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### Research Article

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# Viral Infection-Homograft Interactions in a Murine Model

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**ABSTRACT** The effects on some host defenses of murine cytomegalovirus (MCMV) and(or) EL<sub>4</sub>, a mouse ascites homograft, were studied in mice. Assays of cellular and humoral immunity in response to either or both of these perturbations were carried out by quantitation of various immune activities.

Limited studies demonstrated no effect of EL<sub>4</sub> inoculation on the host response to MCMV by organ viral titer, duration of viral persistence, or anti MCMV complement-fixing antibody titer.

Prior infection with MCMV, however, resulted in greatly reduced numbers of splenocytes, the source in this study of immune effector cells. Residual splenocytes demonstrated less response to both phytohemagglutinin and lipopolysaccharide, particularly in the 2-3-wk interval after infection. Similarly, responder cells in mixed lymphocyte cultures were less reactive when derived from infected animals. Lymphocyte-mediated cytotoxicity of EL<sub>4</sub> was significantly less in mice infected on the day of and 7, 14, and 21 days before the tumor homograft with a return to control levels by 28 days. 90% of the cell-mediated cytotoxicity could be eliminated by treatment with anti-theta serum. Serum-mediated cytotoxicity of EL<sub>4</sub> was also reduced in infected animals. No suppressor cells or serum inhibitory factors could be identified in infected animals. Although alternative explanations exist, this study suggests that in infected animals there is a significant reduction in both the number and function of bone marrow-derived and thymus-derived cells directed against the alloantigens in EL<sub>4</sub>.

## INTRODUCTION

The hosts of infectious agents, homografts, and certain tumors share a partial dependence on cell-mediated immunity and humoral immunity for survival (1-8). It is not certain that all the same recognition and effector

processes are involved but it seems likely that there are common mechanisms and interactions, both enhancing and suppressing the cellular response to these foreign agents.

Clinical evidence exists to support the hypothesis that there are interactions between infections and grafts. For example, the increased risk and variety of infections seen in patients with renal homografts is well known (9, 10). Although this is undoubtedly related to the underlying disease, to increased exposure to potential pathogens, to invasive procedures, and especially to drug-induced immunosuppression, it is possible that immune mechanisms involved in graft acceptance or rejection alter the host's response to infection. Examples are the activation of latent virus (11, 12) and enhancement of infection (13, 14).

Animal studies suggest an effect of bacteria, bacterial products, mycobacteria, viruses, and oncogenic viruses on graft survival (15-22). These studies show increased, decreased, or no effect on graft survival depending on the system. For example, Bacillus Calmette-Guérin reduced graft and tumor survival in both animals and humans (23-30). Simmons et al. (31-33) and Lopez et al. (34-36) suggest that renal homografts fare less well in cytomegalovirus-infected patients than in uninfected patients. Briggs et al. (37) and David et al. (38) present additional cases suggesting that viruses can enhance immunity and accelerate graft rejection. Most data, however, suggest viruses, including cytomegalovirus, as the cause of immunodepression (1, 39-43). Osborne and co-workers (44-46), Selgrade et al. (47), and Howard et al. (48, 49) specifically identified suppression of humoral and cell-mediated immunity in animals infected with murine cytomegalovirus (MCMV).<sup>1</sup> Some tumor viruses (50) and the enterotoxin

<sup>1</sup> *Abbreviations used in this paper:* B, bone marrow-derived; LPS, lipopolysaccharide; MCMV, murine cytomegalovirus; PBS, phosphate-buffered saline; PHA, phytohemagglutinin; T, thymus-derived.

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isolated from *Vibrio cholera* (51, 52) are known to suppress cell-mediated immunity.

This study examines some of the interrelationships between MCMV infection and host immunity to EL<sub>4</sub>, a peritoneal ascites tumor allograft. In particular, the effect on cell function is studied.

## METHODS

### *Animals*

Inbred strains (male and female) of specific pathogen free mice (The Jackson Laboratory, Bar Harbor, Maine) with defined histocompatibility loci were used in all experiments. The 18–20-g mice were housed in cages with four littermates for 2–3 wk before experiments and fed standard mouse chow and water ad libitum. Specific mouse strains included BALB/c (H-2<sup>d</sup>), C57/BL/6J (H-2<sup>b</sup>) and B10.A (H-2<sup>a</sup>).

### *Virus*

MCMV, a subculture of the Smith Strain, was maintained through serial passage in (BALB/c) and mouse embryo tissue culture. The virus was titered by plaque assay, distributed in vials, and held in liquid nitrogen until use. Mice were infected by injection of 10<sup>4</sup> plaque-forming units intraperitoneally and no deaths or apparent disease ensued. Spleen and salivary gland viral titers were performed at weekly intervals after infection on excised, weighed tissue.

