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Induction of Immune Interferon by Murine Cytomegalovirus (39861)

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Immune function in mice infected with murine cytomegalovirus (MCMV) is altered in a number of ways, generally adverse. It has been reported, for example, that MCMV infection suppresses antibody response (1, 2), inhibits induction of interferon by an unrelated virus (2, 3), and depresses cell-mediated immunity (CMI) as determined by prolongation of skin graft survival and inhibition of uptake of [^{3}H]thymidine ([^{3}H]Tdr) by splenic lymphocytes in response to phytohemagglutinin (PHA) (4). According to a more recent report, response of lymphocytes to both B- and T-cell mitogens was similarly suppressed (5). The purpose of this study was to further explore the interrelationship between MCMV and the immune system in the mouse. We hoped to induce cell-mediated immunity to MCMV and thus provide an animal model for the study of CMI to cytomegaloviruses in general. Such a model was felt to be desirable in view of the current interest in immunity to CMV in man where it appears that mechanisms of resistance other than antibody-mediated immunity are of importance. It was decided to employ two *in vitro* parameters of CMI in the murine model: antigen-specific lymphocyte transformation ([^{3}H]Tdr uptake) and immune interferon production.

Materials and methods. Male BALB/c mice, 3 months of age, weighing on the average of 20 g, were employed. Two to three mice were assigned to each experimental group. MCMV (Smith strain from American Type Culture Collection) was grown in primary mouse embryo fibroblast cells (SME). When MCMV-induced cytopathogenic effect involved 90% of monolayer, the cells were disrupted by a cycle of freezing and thawing. MCMV was concentrated 10-fold by ultracentrifugation to a titer of 2.6×10^5 pfu/0.5 ml. Viral antigen preparation was then inactivated by ultraviolet irradiation with a germicidal lamp (GE G15TB) for 10 min at a distance of 15 cm with constant stirring. The preparation was shown to be totally noninfectious in SME cell culture. Control antigens consisted of lysates of SME cells prepared in an identical manner. The T-lymphocyte mitogen used was PHA-P (Difco, lot No. 607701; Detroit, Mich.). MCMV for animal infection was prepared in a similar manner, but was not inactivated. Infection was produced by injecting mice intraperitoneally with 2×10^5 pfu of MCMV in 0.5-ml volume, or with 0.5 ml of Dulbecco's phosphate-buffered saline (PBS).

Lymphocyte transformation studies were done according to the techniques reported in detail previously (6, 7). Briefly, mice were sacrificed by cervical fracture at 7, 10, and 14 days after infection. Splenomegaly and hypertrophy of salivary glands provided evidence of infection. MCMV was recovered from salivary glands in a titer of 8.4×10^4 pfu/ml of 10% (w/v) organ homogenates. Virus was isolated from infected mice at 14, but not at 7, days after inoculation. Spleens were removed aseptically, washed with Roswell Park Memorial Institute (RPMI-1640) medium, minced, and cell suspensions were prepared in the usual manner (7). Cell counts were adjusted to 4×10^6 cells/ml in RPMI-1640. Viability was checked at the beginning and termination of each experiment by trypan blue dye exclusion. Mitogen or antigens were diluted as follows: PHA 1:10 and 1:100; MCMV antigen and control antigen 1:1 (undiluted) and 1:10. Various dilutions were used in all of the experiments; however, in general, optimal results in terms of lymphocyte transformation and interferon production were found to occur with the lower dilutions (i.e., 1:10 of PHA and 1:1 of MCMV). Thus, for the sake of clarity, results presented will be

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those obtained with the optimal dilutions for a given experiment.

Antigen preparations were added in 0.1-ml volumes to 1-ml suspensions of spleen lymphocytes in triplicate. suspensions were enriched with fetal bovine serum (Microbiological Associates, Bethesda, Md.) to a final concentration of 5% after it was found that this serum was nonmitogenic and served equally well as autologous mouse pooled sera. Lymphocyte cultures were incubated at 35-36° in 5% CO$_2$ atmosphere. Based on preliminary studies, optimal production of interferon and [^{3}H]Tdr incorporation were observed after stimulation with PHA for 2 days and with antigen for 4 days. At those times, portions of the supernatant fluid were removed, pooled, and stored at −20° for interferon titration. The volume was replaced with RPMI and cultures were pulsed with [^{3}H]Tdr, 1.0 μCi/ml (initial sp act, 6 Ci/mmole). Lymphocytes were harvested on Universal membrane filtration apparatus and processed according to the technique of Rosenberg *et al.* (7); counts were made on Packard Tri-Carb liquid scintillation spectrometer, Model 3375. Results are presented as stimulation indexes which were obtained by dividing mean counts per minute ($\overline{\text{cpm}}$) in cultures stimulated by antigen by $\overline{\text{cpm}}$ in unstimulated cultures (i.e., incubated with antigen controls or PBS in case of PHA studies). A stimulation index equal to or greater than ($\geq$) 3 was considered significant.

