Protective Effect of Low-Dose Interferon Against Neonatal Murine Cytomegalovirus Infection

JOSE R. CRUZ,† GUSTAVE J. DAMMIN,‡ AND JOSEPH L. WANER‡

Department of Tropical Public Health, Harvard School of Public Health, and Department of Pathology, Harvard Medical School and Peter Bent Brigham Hospital,† Boston, Massachusetts 02115

Mice were injected with 10 or 5,000 reference units of interferon intraperitoneally or subcutaneously within 24 h of birth and reinoculated intraperitoneally 24 h later with 200 plaque-forming units of murine cytomegalovirus. Mock interferon and virus diluent were the control inocula. Infection of mock interferon-treated mice resulted in significant retardation of growth, accompanied by tissue injury and a depressed blastogenic response of splenic lymphocytes. Prophylactic administration of interferon prevented growth retardation and resulted in lower tissue viral titers and diminished injurious effects of the virus. Intraperitoneal inoculation of interferon was more protective than was subcutaneous, and 10 U of interferon was often as effective as 5,000 U. Accelerated maturation and enhanced activity of lymphoid elements were observed histologically in spleens and lymph nodes of interferon-treated mice; supportive of these findings was the greater incorporation of [3H]thymidine of splenocytes from interferon-treated mice. The protective effect of interferon may, therefore, be due to stimulation or accelerated maturation of cellular immune functions.

Immunologically immature and compromised hosts are most at risk of suffering tissue injury due to cytomegalovirus (CMV) infection. Congenital infections usually produce the severest sequelae (15, 23, 27). Natal infections occur more commonly, but are usually asymptomatic (9, 14, 18), although long-term excretion of virus and protracted pneumonitis have been reported (22, 28). Infections of immunocompetent adults are almost always asymptomatic, and when a clinical syndrome results, such as CMV mononucleosis, the prognosis is good. Thus, stimulating or accelerating the maturation of immune functions may be useful in modulating the effects of natal CMV infections.

Although beneficial effects of interferon (IF) as an antiviral therapeutic agent in symptomatic CMV patients have not been demonstrated (1, 7), the encouraging prophylactic use of IF in transplant patients (3) suggests that CMV infections may be amenable to this treatment. However, large quantities of high-titered preparations must be used to demonstrate the antiviral properties of IF. The potential side effects of large doses of IF have not been adequately defined. In newborn mice, multiple injections of high-titered IF may cause glomerulonephritis and death (10). Furthermore, large doses of IF are likely to be immunosuppressive, whereas low doses tend to be immunoenhancing (2, 21, 24).

Murine CMV (MCMV) infection in neonatal mice results in retarded physical growth and temporary immunodepression (5). Using this model, we have tested the value of prophylactic administration of a small dose of IF on the course of natal CMV infection.

MATERIALS AND METHODS

Cell cultures. Primary mouse embryo cell cultures were prepared from 17- or 18-day-old CD-1 embryos. Secondary cultures were grown in 35-mm-diameter petri dishes (Falcon Plastics, Oxnard, Calif.) and maintained in Eagle minimum essential medium supplemented with 2% fetal calf serum, 135 mg of NaHCO3/100 ml, and 100 μg of streptomycin and 100 IU of penicillin/ml; cell cultures were held in a humidified 5% CO2 atmosphere at 37°C.

Viruses. The Smith strain of MCMV was passaged four times, and the Indiana strain of vesicular stomatitis virus was passaged twice in mouse embryo cell cultures to prepare working stocks. Viral titers were determined by plaque assay in mouse embryo cell cultures by a conventional method (12). Plaques were counted with the aid of a dissecting microscope.

Mice. CD-1 mice were bred locally as previously described (6); three females were caged with one male; 30 h later the males were taken away, and four females were quartered per cage until delivery.

IF and mock IF. Mouse fibroblast IF containing no stabilizing protein and mock IF were purchased from Calbiochem-Behring Corp. (La Jolla, Calif.); the titer of the IF preparations varied from 0.56 × 105 to 1.5 × 106 U/mg per ml. In our determinations, vesic-
ular stomatitis virus plaque reduction in mouse embryo monolayers, 1 U of IF equaled 10 National Institutes of Health reference units. Concentrations of IF in the text are given in reference units; mock IF preparations contained the same concentrations of protein as IF preparations.

