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Immunosuppression-Induced Defective Lymphocyte Proliferation to Murine Cytomegalovirus Is Prolonged following the Cessation of Immunosuppression[1] (41525)

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Abstract. Defective murine cytomegalovirus (MCMV) antigen-specific proliferation, induced by treatment of MCMV-infected mice with either antilymphocyte globulin (ALG), prednisolone, or both agents, was eventually restored following the cessation of immunosuppression. At 100 and 278 days following the end of immunosuppressive therapy splenic lymphocytes from infected and subsequently immunosuppressed mice responded significantly *in vitro* to soluble MCMV antigen after having lost this response immediately upon initiation of immunosuppression. Circulating specific antibodies and mitogen-induced blast transformation were comparable between infected mice and infected mice that also were immunosuppressed. At 278 days following the cessation of immunosuppression splenocytes from infected mice that had been treated with ALG yielded greatly increased background proliferation. Nylon-wool adherence was used to obtain enriched populations of T cells, and B cells and monocytes from MCMV-infected mice. While T cells alone did not respond *in vitro* to MCMV antigen, recombining B cells and monocytes with the T cells reconstituted *in vitro* proliferation. Defective lymphocyte proliferation to MCMV for an extended period of time following the end of immunosuppressive therapy indicated a prolonged inability to respond to an active MCMV infection. Identification of the cellular basis for the proliferation defect might lead to the development of effective immunotherapy.

Both cell-mediated (CMI) and humoral immunity play important roles in recovery from acute viral infections (1), although patients with intact humoral immunity but deficient CMI are more susceptible to viral infections than are patients with deficient humoral immunity alone (2). Cytomegalovirus (CMV) is cell associated and may not be available to attack by extracellular antibody; therefore, CMI may be a more important host defense against this virus. Immunosuppressed human allograft recipients suffer substantial posttransplant morbidity and mortality due to CMV, despite evidence of serologic and cellular immunity prior to transplantation. Pollard *et al.* demonstrated a correlation between CMV-specific humoral immunity and CMI in subjects presumed to be naturally immune (3). Immunosuppression to prevent renal allograft rejection resulted in depression of *in vitro* mitogenesis and long-term abrogation of CMV-specific lymphocyte proliferation while levels of circulating CMV-specific complement-fixing (CF) antibodies were transiently reduced (4). The lack of demonstrable CMI to herpes simplex and varicella–zoster viruses correlated with susceptibility to these viruses in cardiac transplant recipients (5).

To help increase understanding of immunity to CMV in the immunocompromised patient, we have been working with a mouse model involving murine CMV (MCMV) infection and have developed a method for evaluating MCMV-specific CMI (6). Using this procedure, we reported that immunosuppression of previously infected mice with either prednisolone or antilymphocyte globulin (ALG) immediately decreased lymphocyte proliferation in response to soluble MCMV antigen (7).

We report here data that complete our

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observations of the duration of depressed MCMV-specific lymphocyte transformation after cessation of immunosuppression. We also present results from experiments designed to identify the lymphocyte subpopulations responding *in vitro* to soluble MCMV antigen.

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Materials and Methods. *Mice.* Six-week-old C57BL/6NCr1BR 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. MCMV was originally obtained from June Osborne, University of Wisconsin, Madison. A pool of MCMV was prepared from the homogenate of salivary glands taken from Swiss–Webster mice 14 days after infection with MCMV (9). The pool had a titer of 2×10^7 plaque-forming units (pfu)/ml when assayed with secondary mouse embryo fibroblast cells.

Preparation of viral antigen. MCMV antigen for the *in vitro* humoral immunity and lymphocyte proliferation assays was prepared as described previously (6). Briefly, secondary Swiss–Webster mouse embryo fibroblasts were grown to confluence in 1000-cm^2 roller bottles. Each bottle was then inoculated with 10^6 pfu of MCMV (a multiplicity of infection of 0.01). When the cytopathic effect involved 80 to 90% of the monolayer, approximately 5 days after MCMV inoculation, adherent cells were scraped off the glass into the medium and the medium was harvested, frozen at −70°, and thawed to release intracellular virus. The tissue culture medium (1000 ml) was clarified of cell debris by centrifugation for 20 min at 4800*g* in a Beckman J-21 ultracentrifuge. The supernatant was carefully decanted and centrifuged for 60 min at 32,296*g* in a Beckman L5-50 ultracentrifuge. The resulting pellet was resuspended in 40 ml of Hanks' balanced salt solution and centrifuged in an SW-27 rotor for 60 min at 131,453*g* and then was resuspended in a final volume of 2 ml (a 500-fold concentration). The pellet suspension was titrated for infectious virus as described previously (6) and contained 7.7×10^5 pfu/ml. Protein content was 2000 μg/ml as measured by the Lowry method (10). The virus suspension was heat inactivated for 60 min at 56°, after which no infectious virus was detectable in cell culture, and then stored at −70° until use. A control antigen was also prepared and processed as described above from fibroblasts not infected with MCMV.