### *Tumor allograft*

EL<sub>4</sub>, an ascites tumor, was maintained by serial weekly passage in C57/B6/6J mice. Before experimental injection, the cells were harvested and washed free of debris in phosphate-buffered saline (PBS), the erythrocytes were lysed with Tris-NH<sub>4</sub>Cl, and the tumor re-suspended in PBS.

### *Experimental protocol*

Animals were infected at various intervals before intraperitoneal grafting of EL<sub>4</sub>. 11 days after grafting with 25 × 10<sup>6</sup> tumor cells, the animals were sacrificed and the following assays performed.

### COMPLEMENT-FIXATION TEST FOR MCMV ANTIBODY

Serum was collected from every test and control animal, heat inactivated at 56°C for 30 min, and kept frozen at -70°C until use in the microtiter complement-fixation test as described by Harris (53). The complement-fixation antigen was prepared by infecting monolayer cultures of secondary mouse embryonic fibroblasts in a roller bottle with MCMV. The infected cells were harvested 48–72 h later when the cell layer showed a 100% cytopathic effect. The infected cell layer was rinsed once with glycine buffer, pH 8.6. 30 ml of fresh buffer was added and the cells were removed from the glass surface by gentle scraping with a rubber policeman. The cell suspension was sonicated and stored in small aliquots at -70°C until used.

### LIGHT AND ELECTRON MICROSCOPY

Spleens were excised after mouse exsanguination and photographed. For electron microscopy, blocks of splenic

tissue were fixed in cacodylate-buffered glutaraldehyde. Multiple thin sections from four Epon blocks (Shell Chemical Co., Houston, Tex.) from two different animals in each experimental group were carefully examined. Areas selected for thin sectioning were chosen by examining by light microscopy the toluidine blue-stained, 1- $\mu$ m sections of Epon-embedded tissue. These toluidine blue-stained 1- $\mu$ m sections were studied together with the larger paraffin-embedded hematoxylin and eosin sections to assure proper sampling. Both red and white pulp were examined by electron microscopy. Peritoneal exudate cells were harvested by three washes with PBS, sedimented by centrifugation, and the pellet fixed in cacodylate-buffered glutaraldehyde. Sections for light microscopy were made from 10% formalin-fixed blocks and stained with hematoxylin and eosin.

### IMMUNOLOGIC ASSAYS

*Lymphocyte stimulation.* This was performed by a modification of the procedure described by Oppenheim and Schecter (54). Spleens from sacrificed mice were harvested, minced gently, washed in RPMI 1640 and 5% fetal calf serum, the erythrocytes lysed with Tris NH<sub>4</sub>Cl, and the splenocytes suspended as 5 × 10<sup>6</sup> viable cells/ml and distributed in 0.2-ml aliquots in Linbro microtiter wells (Linbro Chemical Co., Hamden, Conn.) in triplicate. Control and mitogen-(phytohemagglutinin [PHA-M] from Gibco Diagnostics, Chagrin Falls, Ohio, 5  $\mu$ g/ml, and *Escherichia coli* lipopolysaccharide [LPS-Type I] from Sigma Chemical Co., St. Louis, Mo., 50  $\mu$ g/ml) stimulated cultures were done. Plates were incubated at 37°C in 5% CO<sub>2</sub> for 3 days and labeled with 0.1 ml of 10  $\mu$ Ci/ml [<sup>3</sup>H]thymidine in RPMI 1640 with 5% fetal calf serum 18 h before harvesting with a microtiter harvester. Samples were collected on glass fiber filter disks, placed in Aquasol (New England Nuclear, Boston, Mass.), and counted in a Beckman scintillation counter (Beckman Instruments Inc., Fullerton, Calif.).

*Mixed lymphocyte cultures.* These were performed according to a modification of the procedure described by O'Leary et al. (55). Stimulator cells were incubated with 25  $\mu$ g mitomycin C for each 10<sup>7</sup> cells at 37°C for 20 min to inhibit DNA synthesis and then washed three times, and 10<sup>6</sup> stimulator cells were incubated with equal numbers of viable responder cells in Linbro microtiter plates (Linbro Chemical Co.) in triplicate. These mixed lymphocyte cultures were then incubated at 37°C in 5% CO<sub>2</sub> for 2–3 days and labeled with [<sup>3</sup>H]thymidine for 18 h before harvesting as previously described.

