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Protective Effect of Early Serum from Mice After Cytomegalovirus Infection

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Serum obtained from mice 3 to 5 days after cytomegalovirus infection contained complement-requiring neutralizing antibody, which, when passively transferred, protected mice from lethal infection with the homologous virus. The active substance was largely heat-stable 7s immunoglobulin.

Mannini and Medearis (5) showed that Swiss-Webster mice infected with murine cytomegalovirus (MCMV) did not develop neutralizing antibodies until 19 to 26 days after infection. Starr and Allison (8) showed that serum obtained from mice 6 days after infection did not passively protect mice from death 24 h after inoculation with MCMV. At the same time, immune spleen cells from such animals protected. MCMV has been reported to suppress the primary response to sheep erythrocytes (7) and to destroy germinal centers in the spleen within the first week of infection (4). The question of whether specific humoral immunity is stimulated during the acute infection arises. The following report describes the protective effect of serum obtained 3 to 5 days after infection and shows that protection is probably due to specific immunoglobulin.

MATERIALS AND METHODS

Virus. The Smith strain of MCMV was obtained from June Osborn. Virus stocks were prepared from the salivary glands of mice 3 weeks after infection. Outbred albino CD-1 mice (Charles River Breeding Laboratories, Wilmington, Mass.), 2 to 8 weeks old, were used. To titrate MCMV, 20% organ homogenates were serially diluted with medium 199, and 0.1-ml samples of each dilution were inoculated on duplicate wells of mouse embryo cell cultures (MECC). After 90 min of absorption at 37°C under 5% CO_2, tragacanth gum overlay was added to each well (6). Cultures were incubated for 7 to 9 days. The overlay was aspirated off, and the cell sheets were stained with gentian violet for plaque counting.

Cell culture. Pregnant mice (CD-1 strain) were used for MECC. Primary MECC were prepared from mouse embryos in Povitsky bottles and grown in medium 199, supplemented with 5% newborn calf serum and antibiotics. After 3 days, secondary cultures were made by dispensing 2.5 ml of cell suspension (10^5 cells per ml) into each well of six-well plates (Linbro, Hamden, Conn.). The subcultures were ready for use after 2 to 3 days of incubation at 37°C in humidified 5% CO_2.

Interferon. Serum interferon was prepared by inoculating intravenously 10^8 plaque-forming units (PFU) of Newcastle disease virus and collecting the sera 6 h later. Serum or serum fractions were assayed for interferon on MECC, using the method described by Armstrong (1). Uninfected MECC were pretreated overnight with serial dilutions of samples and were challenged with vesicular stomatitis virus 18 h later. Interferon titer was based on 50% end point dilutions of protective activity of 0.1-ml samples and standardized with the NIH mouse interferon standard.

Immunology. Immune sera were obtained from mice infected with 4×10^4 PFU of MCMV intraperitoneally (i.p.). Blood was collected at indicated intervals by incision of the axillary artery. Unless otherwise specified, all sera were heated at 56°C for 30 min.

Guinea pig complement (Microbiological Associates, Bethesda, Md.) did not have MCMV-inactivating activity at 1:8 and was used at 1:12. It was found to have 33 hemolytic units per 0.1 ml. Dilutions were made in medium 199. For the neutralization test, mixtures of the following were added in order: 0.3 ml of diluted serum or serum fraction, 0.15 ml of a 1:3 dilution of complement, and 0.15 ml of virus (approximately 6×10^2 PFU). In parallel tests complement was replaced with either heated guinea pig serum or medium. Controls contained normal serum treated in the same manner as the test sera. Titer was expressed as the reciprocal of the highest serum dilution causing a 50% reduction of the number of plaques in the neutralization mixture containing normal mouse serum.

When required, serum was treated with mercaptoethanol (final concentration, 0.1 M) for 2 h at room temperature and dialyzed against large volumes of 0.02 M iodoacetamide in phosphate-buffered saline, pH 7.4, overnight at 4°C. The alkylated sera were then dialyzed with four changes of phosphate-buffered saline.

For immunodiffusion tests, goat anti-mouse immunoglobulin G (IgG) and anti-mouse IgM sera (Meloy Laboratories, Springfield, Ill.) were used. Serum or gradient fractions were tested against these anti-mouse reagents by immunodiffusion in 1% agarose gel in 0.9% NaCl on a microscope slide. Preparations were

placed in a moist chamber at 4°C overnight before the precipitin reactions were read.

