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Effect of Immunosuppression on Humoral and Cell-Mediated Immunity to Murine Cytomegalovirus (40549)[1]

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Both humoral and cell-mediated immunity (CMI) are important in protecting animals from viral infections and in ridding the host of established infections. Serum antibody has usually been used to determine the immune status of the host because of the relative ease in collecting serum samples and performing antibody titers. However, cell-mediated immunity (CMI) may be a more important host defense mechanism in viral infections.

Cytomegalovirus (CMV) in transplant recipients can cause substantial morbidity and even fatality. Morbidity or mortality may occur even though the patient has demonstrable serum antibody to the virus. This finding may not be surprising since CMV is a cell-associated virus and may not be subject to attack by extracellular antibodies. Recently Pollard *et al.* (1) found a good correlation between humoral immunity (HI) and CMI to CMV in normal subjects. However cell-mediated immunity to CMV in heart transplant recipients did not appear for 3 yrs following cardiac transplantation even though HI is present much earlier. In another study these authors found that lack of CMI to herpes simplex and varicellazoster virus correlated with susceptibility to these viruses in cardiac transplant recipients (2). Others (3, 4) showed specific transformation to CMV antigen in normal seropositive subjects.

In order to further understand the relationship between immunity to CMV in patients with immune deficiency states and on immunosuppressive drugs we recently described in *in vitro* test for CMI to CMV (5). We report in this paper the effect of immunosuppression with prednisolone and rabbit anti-mouse antilymphocyte globulin (ALG) on HI and CMI to murine CMV and on blast transformation to nonspecific mitogens.

Materials and methods. Mice. Six-week-old C57Bl/6 female mice were obtained from Charles River Breeding Laboratories, Bloomington, Massachusetts. The animals were kept in clean dry cages and were fed water and Purina Laboratory Chow (Ralston Purina, St. Louis, Mo.) *ad libitum.*

Virus. Murine CMV was originally obtained from Dr. June Osborne, University of Wisconsin, Madison, Wisconsin. Pools of virus were made from salivary gland homogenates from outbred Swiss–Webster mice 14 to 20 days after infection and the virus was titered in mouse embryo fibroblasts as previously described (6). The pool used had a titer of 7.9×10^7 plaque-forming units (pfu) per milliliter.

Preparation of viral antigen. CMV viral antigen was prepared as described previously (4). Briefly, confluent monolayers of Swiss–Webster mouse embryo fibroblasts were grown in roller bottles having a surface area of 1000 cm^2. CMV (10^6 pfu/bottle) was inoculated into each roller bottle (approximately 10^8 cells/bottle). This represented 0.01 pfu CMV per cell. When the cells showed 80 to 90% cytopathic effect (approximately 5 days after inoculation), they were scraped off the glass surface. The harvested material (approximately 1000 ml) was frozen and thawed at −70° to release intracellular virus. Cell debris was removed by centrifugation at 4800*g* for 20 min. The supernate was carefully decanted and then centrifuged at 32,296*g* for 20 min in a Beckman L5-50 centrifuge using a T-19 rotor. The virus pellet was resuspended in 10 ml Hanks' balanced salt solution and centrifuged at 131,453*g* for 60 min in a SW-70 rotor and resuspended in

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a final volume of 2 ml (a concentration of 500). The virus suspension was then titrated as previously described (5) and assayed for protein content by the method of Lowry *et al.* (7). The final suspension contained 3800 μg protein per milliliter and had a virus titer of 4×10^6 pfu per milliliter. The CMV antigen preparation was heat-inactivated at 56° for 60 min prior to use and stored at −70° until used. The final CMV antigen contained no viable virus.

Control antigen. As a control for the CMV antigen confluent layer of Swiss–Webster mouse embryo fibroblasts was prepared and harvested as described except that the fibroblasts were not infected with CMV.

