection only in contact with feeder cells, as was the case with the "no chamber" controls, but this was not conclusively demonstrated.

The possibility that B cells produced neutralizing antibody in the chamber to account for the absence of free virus was also studied. Approximately 2 ml of culture fluid from each of five chamber cultures was collected on days 3, 6, and 9 after culturing and pooled. Thus, about 30 ml was obtained from both T- and B-cell cultures. The culture fluid was concentrated to approximately 1.5-ml volume by means of a Minicon B-15 concentrator (Amicon Corp., Lexington, Mass., lot no. CO 162A) at 5°C for 14 h. The concentrated culture fluid was heated at 56°C for 30 min, and its neutralization power was determined as described in Materials and Methods. Media containing 50% normal calf serum served as control. The mean plaque numbers resulting from a virus suspension treated with the control and with culture fluid from T and B cells were 29, 26, and 31, respectively. Thus, no neutralization effect could be demonstrated.

The effect of various sera on IC production by lymphocytes. The effect of normal and hyperimmune mouse sera and normal calf and rabbit sera on IC production by B and T lymphocytes obtained from mice infected 7 days previously in four replicate experiments is summarized in Table 6. After the lymphocytes were prepared as described in Materials and Methods, they were suspended in maintenance medium containing 25% of the serum tested. Each well of

TABLE 6. *Effect of serum treatments on infectious center production by T and B lymphocytes*[a]

Lymphocytes	Expt. no.	Sera							
		Normal mouse		Immune mouse		Normal calf		Normal rabbit	
		n[b]	Mean[c]	n	Mean	n	Mean	n	Mean
T cells	1	4	29.5	5	35.6	5	45.0		ND[d]
	2	3	34.0	4	23.0	5	49.0	6	54.0
	3	5	57.0	5	43.2	5	95.4	6	94.5
	4	6	39.5	5	41.0	5	40.6		ND
	Total	18	40.0	19	35.7	20	57.5		
B cells	1	11	35.5	11	13.5	9	58.8		ND
	2	6	19.2	6	9.0	6	23.7	6	19.3
	3	6	13.8	6	6.0	5	18.2	6	20.7
	4	3	22.7	5	8.0	4	41.3		ND
	Total	26	22.8	28	9.1	24	35.5		

[a] The overlay for IC determination contained 0.25% of stated serum. The neutralization titer of hyperimmune serum was 1:320.
[b] Number of replicates.
[c] Mean number of IC.
[d] ND, Not done.

a 12-well plate culture containing ICR fibroblasts received 10^5 lymphocytes in 0.1 ml. After overnight incubation, 0.9 ml of overlay medium was added (final concentration of test serum in overlay: 2.5%). IC were scored as in previous experiments. Normal rabbit serum was used in only two of the four experiments (experiments 2 and 3) with each type of lymphocyte, and its effect was analyzed statistically only within the two experiments. The action of normal rabbit serum on both T and B cells was the same as normal calf serum in the experiments where it was employed ($P > 0.1$ for T cells, $P > 0.5$ for B cells) (Table 6).

The difference between the numbers of IC produced when normal mouse, hyperimmune mouse, or normal calf serum was added to T cells, adjusted for the variation between experiments, was highly significant ($P < 0.001$). This significant effect among the three serum treatments was due to the reduction in IC caused by normal mouse serum as compared to normal calf serum ($P < 0.02$) and by hyperimmune mouse serum as compared to normal mouse serum ($P < 0.002$). There was no difference in IC formation by T cells treated with normal or immune mouse sera ($P > 0.25$).

The three serum treatments also had a highly significant effect on IC production by B cells, after adjustment for the differences between experiments ($P < 0.001$). As was true with T cells, normal mouse serum significantly reduced B-cell IC production in comparison to normal calf serum ($P < 0.002$). In contrast, however, B-cell infectivity was significantly more decreased by immune than by normal mouse serum ($P < 0.002$).

To determine whether the reduction in IC production by both T and B cells in the presence of normal mouse serum and the additional decrease by B cells with hyperimmune serum were properties unique to lymphocytes, the effect of the three sera on infected fibroblasts was examined. Secondary mouse embryo cells were infected with MCMV at an input multiplicity of 1. After 48 h, the infected cells were trypsinized and washed, and 100 cells were treated with either normal mouse, hyperimmune, or normal calf serum. The number of IC for these three treatments was not found to be different ($P > 0.1$).

Enrichment of MCMV-associated lymphocytes. The depressive effect of normal and hyperimmune serum on formation of infectious centers by latently infected lymphocytes supports the notion that there is an involvement of Fc receptors. Total lymphocytes (T + B) from animals 7 days after infection were fractionated into enriched and deficient rosette-forming lymphocytes as described in Materials and Methods. The IC in each fraction and in unfractionated lymphocytes were determined. Despite the fact that FcR (+)-enriched cells represented only 46% of the total, they accounted for 73% of the IC (Table 7).

DISCUSSION

Viremia resulting from cytomegalovirus infection in mice may take several forms. During early infection (3 to 8 days), free virus was found in plasma, and MCMV could be found in association with granulocytes. One week after infection free virus disappeared, and sonic extracts of granulocytes and lymphocytes revealed no free virus. Infection was only demonstrated in T- and B-type lymphocytes by cocultivation on synge-

TABLE 7. *IC formation in FcR (+) lymphocyte-enriched population*

Cell source	Total cells recovered (%)	IC per 10^5 cells (±SE[a])	IC[b]	
			Total	% Recovered
Unfractionated[c]	2×10^7 (100)	36.2 ± 3.8	7,240	100
FcR (+) deficient	1.2×10^7 (60)	10.6 ± 0.9	1,272	17.6
FcR (+) enriched	9.2×10^6 (46)	57.3 ± 3.2[d]	5,271	72.8

[a] SE, Standard error.