Cell proliferation assay. The cell culture technique that we utilized has been described previously (11). Mice were sacrificed and their spleens were removed aseptically. Splenic lymphocytes were suspended in RPMI 1640 medium containing 10% heat-inactivated fetal calf serum, 100 U of penicillin/ml, and 100 μg of streptomycin/ml. Erythrocytes were removed from the cell suspension by the Ficoll–Hypaque technique of Boyum (12). For all experiments 2.5×10^5 cells in 0.1 ml of medium were seeded in individual wells of microtissue culture plates (Linbro 76-003-05, Flow Laboratories, Inc., Rockville, Md.) and 2-mercaptoethanol was added to the incubation medium at a final concentration of 0.05 m*M*. One microgram (Lowry protein) of MCMV antigen or control antigen was added in 0.1 ml of medium to replicate wells for each group of animals assayed, and the plates were incubated for 6 days at 37° in a 5% CO_2 atmosphere. Blast transformation experiments were conducted as described previously (8). One microgram of concanavalin A (Con A, Sigma Chemical Co., St. Louis, Mo.), 0.5 μg of phytohemagglutinin (PHA purified; Burroughs Wellcome Co., Research Triangle Park, N.C.) or 2.0 μg of *Escherichia coli* 026:B6 endotoxin lipopolysaccharide (LPS, Difco Laboratories, Detroit, Mich.) was added in 0.1 ml of medium to triplicate wells that contained 2.5×10^5 spleen cells. The cultures were incubated for 48 hr at 37° in a 5% CO_2 atmosphere. For the last 6 hr of incubation of the proliferation assays, 1 μCi of [*methyl*-^{3}H]thymidine (sp act, 6.7 Ci/mmole) was added to each well. The plates were frozen at −20° at the end of incubation and then

thawed before individual wells were harvested on glass fiber filter paper with a Skatron cell harvester (Flow Laboratories, Inc., McLean, Va.). The radioactivity of the individual wells was determined as counts per minute (cpm) by liquid scintillation counting with a Beckman LS-3133T scintillation counter. Results were expressed as the mean and standard error of replicate cultures.

MCMV CF antibody. Mice were bled at sacrifice from the axillary artery and vein and the blood was transferred with Pasteur pipets into glass tubes. Sera were separated, heat inactivated for 30 min at 56°, and stored at −70° until all samples had been collected. CF antibody titers were measured in a microtiter system by the standard method of our diagnostic virology laboratory (13). The assay was done by using 4 U of the MCMV antigen, 1% sensitized sheep erythrocytes, and 2 U of guinea pig complement. Results were expressed as reciprocal titers.

Immunosuppression. Mice were immunosuppressed for 13 days with intraperitoneal injections of (i) 0.5 mg (25 mg/kg) of prednisolone, (ii) 5 mg (250 mg/kg) of ALG, and (iii) both prednisolone and ALG. The mouse ALG was prepared in horses by Richard Condie of the Minnesota ALG Program.

Experimental design. Animals that received virus were injected intraperitoneally with 2×10^5 pfu of wild-type MCMV. Immunosuppression was initiated on Day 18 following virus injection and continued through Day 30. Prednisolone was administered daily and ALG was administered every other day; a control group of virus-infected mice did not receive immunosuppression. Two to three animals from each group were sacrificed at each time point and tested for MCMV-specific immunity and mitogen-induced blastogenesis. The data presented here are for time points that occurred at several months following the cessation of immunosuppression. For the nylon-wool separation experiments, mice were injected intraperitoneally with 2×10^5 pfu of wild-type MCMV. At 18 days after infection, the time at which peak MCMV immune responses developed (6), the animals were sacrificed and their Ficoll–Hypaque purified splenocytes were separated into nylon-wool-adherent and nonadherent subpopulations. The separated subpopulations and the subpopulations recombined in different proportions were assayed for mitogen-induced and MCMV-specific lymphocyte transformation.

