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Effect of Immunization of Mothers on Cytomegalovirus Infection in Suckling Mice[1] (40089)

DONALD N. MEDEARIS, JR.[2] AND SANDRA L. PROKAY

Department of Pediatrics, Case Western Reserve University and Cleveland Metropolitan General Hospital, Cleveland, Ohio 44109

Introduction. Human cytomegalovirus (HCMV) infections cause mental retardation and/or deafness in an estimated 1% of the babies born in the U.S. (1). This morbidity is probably the result of an intrauterine HCMV infection of the brain (CNS) (2), and it might be prevented by immunization (3). Immunization of a woman protects her offspring from rubella's teratogenic effects (4); and Brambell's review of passive transfer of antibody from mother to offspring indicates that such transfer often confers protection to the young in a variety of species, including man and mouse (5). Studies of mouse cytomegalovirus (MCMV) have provided information relevant to understanding HCMV infection (2), and mortality in suckling mice due to MCMV was reduced by immunization of the mother before conception (6).

Therefore, the studies to be reported were done to determine whether (a) CNS infection could be induced regularly by intraperitoneal (ip) inoculation of newborn mice with MCMV, (b) this infection and its attendant morbidity and mortality could be prevented by immunization of the weanling female prior to pregnancy, and (c) studies of the immune response in this situation would reveal differences that might help us understand which response(s) conferred protection.

Materials and methods. Mice. Swiss Webster mice obtained from National Laboratory Animal Company (O'Fallon, MO) were used in all experiments.

Virus. The Smith strain of MCMV was supplied by Dr. June Osborn, University of Wisconsin, maintained by mouse passage, and prepared and stored essentially as described by her (7). Uninfected (normal) salivary gland suspensions were prepared in the same way from glands of uninoculated mice and employed as a control.

Cell culture and media. Primary mouse embryo cell cultures (MECC) prepared from 15- to 17-day old embryos, were grown in Medium (M) 199 (GIBCO) containing 10% heat inactivated calf serum (CS) (GIBCO) and maintained in M199 with 5% CS. Secondary MECC monolayers were prepared by seeding plastic tissue culture flasks (Falcon 25 cm^2) with 2×10^6 cells. All media contained antibiotics.

Viral assay. Tissues were assayed for MCMV plaques on secondary MECC flasks using 0.8% tragacanth in maintenance media as overlay. All points represent the results of testing a pool of organs from at least two animals, and all plaque assays were run in duplicate.

Tissues from some animals which had been inoculated ip as sucklings were also tested for virus by an explant method 60 or more days after birth. Organs were removed aseptically, pooled, minced in small amounts of media and extruded through needles into Falcon flasks. Salivary glands were minced finely and seeded into the flasks without forced extrusion. Minimal essential media (MEM) (GIBCO) with 20% fetal calf serum (FCS) was used to promote growth of these explant tissues. Cultures were observed for cytopathic effect (CPE) for 2 months during which they were also subcultured periodically on secondary MECC flasks.

Determination of LD_{50}. Two- to 5-day old sucklings, randomized on the day of injection, were inoculated ip with 0.05 ml aliquots of serial tenfold dilutions of MCMV. The animals were returned to nursing mothers in groups (litters) of nine, and at least two litters were inoculated with each dilution in each experiment. Mice were observed daily for 21

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[2] Address for reprints: Children's Service, Massachusetts General Hospital.

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days for signs of disease and the number surviving. The LD_{50} was calculated by the method of Reed and Meunch.

Immunization. Thirty- to 40-day old female mice were immunized using 3 ip injections given at weekly intervals, 2 of 4×10^4 PFU, and 1 of 4×10^5 PFU. Control mice were immunized on the same schedule and with an equal dilution of mouse salivary gland suspension prepared from uninoculated mice. These two groups of mice became the breeding stock for studies to determine the effects of immunization.