**Histopathology and assay of infectious MCMV.** Portions of spleen, thymus, lymph nodes, submaxillary salivary glands, lungs, liver, pancreas, and kidneys were removed aseptically and fixed immediately in 10% buffered Formalin; sections were stained with hematoxylin-eosin and periodic acid-Schiff. Additional samples of spleens, livers, and salivary glands obtained from the same experimental animals were weighed, placed in minimal essential medium with 2% fetal calf serum, and frozen at -70°C. Once thawed, the tissues were disrupted in Thumbug tissue grinders, and the suspensions were centrifuged for 10 min at 250 × g; serial 10-fold dilutions were made of portions of the supernatants and assayed for plaque-forming units (PFU) on monolayers of mouse embryo fibroblasts. Results are expressed as PFU/gram of tissue.

**IFA assay.** The indirect immunofluorescent-antibody (IFA) assay method described by Waner and Biano (25) was used for the preparation of MCMV antigen. Mouse embryo cells were inoculated with MCMV at a multiplicity of infection of 0.3 PFU/cell and incubated at 37°C. Forty-eight hours later, the monolayers were disrupted with trypsin and the cells were suspended in minimal essential medium to a final concentration of 3 × 10⁶ cells/ml. One drop of the suspension was placed in each area of a fluorescent-antibody microscope slide (Bellco Glass, Inc., Vine-land, N.J.), and the slide was incubated at 37°C in a humidified 5% CO₂ atmosphere for 24 h. The slides were rinsed twice in phosphate-buffered saline, pH 7.2, fixed in cold acetone, and stored at -70°C; uninfected cells were treated similarly and used as negative controls. The procedure for the IFA assay was as previously described (26), except that goat anti-mouse immunoglobulin antisera conjugated with fluorescein (F/P ratio, 2.5 mg/g; Cappel Laboratories, Cochranville, Pa.) was used in the final step. Controls included testing sera from uninfected mice and reacting fluorescein-labeled normal goat serum F/P ratio, 2.4 mg/g; Microbiological Associates, Bethesda, Md.) with all sera examined.

**Blast transformation assay.** Assays of lymphocyte transformation in response to phytohemagglutinin (PHA) or *Escherichia coli* lipopolysaccharide were performed as described (5) by measuring incorporation of [³H]thymidine ([³H]TdR; New England Nuclear Corp., Boston, Mass.) into lymphocyte cultures.

**Statistical analysis.** The Student two-tailed t test of significance was used to compare weights of mice and blast transformation assays.

**Experimental protocol.** Newborn mice were assigned randomly to one of the experimental groups within 24 h of birth; four groups received IF (10 or 5,000 U) intraperitoneally (i.p.), and two groups were injected subcutaneously (s.c.). Mock IF of protein concentrations comparable to those of the IF preparations were administered i.p. or s.c. to six groups of mice. Twenty-four hours later, 50% of the animals treated i.p. and all of those injected s.c. were inoculated i.p. with 200 PFU of MCMV; the remaining mice received virus diluent. Groups of 9 or 10 identically treated mice were caged with one nursing mother. Blastogenic responses, anti-MCMV antibodies, titers of MCMV in representative organs, tissue alterations, and body weights were assessed periodically thereafter for 28 days.

**RESULTS**

**Physical growth.** The study confirmed our previous report (5) that 200 PFU of MCMV given i.p. to neonatal mice results in retarded physical growth (Table 1). MCMV-infected mice that received mock IF gained the least weight during the 3 weeks of observation. Mice treated s.c. or i.p. with IF before infection with MCMV gained weight at the same rate as those that received only mock IF and virus diluent. Infected animals that received IF were demonstrably heavier than those injected with mock IF as shown by the weight indices in Table 1. IF thus counteracted the growth-retarding effects of MCMV. The differences between infected animals receiving IF and those receiving mock IF i.p. were appreciable in essentially all of the mouse groups at the time intervals when mice were weighed.