Separation of lymphocyte subpopulations. Ficoll–Hypaque purified splenocytes were enriched for B cells and monocytes (14) or for T cells (15) by passage through nylon-wool columns. Briefly, nylon wool was obtained from a Leucopak (Fenwall Laboratories, Inc., No. LD-1, Morton Grove, Ill.), washed extensively in deionized water, and dried. Each column consisted of a 20-cc syringe barrel packed with 1.4 g of washed nylon wool; the columns were autoclaved separately from the syringe plungers. A three-way sterile, nonpyrogenic stopcock (Pharmseal, Inc., No. k-75, Toa Alta, Puerto Rico) was added to the column to control the flow of media. Prior to application of the cells the column was washed with Hanks' balanced salt solution, then filled with media containing serum and incubated at 37° in a 5% CO_2 atmosphere for 60 min, dislodging air bubbles as much as possible. A maximum of 3.0×10^8 cells at a concentration of 7.5×10^7 cells/ml were added to each column and the cells were allowed to adhere by incubation at 37° in a 5% CO_2 atmosphere for 60 min. The nonadherent cell population was obtained by carefully washing the column with media. The adherent cells were obtained by compression of the nylon wool with the syringe plunger.

Treatment of splenocytes with anti-theta antiserum and complement. Ficoll–Hypaque purified splenocytes from MCMV-infected mice were incubated for 30 min at 37° in medium containing a 1/32 dilution of rabbit anti-mouse brain antiserum, Balb/c adsorbed (Thy 1.2, Litton Bionetics, Inc., No. 8301-04, Kensington, Md.), and a 1/5 dilution of guinea pig serum (M.A. Bioproducts No. 30-956J, Walkersville, Md.) as the source of complement. Following this treatment, the remaining cells were washed and resuspended in medium and were assayed for MCMV-specific *in vitro* proliferation and compared with the response of untreated splenocytes.

Fluorescence microscopy. Smears of unseparated and separated splenocytes were

made, air dried, fixed with acetone for 5 min, and stored at −70° until they were stained. The slides were rinsed with phosphate-buffered saline after thawing. T cells were identified by staining with fluorescein-conjugated rabbit anti-mouse brain, Balb/c adsorbed (Thy 1.2, Litton Bionetics Inc., No. 8301-64, Kensington, Md.), at a final dilution of 1/20, and B cells were identified by staining with the fluorescein-conjugated IgG fraction of rabbit anti-mouse IgG (Cappel Laboratories No. 0411-1222, Cochranville, Pa.) at a final dilution of 1/20. The slides were rinsed for 60 min after staining, then viewed at 160× in a Zeiss microscope equipped with transmitted light phase contrast and reflected light fluorescence specific for fluorescein excitation. Quantification of fluorescing cells was achieved with the aid of an ocular grid micrometer by counting the total number of cells in the field using phase-contrast illumination and then counting the number of fluorescing cells with incident blue light illumination. Several fields were counted for each group of stained cells and the percentage showing positive fluorescence determined.

Results. *Mice immunosuppressed following MCMV infection.* Lymphocytes from MCMV-infected and subsequently immunosuppressed mice responded strongly *in vitro* to MCMV antigen at 100 days after the cessation of immunosuppression when compared with uninfected mice (30,725 ± 5923 cpm vs 3509 ± 789 cpm, $P < 0.001$) (Table I). Prior to that time lymphocytes from similarly treated animals did not respond to MCMV *in vitro*. Uninfected mice never yielded significant MCMV-induced lymphocyte transformation throughout the duration of these experiments. Infected but unimmunosuppressed mice responded positively *in vitro* to MCMV antigen when compared with uninfected mice (15,435 ± 3182 cpm vs 3509 ± 789 cpm, $P < 0.001$) (Table I) despite the 4-month time since primary infection. In subsequent experiments mice which had been infected for a relatively short time were used as an additional control. All MCMV-infected animals, whether or not immunosuppressed, had a CF titer of 16 at 100 days after cessation of immunosuppression, while none of the uninfected animals had measurable circulating MCMV-specific CF antibodies. Mitogen response data were not obtained at 100 days after completion of immunosuppression.