Plaque reduction neutralization assays. Sera from blood obtained from the retroorbital venus plexus of adults or plasma collected from decapitated sucklings using heparinized capillary tubes were stored at 20°, and heat inactivated at 56° for 30 min prior to use. A volume of 0.3 ml of diluted serum or plasma was mixed with 0.3 ml of virus suspension containing 300–600 PFU MCMV. This mixture was incubated at 37° for 90 min, then 0.2 ml were inoculated into each of two Falcon flasks. Guinea pig complement, 10 units hemolytic activity in 10 μl was added to the reactants in some instances. After an absorption period of 2 hr at 37° the inoculum was removed and replaced with tragacanth overlay. The neutralization (plaque reduction) titer of a serum (or plasma) is the reciprocal of the highest dilution of that serum (or plasma) which reduced control plaque counts 50% or more.

Results. LD_{50}. The LD_{50} for suckling mice of the several stock pools of MCMV used in these experiments ranged from $10^{2.2}$ to $10^{3.2}$ PFU. A representative pool had $10^{7.6}$ PFU/ml, its LD_{50} for sucklings was $10^{2.8}$ PFU, and its LD^{50} for weanlings was 10^5 PFU. The LD^{50} for sucklings of the stock used in most of these experiments was $10^{2.8}$ PFU, and that pool contained $10^{8.3}$ PFU/ml.

CNS infection. The quantity of virus recovered from various organs after ip inoculation of a sublethal quantity of virus (average 36 PFU; range 2–75 PFU) is shown in Table I. Suckling mice were 1–4 days old when inoculated, and their organs were removed and tested at various times between 3 and 127 days thereafter. Virus was first detected in the CNS after it was recovered initially from the liver. Multiplication in the CNS peaked between 10 and 15 days and its extent was less than in other organs. Virus was recovered directly from 3 of 13 brains tested 60 and 80 days after inoculation, and from none of seven brains tested after 120 days. Using tissue explant culture techniques, virus was recovered only once from the brain when it was not detected by inoculation of MECC; CPE was observed in one flask seeded with brain tissue obtained at 127 days.

Histopathological examination of infected brains rarely revealed any changes. Once minimal perivascular cuffing and scattered nodules of glial proliferation with rare typical intranuclear inclusions were seen in a section of brain obtained 10 days after inoculation. Giant cells were not seen. Most brains were normal, including those obtained from mice 15 to 20 days after inoculation.

Effect of immunization on infection and mortality. In order to study the effect of immunization, four groups of suckling animals were tested. The first was born to and nursed by immunized females (I-I); the second was born to unimmunized females (either not injected at all or injected with normal salivary gland) and nursed by immunized females (NI-I); the third was born to immunized females and nursed by nonimmunized nursers

TABLE I. RECOVERY OF MCMV FROM SUCKLINGS AFTER INTRAPERITONEAL INOCULATION.[a]

	Days Post Inoculation							
Tissue	3	5	10	15	20	60	123	127
Brain	N[b]	1.4[c]	3.5	2.8	2.3	1.5	N	N[d]
Liver	2.7	3.6	4.5	3.9	2.6	N	2.6	N
Spleen	—[e]	4.1	4.7	4.0	2.7	3.9	N	N
Salivary gland	—	3.7	7.3	8.0	8.6	7.4	—	5.9

[a] Data are from eight experiments in which inoculum used averaged 36 PFU; the range was 2–75 PFU.
[b] Virus not detected in 10% w/v suspension.
[c] Average $\log_{10}$ PFU/g.
[d] See text (virus was recovered using explant method).
[e] Specimens not tested, or not obtained.

(I-NI); and the fourth group was born to and nursed by nonimmunized females (NI-NI). Litters were switched within 24 hr after birth, and sucklings were inoculated 3 days after switching. All sucklings were challenged ip with approximately 10^4 PFU (30 LD_{50}). The effect of immunization was assessed by determining the extent of virus multiplication in various organs and the mortality rates in the four groups of sucklings.