**Pathology.** Intraperitoneal injection of MCMV caused major alterations in the spleen, lymph nodes, salivary glands, and pancreas; minor and inconstant lesions occurred in the thymus, liver, lung, kidney, and adipose tissue. The following terms were used to classify cells of the mononuclear series as found in lymphoid tissues and infiltrating other tissues: (i) small lymphocytes, (ii) large lymphocytes, (iii) lymphoblasts, and (iv) macrophages.

**Spleen.** MCMV resulted in marked splenic enlargement by day 12 through increased cellular proliferation in the white and red pulp. Small lymphocytes were almost completely replaced by large lymphocytes, with some lymphoblasts,
in the follicles. Large lymphocytes, lymphoblasts, macrophages, and cytolytic foci were prominent in broad marginal zones and the red pulp, although active germinal centers were not observed. The pattern differed considerably from that noted in control spleens (Fig. 1a, b).

![Image of spleen sections](http://iai.asm.org)
By day 28, the cell-stimulatory effect of MCMV was less marked. Follicles were smaller with fewer large lymphocytes, but some of the follicles had active germinal centers with clusters of lymphoblasts. Large lymphocytes, lymphoblasts, and macrophages were less numerous in the red pulp.

Ten units of IF i.p. partially inhibited the proliferative response to MCMV. At day 12, follicles were smaller with fewer large lymphocytes and lymphoblasts. Small lymphocytes constituted more of the cell population of the red pulp, and there were fewer large lymphocytes, lymphoblasts, macrophages, and cytolytic foci (Fig. 1c). By day 28, follicles were unchanged in size but had more prominent marginal zones and small active centers containing lymphoblasts and foci of cytolysis. The red pulp was more congested, and small lymphocytes predominated. There was even less evidence of a proliferative response to MCMV at days 12 and 28 in mice that received 5,000 U of IF i.p.

The splenic pattern at day 12 differed in infected mice given 10 U of IF s.c. rather than i.p., with follicles being smaller and with narrower marginal zones. However, large lymphocytes, lymphoblasts, and macrophages were more prominent in the red pulp, which also contained

**Fig. 1c-d.**
a larger number of cytolytic foci, around which there were often clusters of polymuclear leukocytes.

When 5,000 U of IF was given s.c. before MCMV, there were, at day 12, increased numbers of large lymphocytes and lymphoblasts in the follicles and in marginal zones, some of which had cytolytic foci with macrophages and polymuclear leukocytes. Large lymphocytes were diminished in the red pulp, which had fewer cytolytic foci than were noted after 10 U of IF s.c. These changes suggest a less protective effect of IF given s.c. versus i.p. The protection evident after 5,000 U of IF given s.c. was comparable to that observed when 10 U of IF was given i.p.

IF alone produced distinctive changes in the neonatal mouse spleen. The spleens of mice that received 10 U of IF i.p. were markedly enlarged on day 12, containing numerous large lymphocytes in the follicles, marginal zones, and red pulp. Many marginal zones had small foci with macrophages and karyorrhexis. In the follicles and broad marginal zones, there were also many large lymphocytes and macrophages. The white pulp encroached upon the red, in which there were now more large than small lymphocytes. The appearance of active centers with lymphoblasts in the follicles was more pronounced after injection of 10 U of IF i.p. than after 5,000 U. The spleens of mice given mock IF i.p. appeared normal in size, showing essentially no proliferative activity at day 12 and moderate activity at day 28.

Lymph nodes. A marked stimulation of lymph nodes characterized by enlargement and a preponderance of large lymphocytes and lymphoblasts over small lymphocytes was observed at 12 days in MCMV-infected mice that received mock IF i.p. (Fig. 2a). In the medulla, there were scattered cytolytic foci surrounded by macrophages and polymuclear leukocytes. At 28 days, there were numerous mitoses and further node enlargement, as well as structural differentiation with clusters of large lymphocytes and lymphoblasts ordered into follicular patterns. Cytolytic foci with macrophages and polymuclear leukocytes were more numerous.

A protective effect afforded by 10 U of IF given i.p. was suggested by the cellular pattern observed at 12 days in MCMV-infected mice (Fig. 2b). There were fewer large lymphocytes, macrophages, and cytolytic foci. Additional protection at 12 days was evident after treatment with 5,000 U of IF; an even more normal pattern was seen at day 28 when 5,000 U of IF preceded MCMV challenge. By day 28, there was little proliferative activity, and morphological maturation appeared normal with occasional compact germinal centers in the cortex, surrounded by many small lymphocytes; there were few cytolytic foci and few polymuclear leukocytes.