Splenocytes from MCMV-infected and subsequently immunosuppressed mice also responded *in vitro* to MCMV antigen at 278 days following the end of immunosuppressive treatment (Table II). Mitogen-induced proliferation was comparable for uninfected, infected, and infected-immunosuppressed groups of mice at that time (Table III). MCMV-specific proliferation in mice that had been infected nearly 10 months before was greater than that of uninfected controls (10,227 ± 2051 cpm vs 5156 ± 911 cpm,

TABLE I. MCMV ANTIGEN-SPECIFIC PROLIFERATION OF SPLENOCYTES 100 DAYS AFTER CESSATION OF IMMUNOSUPPRESSION FOLLOWING MCMV INFECTION[a]

Group	Control antigen	MCMV antigen[b]	Reciprocal CF antibody titer[c]
1. Uninfected, not immunosuppressed	1379 ± 171[d]	3,509 ± 789	<2, <2
2. Infected only	1436 ± 196	15,435 ± 3182[e]	16, 16
3. Uninfected immunosuppressed	1076 ± 142	2,712 ± 602[f]	<2, <2
4. Infected immunosuppressed	2490 ± 421	30,725 ± 5923[e]	16, 16

[a] Mice were infected with MCMV then immunosuppressed from Days 18 to 30 after virus infection with daily injections of 0.5 mg prednisolone and with 5.0 mg ALG on the first and every other day. Mice were sacrificed and their splenocytes assayed on the 100th day following the end of immunosuppression (130 days after MCMV infection).

[b] Murine cytomegalovirus.

[c] Complement-fixing antibody titer of individual mice.

[d] cpm ± SE.

[e] $P < 0.001$ compared with Group 1.

[f] $P < 0.001$ compared with Group 2.

TABLE II. MCMV ANTIGEN-SPECIFIC PROLIFERATION OF SPLENOCYTES 278 DAYS AFTER CESSATION OF IMMUNOSUPPRESSION FOLLOWING MCMV INFECTION[a]

Group	Control antigen	MCMV antigen
1. Uninfected, not immunosuppressed	6998 ± 1433[b]	5156 ± 911
2. Infected only, 308 days after infection	6000 ± 534	10,227 ± 2051
3. Infected only, 44 days after infection	5385 ± 522	56,692 ± 7685[c]
4. Infected, ALG immunosuppressed	53,239 ± 19,351	82,440 ± 7379[c]
5. Infected, prednisolone immunosuppressed	8708 ± 1918	16,533 ± 3723
6. Infected, ALG and prednisolone immunosuppressed	11,227 ± 1391	17,073 ± 2416[c]

[a] Mice were infected with MCMV then immunosuppressed from Days 18 to 30 after virus infection with either daily injections of 0.5 mg prednisolone or 5.0 mg ALG on the first and every other day, or with both agents. Mice were sacrificed and their splenocytes assayed on the 278th day following the end of immunosuppression (308 days after MCMV infection) except for Group 3 which had been infected 44 days before this experiment (264 days after infection of Groups 2, 4, 5, and 6 and 234 days after the end of immunosuppression).

[b] cpm ± SE.

[c] $P < 0.001$ compared with Group 1.

$P < 0.025$) (Table II), while cells from mice infected 44 days prior to assaying yielded a strong MCMV-specific response (56,692 ± 7685 cpm vs 5156 ± 911 cpm, $P < 0.001$). Infected animals treated with ALG alone or in combination with prednisolone yielded a significant *in vitro* response to MCMV antigen when compared with uninfected mice (82,440 ± 7379 cpm and 17,073 ± 2416 cpm vs 5156 ± 911 cpm, $P < 0.001$ and $P < 0.001$, respectively). Infected animals that were treated only with prednisolone had a lower response to MCMV antigen than did the ALG-treated animals when compared with the uninfected group (16,533 ± 3723 cpm vs 5156 ± 911 cpm, $P < 0.01$); this observation was duplicated in a separate experiment at 282 days following the cessation of immunosuppression (data not shown). Cells from MCMV-infected and ALG immunosuppressed mice also proliferated significantly in response to control antigen when compared with the two infected but not immunosuppressed groups (53,239 ± 19,351 cpm vs 6000 ± 534 cpm and 5385 ± 522 cpm, $P < 0.01$ and $P < 0.005$, respectively).

