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Prevention of Morbidity and Mortality of Wild Murine Cytomegalovirus by Vaccination with Attenuated Cytomegalovirus[1] (39937)

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Cytomegalovirus (CMV) is an important pathogen in infants and immunocompromised patients (1). It is common in renal transplant recipients in whom it has been associated with fever, leukopenia, transplant rejection, bacterial superinfection, and death (2-7). Recently, Elek and Stern (8) and Plotkin *et al.* (9) reported developing a vaccine against human CMV by multiple passages in tissue culture. These live CMV vaccines have been shown to elicit neutralizing and complement-fixing antibodies in human volunteers. However, their efficacy in preventing congenital CMV infectons or illness in immunologically compromised hosts has yet to be determined. In this report we demonstrate that vaccination of mice with a live, attenuated strain of murine CMV can prevent histologic changes, immunosuppression, and death caused by subsequent challenge with virulent CMV.

Materials and methods. Virulent (wild) murine CMV was originally obtained from Dr. June Osborn, Madison, Wisconsin. The virus was isolated from a wild field mouse by Dr. Margaret E. Smith, and it has been passed in mice since that time. Pools of virus were prepared from the submaxillary glands of Swiss-Webster mice that had been infected 14 to 20 days previously with 2×10^5 plaque-forming units (pfu) of CMV as previously described (10). Pools of virus were stored at $-70°$ until used. The virus was titered using a microplaque method (11). The LD_{50} was approximately 4×10^6 pfu.

Murine CMV was obtained from the American Type Culture Collection, Bethesda, Maryland, after 12 passages in mouse embryo fibroblast cells. This strain (VR 194) has been passed by us three times in mouse embryo fibroblasts. Pools of attenuated CMV were prepared in mouse embryo fibroblasts. The cell-free virus was titered, and stored at $-70°$ as previously described (10).

Female Swiss-Webster mice and male and female C57BL/6 (Jackson Laboratories, Bar Harbor, Maine) were used in these experiments. All animals were inoculated with virus intraperitoneally.

In the first experiment 9- to 12-day-old Swiss-Webster mice were divided into five groups. Group 1 received neither attenuated tissue culture-passaged CMV vaccine nor virulent CMV. Group 2 was not vaccinated, but 2 weeks later was challenged with virulent CMV. Groups 3-5 received doses of 10^2, 10^3, and 10^4 plaque-forming units of attenuated CMV vaccine. Two weeks later they were challenged with wild virus. Mice were observed daily and weights were recorded. Two weeks after virulent viral challenge surviving mice were sacrificed. Spleen, liver, salivary glands, and other organs from control and infected mice were fixed in 10% formalin, embedded in paraffin, sectioned, and stained with hematoxylin and eosin.

In the second experiment, four groups of 4-week-old Swiss-Webster mice were used; each group consisted of eight animals. Groups 3 and 4 were given attenuated CMV vaccine at the start of the experiment. Two weeks later groups 2 and 4 were challenged with virulent CMV. Six days later groups 1-4 were immunized with 0.2 ml of 25% sheep red blood cells (SRBC) intraperitoneally. The animals were bled by cardiac puncture 10 days later and the serum was decomplemented by heating at 56° for 30 min. Hem-

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agglutinating titers were performed as previously described (10). Twofold dilutions of mouse serum were made in phosphate-buffered saline in microtiter plates (Linbro Chemical Company, New Haven, Connecticut). An equal volume of a 1% SRBC was added to each well, and the plates were incubated at room temperature for 2 hr. The titer was read as the last well showing hemagglutination.

The third experiment examined the effect of CMV infection on skin graft survival. Three groups of 12 C57BL/6 female mice were studied. At 4 weeks of age, one group was inoculated with attenuated CMV. Two weeks later this group and an unvaccinated control group were infected with virulent CMV. Seven days later all mice were grafted with C57BL/6 male skin. Male C57BL/6 tail skin grafts were placed on the lateral thoracic wall of C57BL/6 female mice and covered with a gauze dressing and a plaster cast. The casts were removed 6 days later, and the grafts were observed for signs of rejection.

Results. Mice were protected from the morbid and lethal effects of virulent CMV by prior inoculation of attenuated CMV (Table I). Five (55%) of nine mice given an LD_{50} (approximately 4×10^6 pfu) of virulent CMV died between 3 and 7 days after virus infection. They also lost weight compared to the steady gain in weight shown by uninfected control animals. On the other hand, a total of 34 mice administered 10^2, 10^3, and 10^4 pfu of attenuated CMV 2 weeks before inoculation of virulent CMV had no mortality. They also gained weight steadily and showed no signs of illness such as hunched posture, ruffled fur, and crusting about the eyes. The mice given virulent CMV alone showed all these signs of morbidity and all animals lost weight.