When 10 U of IF was given to infected mice s.c. rather than i.p., there was little evidence of a protective effect. There were cortical foci of cytolysis and karyorrhexis, fewer small lymphocytes, and increased numbers of large lymphocytes and macrophages.

IF administered alone had a stimulatory effect on lymphocytes in the lymph nodes at day 12, almost matching that observed in the spleen. Maturation, including the development of follicles, was even more accelerated by 5,000 than 10 U of IF i.p.

Thymus. In contrast to spleen and lymph nodes, cell populations in the thymic cortex and medulla were little changed by MCMV infection. After IF alone, at 12 days after injection of 10 U i.p., the cortex had increased numbers of large lymphocytes. After 5,000 U of IF i.p., the proportion of large to small lymphocytes was similar, but there were cytolytic and karyorrhectic foci in the cortex.

Salivary glands. The mixed or seromucous type of salivary gland was the most severely affected by MCMV, and the serous cell types showed more evidence of injury than did mucus-producing cells. The changes in serous cells ranged from loss of cytoplasm and obliteration of the gland lumen to necrosis with replacement of the tissue by infiltrating mononuclear cells. Cellular infiltration and moderate edema of the interstitium commonly accompanied evidences of glandular injury. Irrespective of the degree of injury, neither nuclear nor cytoplasmic inclusions were noted.

The protective effect of 10 U of IF given i.p. when evaluated at 12 days was marginal. Secretory activity and integrity of serous and mucous cells were partially retained, whereas cellular foci seen replacing necrotic glandular tissue and cellular infiltration of the interstitium were reduced. Further regression ensued, with a near-normal pattern being noted by day 28. More complete protection was afforded by 5,000 U of IF. Both doses of IF given i.p. resulted in morphological evidence of protection, but this was less evident when the s.c. route was used.

Pancreas. Observations on the pancreas were few, since not all dissections yielded this tissue.
MCMV injury was evident, with irregular reduction of cytoplasm and secretion content of the acinar cells and mononuclear cell infiltration of the interstitium and the pancreatic islets. Protection was afforded by prophylactic administration of 5,000 U of IF, with i.p. inoculation being more effective than s.c. (Fig. 3).

Liver. MCMV injury to hepatocellular and hematocytopenic elements was minimal to moderate, with infiltration of occasional foci by mononuclear and polymorphonuclear cells. Protection by IF was evident; in mice so treated, hepatocytes appeared more normal, hematocytopenic foci were more numerous, and there was less cellular infiltration.

**Quantitative distribution of virus.** The re-
FIG. 3. (a) Pancreas (control; 10 U of mock IF i.p., virus diluent). Note the height of the secretory cells and the abundant cytoplasm between the nuclei of these cells and the lumen of the acinus. Bar = 20 μm; hematoxylin and eosin. (b) Pancreas (5,000 U of mock IF i.p.; 200 PFU of MCMV). The acini were reduced in size and distorted, with some lacking lumens. The cytoplasmic/nuclear ratio was reduced, and there was cellular infiltration and edema of the interstitium. Bar = 20 μm; hematoxylin and eosin. (c) Pancreas (5,000 U of IF i.p.; 200 PFU of MCMV). The acinar size and secretion content of the epithelial cells closely matched that of the control. There was less interstitial edema and cellular infiltration than occurred with mock IF and MCMV. Bar = 20 μm; hematoxylin and eosin.
covery of virus was delayed in IP-treated animals. At 7 days of age, MCMV was isolated from 53 of 54 mice that received mock IF, but from only 29 of 60 mice that received IF. A comparison of the patterns of viral recovery showed a more pronounced effect of IF on spleen and liver than on salivary glands. MCMV was recovered from all of the salivary glands of IF-treated mice at day 28 but from only 9 of 19 spleens and 3 of 18 livers of IF-treated mice. The mean viral titers of IF-treated mice were lower than those of comparable mice given mock IF (Table 2). The effects on viral recovery of route of inoculation and concentration of IF varied.