Anti-theta antiserum and complement treatment of splenocytes from MCMV-infected mice. Splenocytes from MCMV-infected mice responded *in vitro* to MCMV antigen at 18 days after infection [12,572 ± 3674 (standard error) cpm vs 531 ± 111 cpm for control antigen, $P < 0.05$]. Anti-theta antiserum and complement treatment of a portion of those splenocytes resulted in a complete loss of MCMV-specific proliferation (961 ± 304 cpm vs 329 ± 53 cpm for control antigen, $P < 0.20$).

Nylon-wool separation of splenocytes from MCMV-infected mice. Separation of splenocyte subpopulations that were enriched for either T cells or B cells and monocytes was achieved using nylon-wool columns. Following separation 90% of the nonadherent cells were theta bearing and 5% were IgG bearing. The nylon-wool-adherent population was 85% IgG bearing and 14% theta bearing, as shown by quantitative immunofluorescent microscopy. Con A stimulation of adherent and nonadherent cells and several recombinations of those subpopulations resulted in uniformly decreasing tritiated thymidine incorporation as the proportion of nonadherent cells was decreased (Table IV). One hundred percent nonadherent cells responded the most to Con A (246,877 ± 10,569 cpm) and enriched B cells responded the least (90,738 ± 3967). LPS stimulation of the recombined cell populations resulted in an increasing blastogenic response as the proportion of nonadherent cells was decreased. However, 100% adherent cells responded less (272,439 ± 22,348 cpm) than the population of 80% adherent and 20% nonadherent cells (402,221 ± 4189 cpm) (Table IV). Following PHA stimulation, nonadherent cells responded the most (267,017 ± 10,196 cpm) (Table IV) and adherent cells the least (100,555 ± 1766 cpm). As the proportion of nonadherent cells was decreased

TABLE III. MITOGEN RESPONSES OF SPLENOCYTES 278 DAYS AFTER CESSATION OF IMMUNOSUPPRESSION FOLLOWING MCMV INFECTION[a]

Group	Media	PHA[b]	LPS[c]	ConA[d]
1. Uninfected, not immunosuppressed	7660 ± 525[e]	153,031 ± 13,406	291,693 ± 13,887	214,677 ± 11,473
2. Infected only, 308 days after infection	9871 ± 206	174,610 ± 4,802	334,061 ± 7,278	194,982 ± 10,138
3. Infected only, 44 days after infection	5953 ± 269	175,763 ± 5,637	283,441 ± 20,328	188,965 ± 309
4. Infected, ALG immunosuppressed	11,729 ± 807	196,886 ± 6,431	228,630 ± 12,324	213,381 ± 12,697
5. Infected, prednisolone immunosuppressed	7870 ± 593	187,191 ± 14,216	284,897 ± 8,810	177,100 ± 14,317
6. Infected, ALG and prednisolone immunosuppressed	5531 ± 71	154,463 ± 9,253	276,647 ± 2,075	220,214 ± 15,467

[a] Mice were infected with MCMV then immunosuppressed from Days 18 to 30 after virus infection with either daily injections of 0.5 mg prednisolone or 5.0 mg ALG on the first and every other day, or with both agents. Mice were sacrificed and their splenocytes assayed on the 278th day following the end of immunosuppression (308 days after MCMV infection) except for Group 3 which had been infected 44 days before this experiment (264 days after infection of Groups 2, 4, 5, and 6 and 234 days after the end of immunosuppression).

[b] Phytohemagglutinin.

[c] *E. coli* lipopolysaccharide.

[d] Concanavalin A.

[e] cpm ± SE.

in vitro, PHA-induced proliferation decreased, but not uniformly in that the population composed of 40% nylon-wool-nonadherent cells incorporated more radioisotope (215,191 ± 30,178 cpm) than did the population made up of 60% nonadherent cells (186,387 ± 14,540 cpm). Prior to nylon-wool separation MCMV-specific transformation was significant compared with control antigen (73,338 ± 7345 cpm vs 9699 ± 2379 cpm, $P < 0.001$) (Table V). Nonadherent cells alone did not proliferate in response to MCMV antigen when compared with unseparated splenocytes (4645 ± 1639 cpm vs 73,338 ± 7345 cpm, $P < 0.001$). Recombining the nylon-wool-adherent and -nonadherent cells resulted in restoration of the MCMV-specific response to levels comparable to unseparated splenocytes. When the *in vitro* population was composed of 20% adherent and 80% nonadherent cells MCMV-induced proliferation was readily apparent (50,171 net cpm) (Table V). Net tritiated thymidine incorporation was greatest when the recombined cells were 60% nonadherent and 40% adherent (92,040 net cpm) and as the proportion of recombined nonadherent cells was decreased from 60 to 0% the net MCMV-induced proliferation declined.