CMV–CMI technique. The cell culture technique was carried out as described previously (4). Mice were killed by cervical dislocation. The spleens were aseptically removed. Splenic lymphocytes were purified by the Ficoll–Hypaque technique of Boyum (8). Spleen cells were resuspended in RPMI 1640 containing 10% fetal calf serum, 100 units of penicillin per milliliter, 100 mg streptomycin per milliliter, and 0.05 m*M* 2-mercaptoethanol. For all experiments 2.5×10^5 spleen cells were suspended in 0.1 ml tissue culture medium and were added to microtissue culture plates (Falcon 3040, Falcon Plastics, Oxnard, Calif.). To each well was added 0.1 ml medium containing 1 μg CMV antigen or 1 μg of the control antigen. The plates were incubated for 7 days and [^{3}H]thymidine (1 μCi; specific activity 6.7 Ci per mmole New England Nuclear, Boston, Mass.) was added to each cell culture and the cells were cultured for an additional 6 hr. The cells were then

TABLE I. HUMORAL IMMUNITY AND CELL-MEDIATED IMMUNITY TO MURINE CYTOMEGALOVIRUS IN MICE IMMUNOSUPPRESSED FROM 2 days BEFORE TO 7 days AFTER VIRUS INFECTION[a]

Group	Control antigen	CMV antigen[b]	Reciprocal CF antibody[c] titer
1 Uninfected	5104 ± 404[d]	6138 ± 743	<2, <2
2 Not immunosuppressed, infected	9569 ± 881	54,685 ± 5451	128, 128
3 Uninfected, immunosuppressed	6884 ± 435	7698 ± 846	<2, <2
4 Infected, immunosuppressed	3617 ± 261	7619 ± 1187[e]	128, 128

[a] Mice were immunosuppressed with 0.5 mg prednisolone intraperitoneally Days −2 to +7 and with 5 mg ALG intraperitoneally Days −2, −1, 1, 3, and 5. Virus inoculation occurred on Day 0. Mice were sacrificed and their immune status was analyzed 18 days later.
[b] Cytomegalovirus.
[c] Complement fixing.
[d] cpm ± SE.
[e] $P < 0.001$ when compared to group 2.

TABLE II. MITOGEN RESPONSES IN MICE INFECTED WITH MURINE CYTOMEGALOVIRUS IMMUNOSUPPRESSED FROM 2 days BEFORE TO 7 days AFTER VIRUS INOCULATION[a]

Group	Media	PHA[b]	Con A[c]	LPS[d]
1 Uninfected not immunosuppressed	8835 ± 566[e]	31,853 ± 2831	314,466 ± 6089	471,928 ± 9307
2 Infected	9384 ± 119	35,962 ± 1591	224,623 ± 5474	478,411 ± 8310
3 Uninfected immunosuppressed	9711 ± 977	15,605 ± 938[f]	130,924 ± 2091[f]	141,277 ± 3461[f]
4 Infected immunosuppressed	4782 ± 324	6621 ± 495[g]	59,782 ± 708[g]	109,624 ± 3240[g]

[a] Mice were immunosuppressed with 0.5 mg prednisolone Days −2 to +7 and with 5 mg ALG Days −2, −1, 1, 3, and 5 intraperitoneally. They were infected with cytomegalovirus on Day 0.
[b] Phytohemagglutinin.
[c] Concanavalin A.
[d] *E. coli* lipopolysaccharide.
[e] cpm ± SE.
[f] Significantly less than Group 1, normal controls, $P < 0.001$.
[g] Significantly less than Groups 1 and 2, $P < 0.001$.

TABLE III. CELL-MEDIATED IMMUNITY TO MURINE CYTOMEGALOVIRUS (CMV) IN MICE IMMUNOSUPPRESSED 18 TO 30 days AFTER VIRUS INFECTION[a]

Group	Day 15		Day 32		Day 46		Day 67	
	Control antigen	CMV antigen	Control antigen	CMV antigen	Control antigen	CMV antigen	Control antigen	CMV antigen
1 Uninfected, not immunosuppressed	4,716 ± 32[b]	4,812 ± 921	2,701 ± 182	3,344 ± 266	7,534 ± 1,086	7,873 ± 1,075	5,225 ± 813	5,581 ± 424
2 Infected only	12,132 ± 1,397[c]	105,232 ± 13,607[c]	3,418 ± 473	22,175 ± 2,904[c]	6,518 ± 1,351	23,681 ± 3,405[c]	4,297 ± 685	43,679 ± 6,796[c]
3 Uninfected immunosuppressed	—	—	1,646 ± 170	2,122 ± 96	4,948 ± 256	5,211 ± 242	4,859 ± 916	7,314 ± 1,498
4 Infected immunosuppressed	—	—	1,122 ± 107	2,366 ± 915[d]	5,801 ± 741	5,479 ± 253[d]	3,939 ± 897	2,151 ± 160[d]