Interferon titration was done as described previously (6). Briefly, serial dilutions of supernatant fluids of lymphocyte cultures were added in duplicate to SME cultures for approximately 20 hr. In some instances, because of insufficient amount of specimen, only one screening dilution (e.g., 1:10 or 1:20) was assayed. Supernatant fluids were removed and monolayers were challenged with approximately 50 pfu of VSV (Indiana strain). Following a 24-hr incubation, monolayers were stained with Giemsa stain and VSV plaques were counted. Interferon titer was recorded as the reciprocal of the highest dilution at which the plaque count was reduced by 50% comparing to controls. Appropriate studies were done to identify the viral-inhibiting activity in the supernatant fluid as interferon (6). Immune interferon differs from standard interferon by being acid labile. Accordingly, pooled supernatant fluids containing interferon from various experimental groups were dialyzed against dilute HCl buffer of pH 2 for 24 hr at 4°. This was then switched to dialysis against sodium chloride of pH 7.2 (with three changes) for an additional 24 hr. Mouse interferon standard which was obtained from Bionetics (Bethesda, Md.) was found to be acid stable, whereas interferon obtained after stimulation of appropriate mouse lymphocyte cultures with PHA or MCMV antigen were found to be acid labile [i.e., were immune or "type 2" interferon (8)].

Results. Interferon response is summarized in Table I (*vide infra*). All the preparations save one were tested and shown to be acid labile. PHA uniformly induced interferon ranging in titer from 256 to 8 units in lymphocyte cultures from both the infected and control mice. MCMV antigens added to lymphocyte cultures from MCMV-infected mice produced interferon titers of from >10 to 32 units with no significant difference at the three postinfection times of sampling. No interferon was produced in lymphocytes from MCMV infected mice on stimulation with MCMV antigen control or PBS *in vitro*. Lymphocytes from control (PBS-injected) mice incubated with PHA, but not those treated with MCMV antigen or PBS, produced immune interferon. There was one exception to this finding in

TABLE I. INDUCTION OF IMMUNE INTERFERON BY MCMV[a] AND PHA IN LYMPHOCYTES OF MCMV-INFECTED AND CONTROL MICE.

		Interferon (units/ml) on days after infection		
Inoculum	Stimulant	7	10	14
MCMV	PBS	0	0	0
	PHA[b]	256	≥10[c]	64
	MCMV ag[d]	32	≥10	16
	Ag control	0	0	0
PBS	PBS	0	0	0
	PHA	32	<10	8
	MCMV ag	≥20	0	0

[a] MCMV = murine cytomegalovirus.

[b] PHA diluted 1:10 on Days 7 and 14, 1:100 on Day 10.

[c] Only screening dilution employed.

[d] MCMV antigen (ag) and ag control employed at a dilution of 1:1.

that splenic lymphocyte cultures obtained from control mice injected 7 days previously with PBS appeared to contain "background" amounts of interferon. This was only true when undiluted MCMV antigen preparations were employed for interferon induction. By contrast, in lymphocyte cultures from MCMV-infected mice a 1:10 dilution of the antigen also resulted in stimulation of interferon to a titer of at least 20 units/ml (data not shown in Table I). Thus, this finding may represent induction of "type 1" or standard interferon by viral or cellular DNA or nucleoproteins. Unfortunately, not enough material was available to test this interferon for acid lability.

There was no antigen-specific lymphocyte transformation by MCMV as measured by [^{3}H]Tdr uptake at 7, 10, or 14 days after established infection. All of the stimulation indexes were <2 (range 1.0–1.8), employing both undiluted and 1:10 diluted MCMV antigen preparations. Stimulation indexes produced by PHA on incubation with mouse spleen lymphocytes from MCMV and control mice are depicted in Table II. It can be seen that, throughout the period of observation, that is, at Days 7, 10, and 14 after infection, there was a significant reduction in the stimulation indexes in the MCMV-infected mice compared to those in the control (PBS-injected) mice.