MCMV was isolated from the spleens of all animals treated with mock IF throughout the 28-day observation period (Table 2); viral titers increased in the mock IF-treated animals during the period, reaching the highest levels at 28 days. In contrast, virus was not detected in the spleens of 13 of 20 and 25 of 37 IF-treated animals at 7 and 28 days of age, respectively (Table 2). Less virus was recovered from the IF-treated mice that yielded virus than from comparable mock IF-injected animals.

The amount of MCMV recovered from livers was not as great as that recovered from spleens (Table 2), with mean viral titers from livers greatest at 12 days of age. MCMV was recovered infrequently from mock IF- and IF-treated mice by the 28th day. Injection of IF i.p. or s.c. was associated with less infectious virus in liver specimens.

MCMV was isolated from the salivary glands of all mock IF-treated and from 14 of 20 IF-treated animals at 7 days of age (Table 2). In contrast to liver and spleen, the amount of virus recovered from salivary glands continued to increase, by day 28 reaching $10^9$ to $10^9$ PFU per g of tissue. Injury to salivary glands did not, interestingly, parallel the increase in virus titers; high titers were found in specimens with minimal virus-related injury. The effect of IF on recovery of virus was most pronounced through the first 12 days in mice that received IF i.p.

MCMV was not isolated from specimens obtained from uninfected controls.

**Antibody levels.** Anti-MCMV antibodies were detected through 12 days of age in 8 of 10 mice that received mock IF and in 2 of 19 mice treated with IF i.p. (Table 3). Comparable antibody titers were detected in all infected animals by 28 days of age. Although the geometric mean titer was lowest in the group of animals that received 10 U of IF, the difference was not statistically significant.

**Blast transformation assays.** Neonatal infection with MCMV depressed the lymphocyte response of mock IF-treated mice to mitogenic
challenges of PHA at all times tested ($P < 0.0005$; Table 4). Administration of IF i.p. before infection did not significantly affect the diminished lymphocyte response during the first 12 days of life. The lymphocytes of IF-treated, MCMV-infected animals responded comparably, however, to those of uninfected mice at 28 days of age, whereas the lymphocytes of infected mice treated with mock IF before infection continued to have diminished responses to PHA ($P < 0.0001$).

In uninfected mice, 10 U of IF i.p. induced an increase in the incorporation of $[^\text{3}H]\text{TdR}$ into cultures of unstimulated (background) lymphocytes at 10, 12, and 28 days of age ($P < 0.005$; Table 4); a similar effect was seen in mice treated with 5,000 U of IF at 12 and 28 days, but not at 10 days of age. This stimulatory effect of IF is reflected in the appearance of active centers with lymphoblasts in the splenic follicles (Fig. 2d), which was more striking after injection of 10 than of 5,000 U of IF. Additionally, injection of 10 or 5,000 U of IF resulted in an enhancement of $[^\text{3}H]\text{TdR}$ incorporation in lymphocytes of PHA-stimulated cultures 10 and 12 days after IF treatment ($P < 0.005$); by 28 days of life, the response to PHA returned to values comparable to those obtained with lymphocytes from mice that received only mock IF.

Incorporation of $[^\text{3}H]\text{TdR}$ by splenocytes obtained from infected or uninfected mice and incubated with lipopolysaccharide was unaffected by IF (data not shown).

**DISCUSSION**

Small amounts of MCMV (200 PFU) given to neonatal mice (>24 h old) were reported to retard physical growth and induce a temporary loss of lymphocyte responsiveness to mitogens (5). Here we report the occurrence of tissue alterations which tended to be moderate, except for lymph node necrosis and a marked splenomegaly. Similar changes in the spleen have been reported (16) after inoculation of large doses of MCMV to newborn mice. MCMV-induced changes in the thymus were minimal in contrast with the severe thymic atrophy reported by Schwartz and colleagues (19) after inoculation through the retina.