Separation of IgG and IgM. The method described by Cowan and Trautman (2), utilizing KBr-$NaNO_3$ gradients, was followed. Gradients were prepared in 5-ml cellulose nitrate tubes (Beckman, Palo Alto, Calif.) by layering 1.5 ml of 65% saturated $NaNO_3$ (vol/vol) on 2.5 ml of 75% saturated KBr (vol/vol). One milliliter of serum was gently delivered on top of the gradient, and the gradient was centrifuged at 35,300 rpm for 20 h at 20°C in an SW 50.1 rotor in a Beckman model L-2 ultracentrifuge. Fractions were collected dropwise from the bottom of the tube. The first 1.0-ml fraction was designated as the high-molecular-weight or IgM fraction, the next 0.5 ml was discarded, and the next 2.0 ml was collected for the low-molecular-weight or IgG fractions.

RESULTS

Protective role of early serum. Adult mice were given 0.5 ml of immune serum i.p. and challenged with 4×10^6 PFU of MCMV 24 h later. The animals were sacrificed 3 days after infection. Their spleens and livers showed lower virus titers than those of controls (Table 1). The 3- and 7-day sera were effective in reducing virus titers in the spleen significantly. Heating for 30 min at 56°C did not remove the antiviral property of the acute serum.

Two-week-old mice were inoculated i.p. with 0.1 ml of serum and were challenged i.p. on the next day with two different doses of MCMV (Table 2). The number of survivors was significantly greater among recipients of immune sera if they received the smaller doses of virus. When the virus challenge was increased to 4×10^5 PFU, the early serum was no longer protective, whereas the late serum still showed good protection.

Role of interferon. It was possible that the protective factor demonstrated by passive transfer of early serum was interferon, which we have shown to be present for several days postinfection (9). To test this possibility, five mice were injected i.p. with 0.5 ml of a serum interferon having a titer of 1:14,260, and five each received 0.5 ml of normal mouse serum. All 10 received 4×10^6 PFU of MCMV 24 h later i.p. The mean virus titers in the livers of the treated and control mice were 1.35 ± 0.33 (standard deviation) and 1.50 ± 0.86 log PFU per 0.1 ml. The respective mean spleen titers were 3.10 ± 0.48 and 3.10 ± 1.24 log PFU. There was no significant difference between the titers in the treated and control mice, showing that a dose of interferon greatly in excess of that injected in the experiment above (Table 1) was not protective. Serum interferon titers in heated sera used in passive immunization experiments did not exceed 1:32.

TABLE 1. *Effect of passive transfer of early sera on virus titer in livers and spleens*[a]

Type of serum (0.5 ml i.p.)	Liver		Spleen	
	P/T	Virus titer[b]	P/T	Virus titer
3-day immune	0/5	None	5/5	2.03 ± 0.56[c]
5-day immune	3/5	0.48 ± 0.45	5/5	2.48 ± 0.37
7-day immune	4/5	0.84 ± 0.60	5/5	1.65 ± 0.68[c]
Normal serum	3/5	1.22 ± 1.16	5/5	3.25 ± 0.52

[a] Mice were challenged i.p. with 4×10^6 PFU of MCMV 1 day after passive transfer with 0.5 ml of heat-inactivated serum. P/T is the ratio of the number of organs with detectable virus to the number of animals examined.

[b] Expressed as mean log PFU per 0.1 ml of 10% organ suspension ± standard deviation in group of five mice.

[c] Significant reductions in virus titer ($P < 0.05$, Dunnett t test); others not significant.

TABLE 2. *Protective effect of immune serum on survival of mice*[a]

Treatment (0.1 ml i.p.)	Survivors/total no. of mice at challenge dose (PFU of MCMV i.p.) of:		
	4×10^4	4×10^4	4×10^5
Normal serum	2/10	8/20	1/10
5-day serum	8/8[b]	17/20[b]	0/10[c]
82-day serum	10/10[b]	Not done	9/10[b]

[a] Each column represents a separate experiment.

[b] In χ^2 test, with Yates correction, $P < 0.01$ in comparison with normal serum control.

[c] Not significant.