*Lymphocyte-mediated cytotoxicity.* This was performed according to the techniques of Berke et al. (56, 57). 25 × 10<sup>6</sup> EL<sub>4</sub> cells were inoculated intraperitoneally into BALB/c mice, some of which had been previously infected. 11 days later, the animal groups were anesthetized and sacrificed by exsanguination. The peritoneal cavity was washed with 10% fetal calf serum in PBS and the absorbance of the suspension measured. Grossly bloody fluids were not encountered. The spleen was removed and minced gently. Clumped debris was removed and the erythrocytes were lysed with Tris-NH<sub>4</sub>Cl. The splenocytes were washed and resuspended for the cytotoxicity assay. Tumor cells were labeled by the addition of 125  $\mu$ Ci of Na<sub>2</sub>CrO<sub>4</sub> (<sup>51</sup>Cr) to 30 × 10<sup>6</sup> EL<sub>4</sub> cells and incubated at 37°C for 45 min followed by three washes with 10% fetal calf serum in RPMI 1640. The splenocytes (4 × 10<sup>6</sup> in 1 ml) were mixed with the labeled EL<sub>4</sub> target cells (5 × 10<sup>4</sup> in 0.1 ml) in triplicate in Linbro flat bottomed wells (Linbro Chemical Company) and included for 3 h on a rocking platform at 37°C. Cytotoxicity was terminated by placing the plates in an ice bath. The contents of each well were aspirated and centrifuged at 2,000 rpm for 10 min at 4°C. An aliquot of each supernate was extracted and

counted in a Nuclear Chicago gamma counter (Nuclear Chicago Corp., Des Plaines, Ill.). Radioactivity measured free  $^{51}\text{Cr}$ , including that released from labeled target cells by cytolysis. Percent cytolysis was calculated from the following equation: percent cytolysis = (experimental - control/total)  $\times$  100, where experimental = counts per min released from labeled target cells in the presence of immune cells, total = counts per minute released from target cells in presence of 1 N HCl, and control = counts per minute released from target cells alone. Cytolysis was nonantibody and noncomplement dependent and is almost completely a thymus-derived (T)-cell function. Susceptibility of splenocytes to anti-theta serum (adsorbed rabbit anti-mouse brain serum from Litton Bionetics, Div. of Litton Industries Inc., Kensington, Md.) was tested by incubation of 0.2 ml serum with  $10 \times 10^6$  splenocytes for 30 min in the presence of guinea pig complement followed by retesting for lymphocyte-mediated cytolysis.

**Serum-mediated cytolysis.** Sera collected from mice sacrificed in the above protocols were tested for lytic activity against  $^{51}\text{Cr}$ -labeled tumor cells as described by Canty et al. (58). 50- $\mu\text{l}$  vol of serially diluted and heat inactivated serum from experimental animals were incubated at room temperature in 13  $\times$  75-mm glass tubes for 30 min with 50  $\mu\text{l}$  PBS that contained  $1 \times 10^6$   $^{51}\text{Cr}$ -labeled target cells. The reaction was stopped with cold PBS. All tubes were centrifuged at 1,000 rpm for 10 min and the supernate discarded. The sediment was further incubated at 37°C for 45 min with 50  $\mu\text{l}$  of rabbit complement which had been adsorbed with EL<sub>4</sub>. Again the reaction was stopped with cold PBS and the tubes centrifuged at 2,000 rpm. Aliquots (0.2 cm<sup>3</sup>) of supernates were placed in counting tubes and counted in a Nuclear Chicago gamma counter. Percent lysis was calculated as follows: percent lysis = 100  $\times$  (counts per minute experimental - counts per minute control [negative control]) / (counts per minute total [positive control]).

**Suppressor cell function in lymphocyte-mediated cytolysis.** Spleen cells from animals infected with MCMV 18 days previously were studied for possible suppressor or helper activity in lymphocyte-mediated cytolysis with sensitized spleen cells from animals only grafted with EL<sub>4</sub> 11 days previously. Appropriate control spleens were also examined. Increasing numbers, from 0 (medium only) to  $4 \times 10^6$ , of cells being assayed for suppressor activity were incubated at 37°C on a rocking platform with standard ( $4 \times 10^6$ ) numbers of sensitized

splenocytes for 3 h in the presence of  $5 \times 10^4$   $^{51}\text{Cr}$ -labeled EL<sub>4</sub> target cells. At the end of the incubation, the  $^{51}\text{Cr}$  was measured in the supernate and cytolysis was calculated as previously noted.

**Serum suppressor factors.** Assays for inhibitory factors were performed by adding 100 or 10  $\mu\text{l}$  (from animals infected 0, 14, and 28 days previously) to sensitized splenic lymphocytes for 1 h at room temperature before performance of the standard cytolysis (lymphocyte-mediated) procedure. Control animal sera and sera from animals only grafted were also tested.

## STATISTICS

The *P* value was calculated with the "t" statistic of means. Further statistical control for multiple comparisons within a mitogen stimulation category was accomplished by adjusting the effective significance level of tests with Bonferroni's inequality correction (59).

## RESULTS

### Viral isolation, distribution, and infection

No deaths or recognized disease caused by MCMV were observed in any of the animals tested. Peak salivary gland virus titers averaged  $2.5 \times 10^6$  plaque-forming units per gram of tissue at 11 days after infection, falling to undetectable levels by 28 days after infection in ungrafted mice. Splenic viral titers were less than those in salivary gland by 25 days after infection. In animals both infected and grafted, salivary viral titers were comparable to those only infected (Fig. 1). In one instance, virus in low titer was detected in the spleen at 8 days in an animal both infected and grafted. Mean anti-MCMV complement-fixing antibody titers were not different for animals infected and grafted or only infected (Fig. 1).