[a] Mice were immunosuppressed with 0.5 mg prednisolone intraperitoneally from Days 18 to 30 and with 5 mg ALG on Days 18, 20, 22, 24, 26, 28, and 30. They were infected with cytomegalovirus on Day 0.
[b] cpm ± SE.
[c] $P < 0.001$ compared to Group 1.
[d] $P < 0.001$ compared to Group 2.

collected on filter paper and radioactivity was determined by liquid scintillation counting using a Beckman L5-330 scintillation counter. Radioactivity was expressed as counts per minute.

Blast transformation experiments were conducted as described previously (9). One microgram PHA-P (Difco Laboratories, Detroit, Mich.) 1 μg concanavalin A (Con A) (Sigma Chemical, St. Louis, Mo.) or 2 μg *Escherichia coli* 026:B6 endotoxin lipopolysaccharide (LPS) (Difco Laboratories, Detroit, Mich.) was added to each well containing 2×10^5 spleen cells in RPMI 1640 containing 10% fetal calf serum (final volume, 0.2 ml). The cultures were incubated for 42 hr at 37°. [^{3}H]Thymidine, 1 μCi, was added and cultures were harvested 6 hr later. Radioactivity was determined by liquid scintillation counting. All cultures were performed in triplicate.

Antibody to CMV. Mice were bled from the retroorbital venous plexus and the serum was separated and heat inactivated at 56° for 30 min. Complement fixing (CF) antibody titers were measured by a microtiter system according to the standard method of our diagnostic virology laboratory (2). The assay was done using 1% sensitized sheep erythrocytes and guinea pig complement. Results are expressed as reciprocal titers.

Immunosuppression. Mice were immunosuppressed with 0.5 mg prednisolone (25 mg/kg) and 5 mg of horse anti-mouse lymphocyte globulin (ALG) (250 mg/kg) given intraperitoneally (ip).

Experimental design. Animals that received virus were inoculated with 2×10^5 pfu CMV on Day 0. Some groups were given immunosuppression with ALG at Days −2, −1, 1, 3, and 5 and with prednisolone at Days −2 through 7. Mice received both prednisolone and ALG. Other groups were not immunosuppressed. Two to three mice were used at each time point. Their spleens were analyzed separately. These mice were immunosuppressed around the time they were inoculated with virus to determine whether or not the development of HI and CMI to CMV could be prevented. They were tested for immunity 18 days following virus challenge, a time when we have previously shown that animals have maximal levels of HI and CMI to CMV.

TABLE IV. COMPLEMENT-FIXING ANTIBODY TITERS IN MICE IMMUNOSUPPRESSED 18 TO 30 days AFTER INFECTION[a]

Group treatment	Reciprocal CF-titer			
	Day 18	Day 32	Day 46	Day 67
1 Uninfected not immunosuppressed	<2, <2	<2, <2	<2, <2	<2, <2
2 Infected	128, 256	64, 64	128, 64	64, 64
3 Uninfected immunosuppressed	<2, <2	<2, <2	<2, <2	<2, <2
4 Infected immunosuppressed	128, 128	64, 64	128, 128	64, 64

[a] Mice were immunosuppressed with 0.5 mg prednisolone intraperitoneally from Days 18 to 30 and with 5 mg ALG on Days 18, 20, 22, 24, 26, 28, and 30. They were infected with cytomegalovirus on Day 0 (cpm ± SE).

In another experiment animals were immunosuppressed with ALG every other day from Days 18 through 30 and were immunosuppressed with prednisolone daily Days 18 through 30. These animals were immunosuppressed when they already had high levels of HI and CMI. Immunity to CMV was tested 32 to 67 days after virus infection to determine whether or not HI and CMI could be abrogated by the immunosuppressive treatment.