IF doses of 10 or 5,000 U administered before virus inoculation prevented the detrimental effects of infection on physical growth; this was accompanied by less virus-induced tissue injury and recovery of less MCMV from the viscera of IF-treated animals. IF appeared to delay and, thereafter, limit visceral involvement by MCMV. After the initial delay, viral titers continued to increase in salivary glands, in contrast to a decrease noted in livers and spleens. Interestingly, injury to salivary glands did not parallel viral titers. The lower proportion of seropositive mice that received IF (2 of 19) as compared with

**TABLE 4. Effect of MCMV infection and prophylactic administration of interferon i.p. on the blastogenic response of lymphocytes to PHA**

<table>
<thead>
<tr>
<th>Status</th>
<th>IF concn (U)</th>
<th>Mitogen</th>
<th>$[^\text{3}H]\text{TdR}$ incorporation (cpm x $10^3$) into lymphocyte cultures at age:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>10 Days</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Mock IF</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Uninfected</td>
<td>10</td>
<td>None</td>
<td>1.4 ± 0.6</td>
</tr>
<tr>
<td></td>
<td>5,000</td>
<td>None</td>
<td>1.9 ± 0.3</td>
</tr>
<tr>
<td>Infected</td>
<td>10</td>
<td>None</td>
<td>1.1 ± 0.5</td>
</tr>
<tr>
<td></td>
<td>5,000</td>
<td>None</td>
<td>1.0 ± 0.2</td>
</tr>
</tbody>
</table>

*Values represent mean ± standard deviation of four to eight animals.

a Geometric mean titer of antibody-reactive animals.

b Number of animals positive/number tested.
those injected with mock IF (8 of 19) through the first 12 days may have been due to a diminished viremia in IF-treated mice. The lower viral titers in IF-treated mice at 7 days may reflect a viremia reduced on this basis.

The recovery of less virus, with time, from livers and spleens could reflect the presence of reticuloendothelial elements stimulated by IF which are not significantly present in salivary glands. Increased size of follicles accompanied by active centers and enhanced cellularity of the red pulp characterized spleens from IF-treated mice. Furthermore, the stimulatory effect of IF on lymph nodes approached that observed in spleens, including follicular development. Complementing these observations was the marked stimulatory effect of IF on the blastogenic response to PHA of splenocytes. Paradoxically, MCMV showed a stimulation of splenic cellular elements, although simultaneously depressing the PHA response.

Two mechanisms may be responsible for the protective effect of IF. (i) The antiviral activity of IF, in particular 5,000 U, may have had an effect on the initial replication of the viral inoculum but would not account for the differences in viral titers observed in spleens and livers at 28 days. Additionally, it seems unlikely that 10 U of IF administered s.c. would have an antiviral effect on the inoculum injected i.p. (ii) Enhanced immune mechanisms may be involved. Local activation of peritoneal macrophages by IF may be responsible for the increased protection afforded by i.p. injection of IF compared with s.c. administration. Interferon may induce enhancement of macrophage endocytosis and cytotoxicity and the release of soluble factors which activate lymphocytes. The finding that 10 U of IF injected s.c. conferred protection, however, suggests that additional defense mechanisms may be operative.

Interferon may potentiate cytotoxic lymphocytes (8, 20). Cytotoxic cells were described during MCMV infection, with their appearance preceded by high levels of IF in serum (17). Additionally, natural killer cell activity, reported in 5-day-old mice, was stimulated 1 day after injection of an IF inducer (13). Enhanced natural killer cell activity after administration of 10^5 to 10^6 U of IF to adult mice has also been reported (20).

Low doses of IF have been associated with immune enhancement. Sonnenfeld and co-workers (21) showed that 10^6 U of type I IF induced increased numbers of anti-sheep erythrocyte plaque-forming cells in the spleens of treated adult mice, whereas 10^8 U was immunosuppressive. Injection of newborn mice with 12.8 U of IF resulted in augmented numbers of anti-sheep erythrocyte plaque-forming cells in the spleen (24). In vitro studies have shown that IF, in doses ranging from 10 to 10^8 U, generates natural killer cell activity in cultures of murine spleen cells within the first 24 h (4).

Our histopathological observations and blastogenic assays indicate that administration of a small quantity of IF induces changes in the lymphocyte populations of the spleen accompanied by increased uptake of [3H]Tdr which may be responsible for the limitation of viral replication in the organs examined. These observations may reflect enhanced lymphocyte cytotoxicity as a mechanism that is responsible for the protection afforded. The data suggest a possible effective prophylactic role for IF as a potentiator of immunity to modulate viral infections, particularly those which may be acquired as natal infections.

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LITERATURE CITED