### Morphology and microscopy

The spleens from infected animals were smaller than those from control and grafted animals. Total cell counts confirmed significant (*P* < 0.01) differences (Fig. 2). Light microscopic examination of the spleens from animals infected 18 days before showed massive necrosis and no viral inclusions, whereas the grafted only spleens showed increased cellularity and no identifiable tumor. Spleens from animals both infected and grafted had some necrosis and disorganization of normal architecture but less than in the spleens from animals only infected. No MCMV was seen by electron microscopy.

Light and electron microscopy examination of peritoneal cells from animals grafted and infected revealed many tumor-like cells in mitosis, comparatively few macrophages, prominent viral "C"-type particles, and no MCMV. In contrast, peritoneal exudate cells from grafted but uninfected animals revealed many macrophages, no tumor-like cells in mitosis, few viral C-type particles, and no MCMV.

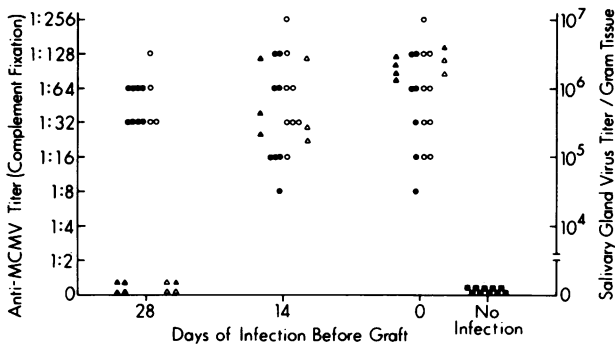


FIGURE 1 MCMV antibody titers and salivary gland virus titers. Uninfected animals (■) had neither detectable virus nor anti-MCMV antibody. Infected only animals had decreasing salivary gland virus titers (▲) over the 28-day period and constant mean MCMV antibody titers (●). Animals both infected and grafted had statistically the same virus titers (Δ) and antibody titers (○) as their infected counterparts.

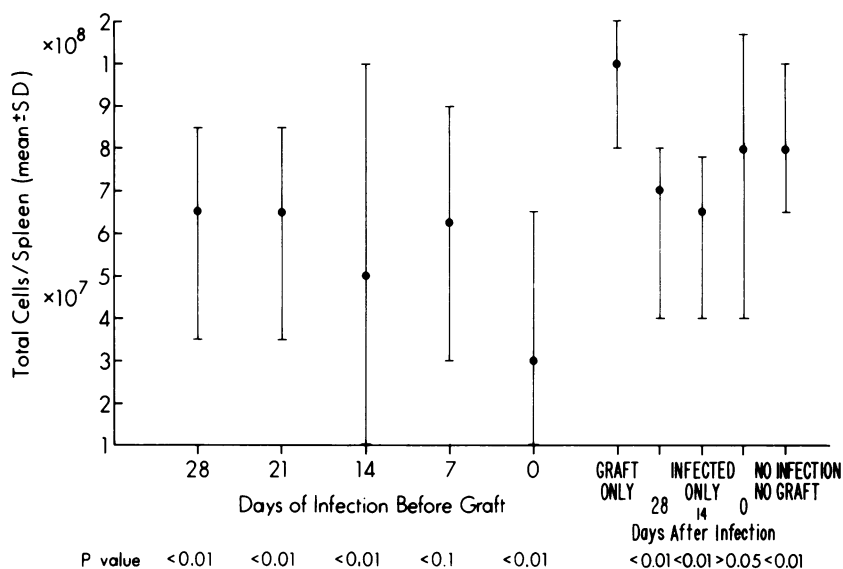


FIGURE 2 Total spleen cells. As compared to grafted animals, infected and grafted animals at every interval studied had mean total spleen numbers significantly less, the maximal reduction beginning on day 0. Animals only infected at days 14 and 28 as well as uninfected and ungrafted animals had significantly reduced cell numbers as compared to the grafted controls.

### Lymphocyte stimulation

Table I shows the results in mitogen stimulated cells from animals infected and(or) grafted as compared to controls. Grafted animals have a significantly increased uptake of tritiated thymidine with lipopolysaccharide (LPS) stimulation but are otherwise similar to controls. In contrast, animals infected 11 days previously have significantly ( $P < 0.05$ ) reduced uptake of thymidine as compared to controls in unstimulated cultures and those stimulated with phytohemagglutinin (PHA) and LPS. Responsiveness to LPS remains significantly reduced in animals infected 18 days before. When the animals have been both infected and grafted, differences from controls seem to represent the additive effects of the two perturbations expressed individually. That is, unstimulated cultures are the same as controls, even when infection preceded the assay by 11 days. PHA and LPS responsiveness in this latter group remained reduced but less so than when the animals were only infected. In the animals infected 18 days previously, uptake was still increased in the LPS stimulated group but less so than when the animals were only grafted.