Results. Mice immunosuppressed within 1 week of CMV inoculation. Infected mice that were immunosuppressed during the period 2 days before to 7 days after virus inoculation failed to develop CMI to CMV (7619 ± 1187 cpm) compared to mice that were infected but not immunosuppressed (54,685 ± 5451 cpm, $P < 0.001$) (Table I). Mice not inoculated with CMV also failed to respond to CMV antigen. However, virus-infected mice did mount a significant CF-antibody response (1:128) to CMV whether or not they were immunosuppressed (Table I). Thus, immunosuppression administered during the period shortly before and after virus inoculation prevented development of CMI but not of HI to CMV.

Blast transformation to T- and B-cell mitogens was also significantly suppressed in immunosuppressed mice compared to nonimmunosuppressed mice (Table II). If the mice were inoculated with CMV in addition to being immunosuppressed, the ability of their spleen cells to transform to PHA, Con A, and LPS was further suppressed when compared to mice that were immunosuppressed, but not infected. Spleen cells of mice that were infected with CMV but not immunosuppressed with ALG and prednisolone did not transform to PHA and LPS significantly differently from normal control animals. However, there was a significantly ($P < 0.001$) lower response to Con A-infected mice compared to the controls.

Mice immunosuppressed 18 to 30 days following virus inoculation. In a second experiment some mice were immunosuppressed for a 12-day period 18 to 30 days following virus inoculation—a time they are known to have high levels of HI and CMI to CMV. Mice were tested 32, 46, and 67 days following infections for HI and CMI to CMV (Table III). Spleen cells from infected mice tested 15 days following virus inoculation—prior to beginning immunosuppression—had 105,232 ± 13,607 cpm. When infected mice were immunosuppressed 18 to 30 days after virus infection and tested 2 days later, there was complete inhibition of CMI to CMV (2366 ± 915 cpm). Forty-six days after infection immunosuppressed, infected mice were still significantly ($P < 0.001$) inhibited in their ability to mount a cellular immune response to CMV (5479 ± 253 cpm) compared to nonimmunosuppressed infected mice (23,681 ± 3405 cpm). CMI to CMV was still inhibited 67 days after virus inoculation in immunosuppressed mice (2151 ± 160 cpm) compared to infected, nonimmunosuppressed animals (43,679 ± 6796 cpm).

Immunosuppression, however, did not affect HI to CMV as it did CMI. Eighteen days after virus inoculations (before immunosuppression) mice had a CF-titer of 128 to 256 (Table IV). Two days after cessation of immunosuppression, 32 days after virus inoculation, the CF titer was 64 in immunosup-

TABLE V. MITOGEN RESPONSES IN MICE INFECTED WITH MURINE CYTOMEGALOVIRUS IN MICE IMMUNOSUPPRESSED FROM 18 TO 30 days AFTER INFECTION[a]

Group treatment	Day 32			Day 46			Day 67		
	PHA	Con A	LPS	PHA	Con A	LPS	PHA	Con A	LPS
1 None	54,660 ± 1530[b]	281,864 ± 7256	378,631 ± 9431	78,972 ± 4535	307,206 ± 16,298	457,301 ± 12,700	87,148 ± 5087	362,500 ± 19,207	216,773 ± 2071
2 Infected	54,451 ± 2061	191,044 ± 2132	341,438 ± 16,923	64,358 ± 3861	219,258 ± 3714	441,740 ± 10,964	174,174 ± 10,964	323,604 ± 15,304	156,052 ± 38,372
3 Not infected immunosuppressed	14,183 ± 6634	7242 ± 779	32,727 ± 2295[c]	5076 ± 253[c]	86,356 ± 866[c]	394,288 ± 7526	55,264 ± 5470[c]	300,696 ± 18,261	113,443 ± 2988[c]
4 Infected immunosuppressed	2281 ± 65[c,d]	2816 ± 536[c,d]	7434 ± 274[c,d]	4122 ± 91[c,d]	34,371 ± 1897[c,d]	464,226 ± 19,359	40,669 ± 4533[c,d]	176,032 ± 6979	102,784 ± 3309[c,d]

[a] Mice were immunosuppressed with 0.5 mg prednisolone intraperitoneally from Days 18 to 30 and with 5 mg ALG on Days 18, 20, 22, 24, 26, 28, and 30. They were infected with cytomegalovirus on Day 0.
[b] cpm ± SE.
[c] Significantly less ($P < 0.001$) than Group 1.
[d] Significantly less ($P < 0.001$) than Groups 1 and 2.

pressed infected mice and 64 in immune mice that were not immunosuppressed, and non-immunosuppressed mice had a CF-antibody titer of 128. Thus, when mice were immunosuppressed at a time when they already had HI and CMI to CMV, CMI was completely abrogated, but HI was relatively unaffected.