These results are provided in Tables II and III. In several experiments, each utilizing several pools of two or more organs, virus was not recovered from the brains or livers of animals in groups I-I or NI-I (Table II). Virus was recovered from but a single pool of spleens of animals in group NI-I which were sacrificed on day 10. Virus was recovered from 5 of the 12 pools of salivary glands obtained from mice of groups I-I and NI-I between days 10 and 20.

In striking contrast, virus was frequently recovered from the brains, livers, and spleens of that group of sucklings born to immune females but nursed by nonimmune, foster mothers (group I-NI). The quantity of virus was usually less than that recovered from sucklings born to and nursed by nonimmune females.

The reduction in mortality afforded by immunization is shown in Table III. Mortality in groups I-I, NI-I, and I-NI was 19, 16, and 26% respectively, in contrast to the 100% mortality induced in the NI-NI group; all were inoculated with 30 LD_{50}.

Serology. Plaque reducing antibody was detected in sera of females which were immunized, but not in those which had not been so treated. The median titer was 8.

Plasma obtained from three groups of I-I sucklings 19, 12–22, and 13 days after birth had titers of 8, 4, and <4 respectively. A pool of plasma obtained from suckling group NI-I 4 days after birth had no activity, whereas a pool obtained 14–19 days after birth had a titer of 4. The titer of another pool obtained 13 days after birth from that group (NI-I) was 4. Plasma from sucklings in groups NI-NI and I-NI had no plaque reducing activity. Complement had no discernable effect on the observed titers.

Discussion. The observation that CNS infection can follow ip inoculation of MCMV is not new. Selgrade and Osborn (7), and Kelsey *et al.* (8) have reported that CNS infections occur after ip inoculation of MCMV in both weanling and suckling mice. Selgrade and Osborn stated that no histopathological changes were seen in the brains of inoculated weanling C57BL mice. Our observations are similar in that very little was seen. The apparent paucity of white cells suggests that in these animals little inflammatory or immune response was present in the CNS at the time.

The immediate reason for documenting this CNS infection was to establish a baseline for ascertaining whether immunization of the mature female would confer protection from CNS infection to her offspring. Protection was conferred, and it is important that suck-

TABLE III. REDUCTION IN MORTALITY AFFORDED BY IMMUNIZATION.

Suckling group	No. dead / No. inoculated	Percent mortality
I-I	11/59	19
NI-I	22/137	16
I-NI	37/142	26
NI-NI	36/36	100

TABLE II. SUPPRESSION OF INFECTION AFFORDED BY IMMUNIZATION.[a]

Suckling group Mother–nurser	Organ tested and day postinoculation							
	Brain		Liver		Spleen		Salivary gland	
	Day 10	Day 14–20	Day 10	Day 14–20	Day 10	Day 14–20	Day 10	Day 14–20
I-I	0/6[b]	0/6	0/3	0/4	1/3 (2.0)[c]	0/4	1/2 (3.6)	2/3 (4.1)
NI-I	0/7	0/5	0/4	0/4	0/4	0/3	0/3	2/4 (4.4)
I-NI	4/7 (3.0)	2/2 (2.8)	3/4 (3.6)	3/4 (3.6)	4/4 (4.8)	2/2 (4.4)	3/4 (7.8)	3/3 (8.2)
NI-NI	Day 6[d] 1/1 (3.0)		Day 6 1/1 (5.9)		Day 6 1/1 (6.3)		Day 6 1/1 (4.6)	

[a] Challenge inoculum = 30 LD_{50}.
[b] Number pools positive/Number pools tested.
[c] Average titer (log PFU/g) of positive pools.
[d] All mice in group NI-NI died by end of 6th day.

lings born of females which were not immune, but nursed by immune foster mothers were afforded as much protection as those born to and nursed by immune females. Moreover, they received more protection than those sucklings which were born to immune mothers but nursed by nonimmune foster mothers. Organs of sucklings born to mothers which had been immunized but nursed by those who had not been, generally contained less virus, and their mortality was less than that of controls. They were, nevertheless, much less protected than suckling mice which had been nursed by immune mothers. Mortality has been directly associated with the extent of viral multiplication in the viscera in MCMV (9). The presence of a reduction in mortality in animals in which antibody was not detected is interesting, but not surprising. Our methods may be unable to detect significant quantities of protective humoral antibody, or it may be that plaque reducing antibody is not a significant protective factor (10).