### Mixed lymphocyte cultures

Table II shows the effects of infection or grafting on mixed lymphocyte cultures. Responder cells, when derived from animals infected 3 wk previously, have a significantly decreased uptake of tritiated thymidine as compared to cells from uninfected animals. Stimulator

cells, however, did not measurably alter the mixed lymphocyte cultures when derived from infected animals. When responder cells from animals grafted 11 days previously with EL<sub>4</sub> were used, uptake was reduced but not significantly.

### Cytolysis

In the model system used, cell- and serum-mediated cytolysis and peritoneal cell counts developed normally as outlined in Fig. 3. Measurable serum-mediated cytolysis developed on day 5 after EL<sub>4</sub> injection and rose rapidly to a plateau by day 7, where it remained through day 14. Cell-mediated cytolysis was measurable in the spleen by day 7 and progressively increased to day 14. Peritoneal cell counts, which were mostly proliferating EL<sub>4</sub> tumor cells, peaked between days 4 and 7 and then, cell counts decreased to base-line levels. Day 11 was selected as an appropriate interval for most subsequent studies in which the system was perturbed by superimposed cytomegalovirus infections at various intervals.

*Lymphocyte-mediated cytolysis.* Neither normal controls nor infected only animals had any demonstrable lymphocyte-mediated cytolysis against EL<sub>4</sub> target cells at any intervals after infection. A dose-response study showed increasingly suppressive effects with doses of MCMV from 10<sup>2</sup> to 10<sup>5</sup> plaque-forming units. A dose of 10<sup>4</sup> particles was selected for later experiments.

Fig. 4 shows the results of splenic lymphocyte-mediated cytolysis in mice infected at various intervals

TABLE I  
Mitogen Stimulation of Splenocytes from Infected  
and(or) Grafted Animals

Experimental group	Tritiated thymidine uptake		
	No mitogen	Mitogen stimulation	
		PHA	LPS
	<i>mean cpm ± SD (× 10<sup>3</sup>)</i>		
Control	15.2 ± 9.6	30.6 ± 21.5	67.6 ± 21.6
Grafted*	25.4 ± 11	42.4 ± 14.5	120.6 ± 17.6 ‡
Infected			
11 days before assay	1.4 ± 1.6 †	0.9 ± 0.7 †	1.8 ± 2.8 †
18 days before assay	8.1 ± 10.6	25.3 ± 20.5	18.9 ± 22.5 †
Grafted* and infected			
11 days before assay	10.2 ± 4.4	8.9 ± 9 §	17.9 ± 12.2 †
18 days before assay	19.5 ± 8.8	41.8 ± 7.6	95 ± 21.5 §

Tritiated thymidine uptake in the splenocytes from infected and(or) grafted animals was compared to control splenocytes in the presence and absence of mitogen (PHA and LPS) stimulation after 3 days of culture. Uninfected but grafted animals had significantly increased uptake in response to LPS. Animals infected 11 days before, however, had significantly reduced uptake as compared to controls with and without mitogen a and with and without grafting. Only the response to LPS was reduced in those animals infected 18 days before.

\* Grafted with EL<sub>4</sub> 11 days before assay.

†  $P < 0.01$  for  $t$  test;  $P < 0.05$  after controlling for multiple tests.

§  $P < 0.05$  for  $t$  test;  $P < 0.10$  after controlling for multiple tests.

before grafting. These data show a statistically significant depression of cytolysis in animals infected at the time of, or 7, 14, and 21 days before grafting as compared to grafted controls. The greatest depression was noted in animals infected 7 days before grafting. Cytolysis progressively increased in animals infected at longer intervals before grafting, and by 28 days no significant effect of prior infection was detectable.

Cytolysis in animals grafted or grafted and infected was 90–100% eliminated by prior treatment of the splenocytes with anti-theta serum plus complement.

*Serum-mediated cytolysis.* Fig. 5 illustrates the results of serum-mediated cytolysis in the same animals. Controls were appropriately negative or positive in ungrafted and sensitized or hypersensitized animals, respectively. Animals only infected but not grafted had no detectable anti-EL<sub>4</sub> activity at the intervals 0, -14, and -28 days. In comparison to grafted animals, however, infected and grafted animals had significantly

lower titers at the -7, -14, -21, and -28 days intervals. As with lymphocyte-mediated cytolysis, maximal depression occurred at -7 days with less depression by day -28.

Peritoneal cell numbers, all in nonbloody fluids, were measured by the absorbancy of an aliquot of aspirated peritoneal fluid. Animals both infected and grafted had more persistent cells but at no interval was the difference statistically significant from grafted only animals. The difference in types of persistent cells was previously noted. Animals that had not been grafted but were infected 0, 14, and 28 days previously had few peritoneal cells (mostly lymphocytes on smear) and the same was true of animals that had been neither infected nor grafted.