Immunosuppression with ALG and prednisolone completely abrogated blast transformation to PHA, Con A, and LPS 2 days after cessation of immunosuppression (32 days after virus inoculation) (Table V). CMV which can decrease HI and CMI in the early postinfection period, did not affect responsiveness to PHA or to LPS but did cause a slightly lowered response to Con A ($P < 0.01$). Mice that were infected in addition to being immunosuppressed had lower blast transformation to PHA, Con A, and LPS than did uninfected immunosuppressed mice ($P < 0.01$). When mice were tested 46 and 67 days after virus inoculation they were still partially immunosuppressed by the ALG and prednisolone they had received 16 and 37 days earlier.

Lethal challenge with CMV in immunosuppressed mice. Mice that had previously been inoculated with CMV and immunosuppressed were challenged with 10^6 pfu ip at a time (2 days after immunosuppression) when they had previously been shown to have HI to CMV but no demonstrable CMI. Thirteen of fifteen (87%) uninfected, control mice and 9 of 10 (90%) uninfected, immunosuppressed mice succumbed to the viral challenge. However, mice previously immunized to CMV did not succumb to a second CMV challenge whether or not they were immunosuppressed, indicating that HI is sufficient to protect mice from an ip challenge with CMV.

Discussion. Using an *in vitro* system for studying cellular immunity to CMV we showed that a brief course of immunosuppression given at the time of virus infection prevents the development of CMV but not of HI to CMV. Similarly, if immunosuppression was administered at a time when mice already demonstrated HI and CMI to CMV, CMV was abrogated but HI was not. This model provides an experimental method for studying the relationship of immunity to virus infections and immunosuppression.

Infections with viruses—especially with

members of the herpes virus family—have been seen much more frequently since the widespread use of chemotherapeutic agents and immunosuppression for transplantation (10–12). Infections with varicella-zoster are especially common in patients with neoplasms of the lymphoreticular system and in patients receiving cancer chemotherapy for other disorders. Similarly, CMV occurs in up to 96% of renal transplant recipients (12). The origin of the CMV infections has been attributed to donor kidney tissue and to reactivation of a latent infection of the recipient caused by the immunosuppressive drugs and/or the ever-present immune response associated with transplant rejection.

Whatever the origin of the virus, there has been little relationship between presence or valve of the CF-antibody titer and CMV infection—whether clinically apparent or not. CMI to CMV has not been well studied in transplant recipients, and yet evidence indicates that CMI—especially to herpes group viruses—is probably a more important host defense than HI in ridding the host of an established infection (12). Herpes viruses are cell-associated viruses and may not exist in the plasma where they are subject to serum antibody. In this regard when CMV is present in the blood it can be cultured from the buffy coat but only rarely from the plasma. In an *in vitro* system Notkins (13) showed that immune lymphocytes were able to prevent the spread of herpes simplex virus in a fibroblast monolayer whereas antibody was not. This study indicates that animals receiving immunosuppression have defective CMI to CMV but the HI remains normal. This loss of CMI in immunosuppressed transplant recipients may explain in part the high increase of CMV infections in these patients.

Summary. C57Bl/6 mice were immunosuppressed with antilymphocyte globulin and prednisolone around the time of infection with cytomegalovirus (CMV) or from 18 to 30 days after infection. Mice immunosuppressed around the time of CMV infection developed humoral immunity (HI) but not cell-mediated immunity to CMV. The ability of spleen cells to transform to nonspecific mitogens was also depressed in immunosuppressed mice. If mice were immunosuppressed from 18 to 30 days after infection, a time they are known to have CMI to CMV, the CMI disappeared and remained low for the duration of the study (67 days after infection). However, HI was not affected. Transformation of spleen cells to nonspecific mitogens similarly remained low. These studies demonstrate that immunosuppression can abrogate CMI but not HI to CMV.

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