Discussion. Cell-mediated immunity (CMI) was studied in MCMV-infected mice by lymphocyte transformation ([^{3}H]Tdr uptake) and immune interferon induction. There was no evidence of antigen-specific lymphocyte transformation at 7, 10, and 14 days following infection. These data are similar to those of Lang and associates who found that MCMV (infectious or inactivated) was not mitogenic for lymphocytes from infected mice (personal communication). Our results that PHA response was diminished in lymphocytes from MCMV-infected mice confirm those of Howard *et al.* (4) and Selgrade *et al.* (5). MCMV infection appears to differ with respect to lymphocyte transformation from clinical infections with the human strain of CMV. Both Thurman *et al.* (9) and Rytel (10) reported antigen-specific lymphocyte transformation in patients infected with CMV. Similarly Thong *et al.* (11) reported detection of CMI to human CMV with the ^{51}Cr-release microassay.

TABLE II. PHA-INDUCED [^{3}H]TDR INCORPORATION IN SPLEEN CELLS FROM MICE INFECTED WITH MCMV AND FROM CONTROL MICE.

	S.I.[a] on days after infection		
Inoculum	7[b]	10[c]	14[b]
MCMV	1.7[d]	2.5	3.4
PBS	5.4[d]	5.9	7.2

[a] S.I. = stimulation index.

[b] PHA diluted 1:10.

[c] PHA diluted 1:100.

[d] Differences in S.I. ≥ twofold are considered significant.

Immune interferon production is considered to be a valid assay of CMI *in vitro* (12). Employing this parameter we have succeeded for the first time in demonstrating development of CMI to MCMV in mice. Thus, although by a different assay system, this finding supports the human studies indicating that CMI response does indeed develop in the course of a CMV infection, both in man and in mice. In addition, the finding that immune interferon is produced in the course of a CMV infection is of special importance in view of the possibility that interferon constitutes an important effector moiety by which CMI counteracts infection with viruses which spread directly from cell to cell. Notkins referred to this type of dissemination as "type 2 spread" (13), and Lodmell and Notkins provided direct evidence that the spread of herpes simplex virus may indeed be halted by immune interferon (14).

The finding that immune interferon production did not correlate with lymphocyte transformation may be due to the fact that heterogenicity of T cells exists and different subpopulations of T cells may be involved in these assays of CMI. A dichotomy has been reported in the production of interferon, lymphotoxin, and DNA synthesis (15), and in production of macrophage migration inhibitory factor and interferon (12).

The ability of murine and human CMV to induce interferon and the relative sensitivity of the virus to interferon are subjects of some controversy in the literature. Though several reports suggest that CMV is a poor interferon inducer and is relatively insensitive to it (3, 16), other reports indicate that these characteristics may be a variable of the system employed in the studies of induction or sensitivity (17–19). More recently, Oie and associates reported that there is interferon production in the cycle of MCMV replication in mouse embryo cultures which occurs as early as 4 hr after infection (20). In addition, they showed, using *in vitro* sensitivity studies, that MCMV in low concentrations was highly sensitive to the antiviral action of interferon, but higher concentrations of virus proved to be markedly resistant. Though these observations dealt mainly with standard or "type 1" interferon, they may be applicable to immune interferon. Thus, a critical role is suggested for immune interferon which may be produced within hours at the initial site of viral replication at a time where the local CMV "inoculum" may still be small. Should this be the case, it would offer a possible explanation of why human renal allograft recipients in whom CMI is iatrogenically suppressed have enhanced susceptibility to dissemination with CMV and are unable to abolish the infection once it becomes established.

Summary. Studies were initiated in mice to develop a model of cell-mediated immunity to murine cytomegalovirus (MCMV). Immune interferon was produced in response to MCMV antigens in cultures of splenic lymphocytes from mice infected 7, 10, and 14 days previously. However, there was no lymphocyte transformation as measured by antigen-specific enhancement of [^{3}H]thymidine uptake. Lymphocyte transformation in response to phytohemagglutinin (PHA) was transiently suppressed in lymphocytes of MCMV-infected mice.

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