*Modifier cells.* The results of the assay for suppressor cells are noted in Table III. They show the expected increased cytolysis when grafted only spleens are added to already sensitized lymphocytes (grafted only). Although the addition of cells from infected animals invariably resulted in a decrease in cytolysis as compared to grafted controls, this was not a dose related response and furthermore, spleen cells from control animals inhibit cytolysis when added to sensitized lymphocytes also. Thus, no cells that tended to modify the response of grafted, sensitized splenocytes could be found.

*Serum suppressor factors.* Assays for serum factors that might account for the suppression of cytolysis were performed. No significant suppressive serum constituents from heat inactivated serum could be demonstrated when the serum was derived from animals infected at -28, -14, and 0 days as compared to grafted controls.

## DISCUSSION

This study demonstrates an association of MCMV-induced effects on the normal immune defense mechanisms of the mouse against EL<sub>4</sub>, an ascites allograft. Whereas normally EL<sub>4</sub> in an allogeneic system evokes a positive immunologic response tending toward its elimination by the recipient host, specific measurements demonstrated deficiencies induced by concomitant or prior MCMV infection. The spleens from grafted animals usually appeared hypercellular as compared to controls. Infection alone or in combination with an EL<sub>4</sub> allograft, however, consistently resulted in necrosis of spleen with fewer numbers of cells. In our experiments, viral replication in the host peaked at nearly the same time interval as the most severe splenic necrosis and as the depressed immunologic functions. Thereafter, viral replication waned and the spleen morphology and immunologic function returned towards normal.

Schwartz (60) documented the morphologic conse-

TABLE II  
Mixed Lymphocyte Cultures of Splenocytes from Infected and(or) Grafted Animals

Source of cells		Tritiated thymidine uptake			P value
Responder	Stimulator	Responder (R)	Stimulator (S)*	MLC (R × S)	
<i>mean cpm ± SD</i>					
I. Uninfected B10.A	Uninfected BALB/c	860±406	335±195	2,538±601	—
Infected B10.A†	Uninfected BALB/c	347±51	465±93	766±407	<0.01
Uninfected B10.A	Infected BALB/c	807±245	300±75	2,804±411	NS
II. Ungrafted BALB/c	Ungrafted C57	1,680±604	747±166	2,979±1,579	—
Grafted BALB/c‡	Ungrafted C57	2,069±777	747±166	1,690±931	NS

Mixed lymphocyte cultures (MLC) of responder splenocytes from uninfected, infected, ungrafted, and grafted mice were performed measuring tritiated thymidine uptake after 3 days in culture with H-2 incompatible donor splenocytes previously treated with mitomycin. Compared to uninfected control responder cells, splenocytes from infected animals had significantly reduced thymidine incorporation whereas no effect was observed if the stimulator cells were from infected animals. Grafted animals' splenocytes did not have the expected response to mitomycin-treated stimulator cells seen with controls.

\* Mitomycin treated.

† Infected with  $10^4$  PFC 3 wk earlier.

‡ Grafted with EL<sub>4</sub> 11 days earlier.

quences of MCMV infection in newborn mice, demonstrating necrosis in spleen and other lymphoid tissues. This confirmed even earlier work by Rowe and Capps (61). Data are available to suggest that human lymphocytes and polymorphonuclear leukocytes are among the cells susceptible to infections with cytomegalovirus (62), and lymphoblastoid cells in particular, have been postulated to be the reservoir for

persistent infections (63). In studies of fractionated splenocytes of adult mice infected at birth or in utero, bone marrow-derived (B) cells were implicated as the source of latent virus although no attempts were made to exclude more generalized (T, B, or null lymphocytes or macrophages) cellular infection in the acute phases of the disease (63). Our studies clearly support the reported susceptibility of splenocytes to infection with