Neutralizing antibody. Sera were collected 1, 3, 5, 7, 14, and 17 days after i.p. infection with 4×10^4 PFU of MCMV. They were heated at 56°C for 30 min and assayed for neutralizing antibody in the presence and absence of complement. Without complement, no neutralizing antibody could be detected in any of the sera until 21 days after infection, when a titer of 1:4 was found. However, in the presence of complement, neutralizing antibody titers of 1:4 and 1:8 were demonstrable as early as days 3 and 5 after infection.

To characterize complement-requiring antibodies, 5- and 39-day anti-MCMV sera were treated with mercaptoethanol and tested for neutralizing activity in the presence and absence of complement. Neutralizing activity was not significantly reduced after treatment with mercaptoethanol, suggesting that the neutralizing antibodies were largely associated with the 7s immunoglobulins.

IgM and IgG fractions were obtained from early and late antisera by KBr-$NaNO_3$ gradient centrifugation. By immunodiffusion, the low-molecular-weight or IgG fraction contained IgG without IgM. The high-molecular-weight or IgM fraction contained IgM and trace amounts of

IgG. No interferon was found in the IgG fraction of either the 5- or the 47-day serum. The IgG and IgM fractions were tested for neutralizing activity in the presence of complement (Table 3). In the early serum, neutralizing antibody activity was detected in the IgG fraction only. In the late serum, neutralizing activity was demonstrated in both IgG and IgM preparations, although the activity was lower in the IgM than in the IgG fraction.

To test the protective activity of the serum fractions, weanling mice received 0.2 ml of IgM fraction and 0.4 ml of IgG fraction (Table 3). They were challenged 1 day after passive transfer of fraction. Analysis of data showed that the IgG fractions of the 5- and 47-day antisera were protective. The IgM fractions did not protect.

TABLE 3. *Neutralizing and protective action of early and late immune serum*[a]

Type of serum	Fraction	Neutralizing titer (with complement)	Protective effect in mice (survivors/total)
Early (5 day)	IgG	4, 8	8/10[b]
Early (5 day)	IgM	<2, <2	1/10[c]
Late (47 day)	IgG	16, 32	10/10[b]
Late (47 day)	IgM	2, 4	3/10[c]
Normal		<2	5/20

[a] IgG and IgM obtained from KBr-$NaNO_3$ gradient centrifugation as described in Materials and Methods, reconstituted to original volume. Each serum titer represents a separate assay. Protective effect was determined by passive transfer of 0.4 ml of IgG or 0.2 ml of IgM to weanling mice, which were challenged with 4×10^5 PFU of MCMV 24 h later.

[b] $P < 0.02$, compared to mice injected with normal serum, χ^2 test with Yates correction.

[c] Not significantly different from controls.

DISCUSSION

The above results show that infection of mice with a sublethal dose of MCMV stimulated early production of neutralizing antibody detectable in the presence of complement. Passive transfer of the acute antiserum showed low but significant protection of the recipients from lethal challenge with the homologous virus.

The present results on the neutralization assays in the absence of complement are similar to those reported by Mannini and Medearis (5), who did not detect neutralizing antibody during the first 2 weeks of MCMV infection. However, in the presence of complement, neutralizing activity was detected as early as 3 days after infection. Complement-requiring neutralizing antibodies were also found 6 days after infection by Starr and Allison (8). It is of interest to note that contrary to expectations, complement-requiring antibodies were in the IgG fraction of the early serum, although activity was detected in both IgG and IgM fractions of the late serum. These results are similar to those reported for herpes simplex virus in rabbits by Yoshino and Taniguchi (10), who also found that complement-requiring antibody in early serum was in the 7s globulin fraction. Whereas late antisera contained specific IgM and IgG fractions, our data indicate that both neutralizing and protective activities were largely accounted for by the heat-stable IgG fractions.

The difference between our results and those of Starr and Allison (8), who found that early immune serum was not protective, may be due to the difference in the strains of mice or the procedures used. In the present study, serum treatment was given before virus challenge, whereas in their study mice were infected first.

These results suggest that despite increasing evidence of the importance of cell-mediated immunity in the defense against cytomegaloviruses of mice and humans (3), effective specific humoral immunity is developed very early in this infection and should be taken into account in research in the cytomegalovirus area.

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