[b] Total lymphocytes (T+B); cell concentration was adjusted, 10^5 lymphocytes were inoculated, and IC were determined as described in the text. For FcR (+)-deficient and -enriched cells, 9 and 11 replicates were used for IC determination.

[c] Starting material contained 2×10^7 cells.

[d] $P < 0.05$ by F test.

neic or allogeneic mouse fibroblasts. By 4 weeks after infection, only one out of eight replicates of embryonic fibroblasts cocultivated with 10^5 lymphocytes revealed eventual cytopathology. Superficially, this is analogous to the situation in infectious mononucleosis (24), where infectious virus is not found in the blood of the convalescing patient, but only when lymphocytes are cultured in vitro in an antibody-free environment.

Cell-associated viremia, whether in the early stages (1 to 10 days after infection) when free viremia was present, or later, was detected equally well with syngeneic or allogeneic fibroblasts. This is consistent with our recent work in which we showed that spleen cells from DBA/2 mice infected more than 4 months previously could be activated equally well on syngeneic or allogeneic fibroblasts (18).

We found an important difference between infectivity associated with T and B lymphocytes. When cells with comparable numbers of IC were cultured in chambers overlaid with fibroblasts, T lymphocytes eventually produced free virus without direct contact with fibroblasts. On the other hand, B lymphocytes did not produce productive infection when held in isolation in chamber cultures without direct contact with fibroblasts. It is possible that B lymphocytes produced a small amount of lytic virus, which was trapped in the chamber or filter and was not detected despite the fact that cells overlaid the filter. We find a 1.7-fold gradient of diffusion of free MCMV from the medium into the chamber (M. Ho, unpublished data). Also it is possible that the survival and hence the reactivability of these two types of lymphocytes differ. Our data do, however, show that the two cell types behave differently. Further experiments are needed to prove that only T and not B cells can be lytically infected. If this hypothesis is correct, T cells may be more readily eliminated in the host either by viral cytopathology or by immune mechanisms. On the other hand, since B cells are nonproductively infected, they may persist in circulation, transmit the virus genome to other tissues, and function as the vehicle of latency (21).

Keller et al. (13) found that human fibroblasts infected with human cytomegalovirus developed Fc receptors that bind with human immunoglobulins. This binding is species specific, since rabbit immunoglobulins did not compete with human immunoglobulin for such receptors (26). Oleszko and Minowada (Abstr. Annu. Meet. Am. Soc. Microbiol. 1977, S40, p. 285) showed that continuous lymphoid cell lines infected with herpes simplex virus also developed Fc receptors on both T and B cells, which are different from normal IgG-Fc receptors in being trypsin resistant. Costa et al. (6) reported that IgG and Fc fragments inhibited replication of herpes simplex virus. Comparable data are not available for MCMV. We showed that MCMV IC formation by both B and T cells was depressed by mouse serum but not by heterospecies serum. We also showed that, when a population enriched with lymphocytes containing Fc receptors was obtained and cocultivated, infectivity was largely in receptor-bearing lymphocytes. The mechanism of inhibition by serum may be binding of Fc receptors on these cells, although this is speculative at this point. What is not known is whether these receptors developed de novo after infection or were present prior to infection, and we have not tested their trypsin sensitivity.

Another finding that differentiated infected B lymphocytes from T lymphocytes was that the infectivity of B cells, expressed in the formation of IC, was inhibited by antiserum against MCMV. It should be noted that productively infected cells, whether fibroblasts or T lymphocytes, were not inhibited by antiserum. It appears, therefore, that nonproductively infected B cells might carry virus-specific antigen(s) on their surface, the binding of which inhibits transmission of infection, possibly by a provirus, from B cells to a permissive feeder layer. This inhibition of B-cell infection by antiserum suggests a mechanism by which specific antibody may prevent activation of B lymphocytes. Obversely, cocultivation of latently infected B cells on per-

missive cells in the absence of antibody may result in viral activation.

We also found that treatment with anti-theta serum had no additional effect in reducing IC formation by cells compared to syngeneic mouse serum. Apparently, theta antigen is not site essential for virus release, nor is theta antigen needed for transmission of the infection to feeder cells.

Our findings may also bear on the mechanism of immunosuppression by MCMV, such as its effect on antibody synthesis and on lymphocyte response to mitogens (3, 22). Selgrade et al. (25) suggested that virus infection of lymphocytes may suppress the mitogenic responses of both B and T cells. Although we find that only a small fraction of these cells ($1/10^4$ to $6/10^4$) are infected in the blood at any one time, infection in the majority of cells may be defective or abortive and may not be detected by cocultivation.

In addition, recent evidence shows that suppressor T cells have Fc receptors for IgG (20). Since we find infectivity to be associated with Fc receptor-bearing cells, it is possible that normal T lymphocytes develop receptors after being infected, and differentiate into suppressor cells.

Perhaps our findings have raised more questions than answers at this point. They do show that various types of relationships exist between cytomegalovirus and lymphocytes, which vary not only with the type of cell but perhaps also with the stage of infection. Further studies would be facilitated if lymphocytes could be readily infected with MCMV in cell culture. Such infection is at best still inefficient (10).

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