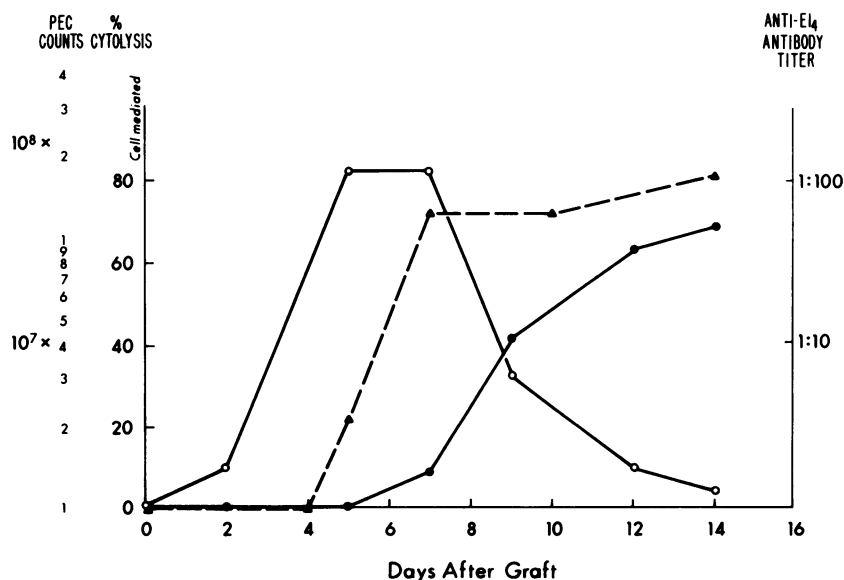


FIGURE 3 Normal immune response to intraperitoneal EL<sub>4</sub> tumor. Peritoneal exudate cell (PEC) (○ — ○) counts increased from  $1 \times 10^7$  to  $2 \times 10^8$  by 4–6 days with return to base line by the 14th day. Cell-mediated cytotoxicity (● — ●) became detectable by day 7 and rose to 65% specific cytotoxicity by day 14. Serum-mediated cytotoxicity (▲ — ▲) was measurable by day 5 and by day 14, had a mean anti-EL<sub>4</sub> titer of 1:100.

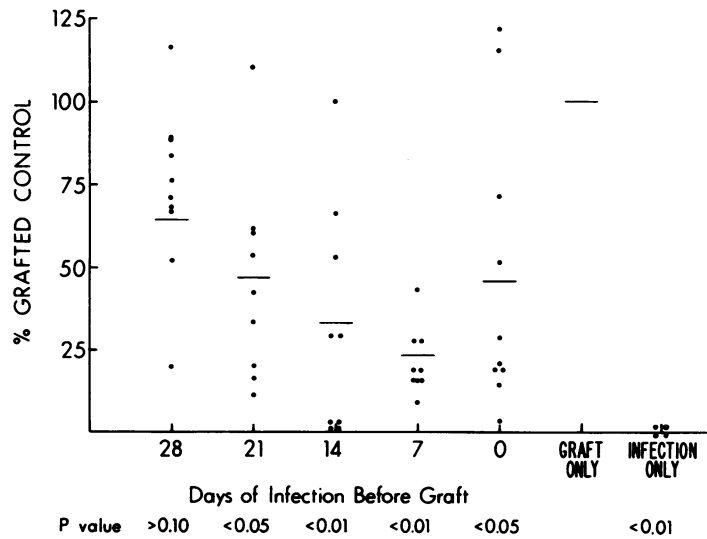


FIGURE 4 MCMV effect on cell-mediated cytotoxicity over time. Expressed as % of grafted control, infected animals had no cytotoxicity and animals infected and grafted had significantly suppressed cytotoxicity when the infection occurred 0 to 21 days before graft with return to control levels by the 28-day interval.

MCMV, but on morphologic bases, do not discriminate between subpopulations.

The finding of C-type virus particles in the peritoneal cells of grafted animals and of animals also infected raised the possibility of immunodepression as a result of induction of these endogenous viruses. Although the Friend-Moloney-Rauscher subgroup of oncogenic murine C-type viruses is a cause of immunodepression, Stutman (64) was unable to detect either cellular or humoral immune depression caused by Gross' leukemia virus and Moloney's sarcoma virus in different strains of mice. Furthermore, Reed and Rapp (65) reported that monolayer cultures of Swiss 3T3 A cells infected

with MCMV were negative for p30 induction, a marker for endogenous virus, by indirect immunofluorescent tests. These data suggest that induction of C-type virus by Herpes virus infection does not result in C-type core shell production. In light of these reports and the facts that the induction of C-type virus particles does not necessarily lead to clinical leukemia and that many agents (chemical, physical, and biological) are capable of C-type virus induction, it is most unlikely that the C-type virus observed in our studies contributed to the immune depression.

Responsiveness to mitogens was used to assess the effects of MCMV on subpopulations of splenocytes.

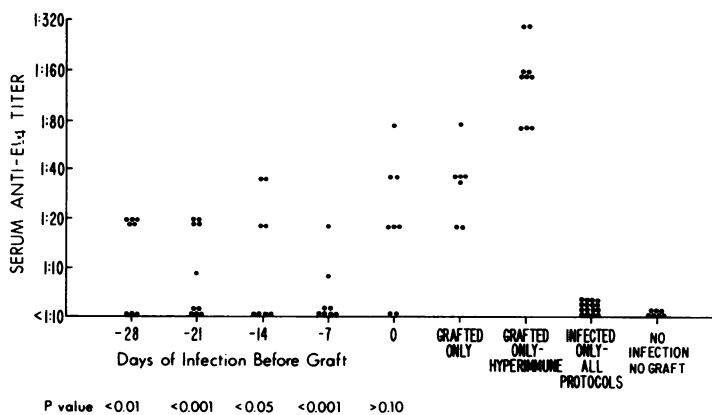


FIGURE 5 Anti-EL<sub>4</sub> serum titer in grafted and/or infected mice. As compared to animals only grafted, those animals both infected and grafted had significantly ( $P < 0.01$ ) decreased activity from 7 to 28 days. Maximal depression was noted at 7 days. Hyperimmunized (injected  $\times 5$  with EL<sub>4</sub>) animals had significantly higher titers than single grafted controls. Animals infected but not grafted and uninfected and ungrafted animals had no anti-EL<sub>4</sub> activity.