It does seem reasonable to conclude that the suckling obtained an essential protective factor (or factors) from an immune foster mother. This observation is like that reported by many authors for many infections in mice as reviewed by Brambell (5). It is known that immune globulins are secreted in murine breast milk and are absorbed by the suckling mouse. Fahey and Barth found that IgG was present in low but detectable amounts in newborn mice, and in colostrum (11). The levels in the suckling rose rapidly during the first week then fell rapidly between the 2nd and 4th weeks of life. By 8 weeks, the level had risen to that of the adult mouse. Our results are consistent with those observations in that the sera of immune mothers contained plaque reducing activity as did the plasma of sucklings in the protected group(s) when it was obtained after a period of nursing.

Other factors might be operating. There is evidence in mice that maternal cells can cross the placenta (12), and that other factors may induce immune alterations (13). Moreover, cells in breast milk capable of mediating cellular immunity can cross the neonatal gut in rats (14). Our crude attempts to obtain sufficient quantities of milk to study cellular and humoral factors were unsuccessful, and it should be noted that we did not determine whether MCMV was being excreted in breast milk.

There is some knowledge of the nature of immunity to MCMV. Selgrade and Osborn have shown that CBA/J mice were more resistent to MCMV than C57BL6J mice, that suckling C57BL6J mice were more susceptible than adults, and that the susceptibility of the suckling was reduced by experimental transfer of syngeneic macrophages or nonimmune lymphocytes from adult mice (7). There was no difference in the *in vitro* response to MCMV of macrophages from these various sources, thus this was different than what Johnson had shown for herpes virus (15). Although differences in susceptibility to MCMV (as measured by comparison of LD_{50}s) were shown to exist between animals that did not apparently differ in terms of their B and T cell function, subsequent studies by Selgrade *et al.* indicated that both T and B cells have some role in host defense against MCMV (16).

Booss and Wheelock have shown that acute MCMV infection impairs T-cell function (17) and that MCMV itself may be the significant suppressive factor (18). Starr and Allison have shown that MCMV induced death of congenitally athymic nude mice is prevented by T-lymphocytes (19).

Protection from human CMV infection and disease seems as complex as MCMV infection. Cytomegalic inclusion disease rarely, if ever, occurs in infants born after an affected baby (20). Virus excretion does occur in mothers who have complement fixing and neutralizing antibody, and their babies can become infected *in utero* with virus susceptible to those antibodies (10). Cell mediated immunity as measured by cytotoxicity or lymphocyte proliferation is suppressed in HCMV infection, but these findings have not been made in situations which could help explain what factors are essential for protection of unborn or neonatal infants.

The purpose of this discussion is not to provide a detailed analysis from which one might conclude which immune response is the more likely to be operative, but rather to review the possible factors which will have to be evaluated in order to learn how protection was conferred.

The experimental model described provides an opportunity to study the immune

response and protection to MCMV in a context analogous to the human situation which results in mental retardation.

Summary. Female weanling mice were immunized with suspensions of MCMV infected salivary glands, then mated. Protection was conferred to their young by breast feeding, and to a lesser extent it was acquired *in utero.* Protection was manifested by a reduction in mortality, and by a reduction in the quantity of virus recovered from brain, liver, spleen, and salivary gland after challenge with 30 LD_{50}. Neutralization antibody (plaque reducing activity) was present in serum of immunized females and in the plasma of sucklings born of and nursed by them, or those only nursed by them. It was not present in unimmunized females or in sucklings born of but not nursed by immunized females.

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