Discussion. Using viral antigen-induced lymphocyte proliferation as a measure of MCMV-specific cellular immunity, we showed that MCMV-specific CMI is eventually restored following the cessation of immunosuppression. Restoration of the MCMV-specific response occurred well after the time at which mitogen-induced lymphocyte proliferation had returned to normal levels (8) and at a time when the primary response of infected but unimmunosuppressed mice had declined to a low level. The data presented here extend our observations of the effect of immunosuppression on immunity to MCMV infection.

Utilizing the same lymphocyte proliferation procedure, we showed that pretreatment of the cells with anti-theta serum and complement abrogated MCMV-specific transformation as did removal of nylon-wool-adherent cells. Readdition of nylon-wool-adherent cells to the nonadherent cells restored viral antigen-induced proliferation.

Several clinical and experimental obser-

TABLE IV. MITOGEN RESPONSES OF UNSEPARATED, AND NYLON-WOOL-SEPARATED AND RECOMBINED SPLENOCYTES FROM MCMV-INFECTED MICE[a]

Cells	PHA	Con A	LPS
100% nonadherent	267,017 ± 10,196[b]	246,877 ± 10,569	135,519 ± 17,603
80% nonadherent 20% adherent	206,339 ± 18,569	219,774 ± 16,441	301,323 ± 12,752
60% nonadherent 40% adherent	186,387 ± 14,540	208,690 ± 37,137	321,397 ± 4,883
40% nonadherent 60% adherent	215,191 ± 30,178	181,940 ± 15,512	342,837 ± 20,287
20% nonadherent 80% adherent	202,872 ± 7,212	157,840 ± 19,416	402,221 ± 4,189
100% adherent	100,555 ± 1,766	90,738 ± 3,967	272,439 ± 22,348

[a] Mice were infected intraperitoneally with 2×10^5 pfu of wild-type MCMV 19 days prior to sacrifice. Nonadherent (T cells) and adherent (B cells and monocytes) cell populations were obtained using nylon-wool columns.
[b] cpm ± SE.

vations have indicated that CMI may be a more important host defense than humoral immunity against herpes group virus infections. Patients with cell-mediated immune defects are subject to severe virus infections (2). Also, CMV-infected renal allograft recipients suffer morbidity and mortality despite demonstrable circulating specific antibodies (4, 16). CMV is a cell-associated virus and as such may not be accessible to attack by serum antibody (17). Notkins showed that the spread of herpes simplex virus in a fibroblast monolayer was prevented by immune lymphocytes but not by antibody (18). In addition, Ho demonstrated the protective effect of passively transferred MCMV-specific cytotoxic lymphocytes and loss of that protection by pretreatment of the cells with anti-theta serum and complement (19).

In a study of persons presumed to be naturally immune, none of those seronegative to CMV and 90% of those seropositive to CMV demonstrated significant *in vitro* antigen-induced proliferation (3). Following renal transplantation, humoral immunity and CMV-induced blastogenesis became dissociated (4). Lymphocyte proliferation to CMV was absent for up to 3 years following cardiac transplantation and immunosuppression (3). Since there is little relationship between CF antibody titer and protection from CMV infection (16), immuno-

TABLE V. MCMV ANTIGEN-SPECIFIC PROLIFERATION OF UNSEPARATED, AND NYLON-WOOL-SEPARATED AND RECOMBINED SPLENOCYTES FROM MCMV-INFECTED MICE[a]

Cells	Control antigen	MCMV antigen	Net
Unseparated	9,699 ± 2379[b]	73,338 ± 7345	63,639
100% nonadherent	463 ± 67[c]	4,645 ± 1639[c]	4,182
80% nonadherent 20% adherent	36,396 ± 12,518	86,567 ± 9550	50,171
60% nonadherent 40% adherent	9,308 ± 1327	101,348 ± 11,330	92,040
40% nonadherent 60% adherent	15,897 ± 1564	103,801 ± 7885	87,904
20% nonadherent 80% adherent	15,093 ± 1985	93,620 ± 4979	78,527
100% adherent	10,358 ± 654	35,872 ± 2987	25,514

[a] Mice were infected intraperitoneally with 2×10^5 pfu of wild-type MCMV 19 days prior to sacrifice. Nonadherent (T cells) and adherent (B cells and monocytes) cell populations were obtained using nylon-wool columns.
[b] cpm ± SE.
[c] $P < 0.001$ compared to unseparated cells.

suppression following transplantation with its attendant effect on CMV-specific CMI may render the recipient incapable of responding to either exogenous or endogenous CMV infection. In this regard, the use of antithymocyte globulin (ATG) has been correlated with loss of the *in vitro* lymphocyte response to CMV antigen and ATG-treated renal transplant recipients had more frequent viremia and CMV-related illnesses than did recipients that did not receive ATG (20).