TABLE III  
Assays of Suppressor Cells

Source of responder	Number suppressor cells	Source of suppressor cells		
		Grafted	Infected	Control
		% cytotoxicity		
Grafted	0	28±2		
Grafted	5 × 10 <sup>5</sup>	26±1	24±3	25±1
Grafted	1 × 10 <sup>6</sup>	32±4	20±3	22±2
Grafted	2 × 10 <sup>6</sup>	32±2	24±3	21±1
Grafted	4 × 10 <sup>6</sup>	36±2	23±2	22±1

Spleen cells in increasing numbers from infected, grafted, or control animals were added to a normal cytotoxicity experiment to assess the presence or absence of cells which might be suppressive. Cells from grafted animals simply added cytolytic potential whereas cells from infected animals and controls had similar nonspecific inhibition patterns.

Whereas grafted animals had a significantly enhanced response to a B-cell mitogen (LPS), infection, particularly in the early acute phase, significantly reduced the responsiveness to both T-cell (PHA) and B-cell (LPS) mitogens. In those animals both infected and grafted, the mitogen response appeared to be largely dictated by the viral effects; namely, depressed responses. Because the mitogen stimulation tests are performed on standardized numbers of cells and because the numbers of splenocytes have already been demonstrated to be reduced, it is concluded that the residual T and B cells have been altered to effect a reduced responsiveness. Not excluded, however, is a defect in macrophage function which, according to Roenstreich et al. (66), is necessary for T-cell activation by PHA.

Splenocytes from infected animals had a diminished proliferative cell response when tested in mixed lymphocyte cultures but behaved similarly to controls when used as stimulator cells. These data suggest a virus-induced defect in T-cell and/or macrophage function based on the report by Hayry et al. (67) which notes the requirement for both cells in this assay. Grafting alone did not significantly alter the proliferative response.

Focusing more on the interrelationships of the host response to infections and homografts together, rather than individually, the EL<sub>4</sub>-MCMV mouse model provides some useful data. The mouse presumably uses a number of defense mechanisms *in vivo* to resist the development of progressive tumor. The measurement of the peritoneal cell counts in infected and grafted animals represents only the end result of these defense mechanisms and it is perhaps not surprising that no significant difference can be detected compared to grafted controls. If deficiencies in some mechanisms

of response are induced by infection, some evidence for which has been presented, it is not unreasonable that other compensatory mechanisms exist. This paper has attempted to quantify some of these mechanisms as regards T-cell function (cytotoxicity) and B-cell function (antibody), suppressor cells, and suppressor factors.

Lymphocyte-mediated cytotoxicity and EL<sub>4</sub> antibody plus complement studies demonstrated conclusively a time-dependent depression of the cellular and humoral response to the allograft. The time sequence, as with the mitogen response, correlated with the time of maximal organ virus proliferation and splenic damage. Treatment with anti-theta serum revealed that the cytotoxicity generated in both grafted infected and grafted only animals was a T-cell-dependent process. Berke et al. (56) reported that the cytotoxicity against EL<sub>4</sub> was almost exclusively a T-cell function with only a small contribution from the macrophage. It is noteworthy that the viral effects on cytotoxicity (T cell) and anti EL<sub>4</sub> antibody (B cell) are self limited with a return to graft control levels by 4-5 wk after infection.

Other explanations for the observed immunosuppressive effects of MCMV on the host response to alloantigens were sought, including suppressor cells and suppressor factors, but not found. Still other possibilities exist including alteration of macrophage function, alteration of adrenal cortical function through stress, competitive inhibition of antiallograft activity by concomitant anti-viral activity, or the contribution of virally induced altered H-2 antigenic determinants.

The hypothesis that allograft may alter the host's capacity to deal with a concomitant viral infection was examined in a limited fashion. Organ viral titers, duration of viral persistence, and anti-MCMV complement-fixing antibody titers were found to be similar in animals either infected or infected and grafted. Certainly no excess mortality caused by virus was observed in those infected animals which were also grafted. Although this contrasts with the work of Wu et al. (13) a difference in original viral dose and age of animals infected could reconcile the two views.

In view of the clinical observation that viral infections and homograft rejection commonly occur simultaneously, this animal model study offers some additional but conflicting data. No evidence was found to suggest that an enhanced immunologic response to a homograft was induced by a prior infection with MCMV. Rather, a depressed response was found which was felt to be mediated by a severe reduction in the number and functional capacity of immune competent cells. The consequences of viral infection that occur after the homograft have not been determined and may yet help to explain the clinical observation.

It is nonetheless clear that both MCMV and EL<sub>4</sub> exert measurable influences on host defenses. Some of the interrelationships have been described here.

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