Using naturally chronically infected wild mice, Gardner *et al.* observed systemic, disseminated MCMV infection following treatment of those mice with injections of rabbit anti-mouse thymocyte sera (21). Jordan *et al.* showed that ALG treatment of mice which had been latently infected with MCMV resulted in reactivation of the infection and that combining cortisone treatment with ALG resulted in reactivation and dissemination (22). In our model, mice were immunosuppressed shortly after virus infection at a time when infectious virus could be readily isolated in tissue culture from the salivary glands (unpublished data). Histologic examination of stained tissue sections of several organs and tissue culture isolation techniques did not disclose a difference between infected and infected-immunosuppressed mice during the period of immunosuppression and early during the postimmunosuppression period (unpublished data).

The data presented here revealed that while the MCMV-specific proliferation response (Table II) of infected, ALG-treated mice (Groups 4 and 6) was significant, the primary responses of mice infected at the same time but that were not immunosuppressed (Group 2) or that were treated only with prednisolone (Group 5) were not significant. These results may have provided evidence that ALG immunosuppression altered lymphocyte responses to the extent that the primary MCMV infection did not resolve until months after cessation of immunosuppressive therapy. Further support for the persistent alteration of immune function by ALG administration was the high background counts (Table II, Groups 4 and 6; and Table III, Group 4) obtained from infected mice that had been treated with ALG. While *in vitro* virus replication and incorporation of the radioactive label into viral DNA might account for the increased background cpm, this seems unlikely since Booss and Wheelock found that the presence of infectious MCMV suppressed *in vitro* DNA synthesis by spleen cells (23). Also, microscopic examination of the lymphocyte cultures at the time of radioisotope addition revealed many clones of cells in the wells that yielded high background cpm but not in the wells showing a lower, more normal background cpm. We have previously reported increased *in vitro* proliferation of unstimulated splenocytes during the first 10 days following MCMV infection (6), and Wu and Ho (24) as well as Osborne and Walker (25) demonstrated that viremia and virus dissemination occurred during the first 10 days after experimental MCMV infection. Therefore, the high background proliferation observed in the present study for MCMV-infected mice subsequently treated with ALG may have been evidence of prolonged virus dissemination to which the mice were immunologically responding at the time of sacrifice 9 months after cessation of immunosuppression.

Having identified a prolonged but reversible *in vitro* MCMV-specific immune defect induced by immunosuppression, we turned our efforts toward identifying the lymphocyte subpopulation(s) responsible for intact MCMV-specific proliferation by nylon-wool separation of splenocytes from MCMV-infected mice. While T cells (nylon wool nonadherent) enriched by this technique responded to PHA, Con A, and LPS (Table IV), they did not respond to MCMV antigen (Table V). MCMV blastogenesis was restored by readdition of adherent cells to the nonadherent cells and levels of radioisotope incorporation were similar over a wide range of proportions of adherent and nonadherent cells. Pass *et al.* observed a similar effect on CMV-specific proliferation when human mononuclear cells were fractionated using adherence to plastic and then recombined (20). Following nylon-wool separation, adherent cells were able to respond significantly to MCMV antigen. Treatment of splenocytes from MCMV-infected mice with anti-theta antiserum and complement completely ablated the MCMV-specific response, suggesting that the response to viral antigen of the nylon-wool-adherent cells was due to the

presence of theta-bearing cells. Our data indicated that while nylon-wool-adherent and -nonadherent cells both may contribute to intact MCMV-induced proliferation, neither T cells alone nor B cells and monocytes alone could support significant antigen-specific proliferation.

Defective CMV-specific lymphocyte transformation following immunosuppression of allograft recipients could facilitate reactivation of CMV and prevent the host from responding to active infection. Identification of the defective immune component could result in the development of specific immunotherapy.

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