TABLE I. MORBIDITY AND MORTALITY OF MICE VACCINATED WITH ATTENUATED CYTOMEGALOVIRUS (CMV) AND CHALLENGED WITH VIRULENT CMV.[a]

Group	Attenuated CMV (pfu)[b]	Virulent CMV (pfu)	No. of mice	No. dead
1	–	–	21	0
2	–	4×10^6	9	5*
3	10^2	4×10^6	9	0
4	10^3	4×10^6	10	0
5	10^4	4×10^6	15	0

[a] Nine- to twelve-day-old Swiss-Webster mice were vaccinated with attenuated virus. Two weeks later they were challenged with virulent CMV.

[b] Plaque-forming units.

* $P < 0.05$ compared to any of the other four groups.

Primary inoculation of 2×10^5 pfu wild CMV results in intranuclear inclusions in spleen, salivary glands, liver, and, rarely, in lymph nodes. Occasionally periportal infiltration of mononuclear cells can be identified within the liver. The intranuclear inclusions in the spleen are around lymphoid nodules. There is depletion of lymphocytes around the lymphoid nodules and occasional destruction of the nodules as well. The spleen and liver pathology is most marked 2 to 8 days after infection. There is gradual repopulation of the spleen thereafter, with restoration of normal cellularity 14 to 21 days after infection. Attenuated virus caused no cellular destruction. Intranuclear inclusions were only rarely seen in the salivary glands. Inoculation of mice with attenuated CMV prior to infection with wild CMV resulted in protection of the mice from the histologic changes in the spleen and liver caused by inoculation of wild CMV alone. These organs remained histologically normal in mice vaccinated with attenuated CMV. There was no cellular destruction, inflammation, or intranuclear inclusions.

Virulent CMV is immunosuppressive (10, 12–14), whereas attenuated murine CMV alone is not. Mice given 2×10^5 pfu of virulent CMV (a virus inoculum that does not cause weight loss or mortality) 6 days before immunization had a decreased hemagglutinating antibody response to SRBC compared to control animals (Table II). The antibody response to SRBC in mice given attenuated CMV was similar to that of controls. When attenuated CMV was given before virulent CMV, mice had increased antibody responses compared to those of control animals.

Virulent CMV also causes suppression of cell-mediated immune responses. Virulent CMV increased graft survival across the weak male-female barrier in C57BL/6 mice from a median of 15.5 days for control mice to 60.5 days for mice infected 7 days

prior to grafting (Table III). Attenuated virus alone did not affect skin graft survival. However, mice inoculated with attenuated CMV 2 weeks before infection with virulent CMV were protected from the immunosuppressive effects of virulent CMV. Indeed, vaccination seemed to enhance cell-mediated responses. The skin graft survival was 9.3 days, compared to 15.5 days for uninfected control mice ($P < 0.01$).

Discussion. These experiments show that a tissue culture-passaged strain of murine CMV can protect mice against the morbidity and mortality of challenge with virulent CMV. In that regard, our findings confirm and extend those of Osborn and Walker, who previously demonstrated that tissue culture passage rapidly attenuated murine CMV for suckling mice (11). In addition, our experiments indicated that vaccination with attenuated CMV protected animals from the immunosuppressive effect of virulent CMV, and, in fact, enhanced both humoral and cell-mediated responses.

A live tissue culture-passaged CMV vaccine has been tested in human volunteers by Elek and Stern (8) and Plotkin *et al.* (9). These investigators have followed CMV antibody titers in their volunteers, but have not challenged them with active virus. In our study we demonstrated that attenuated murine CMV could protect mice from later challenge with virulent virus.

CMV replicates in lymphoid cells. The immunosuppressive effect of primary CMV infection may be due to viral replication which is effectively a takeover of the cells' metabolic machinery. With this redirection of their synthetic processes, the lymphoid cells can no longer carry out their normal immunologic function. The manner in which vaccination with attenuated CMV followed by challenge with wild CMV leads to enhanced humoral and cell-mediated responses is not understood, but is currently under investigation.

TABLE II. HEMAGGLUTINATING TITERS TO SHEEP RED BLOOD CELLS (SRBC) IN MICE INFECTED WITH CYTOMEGALOVIRUS (CMV).[a]

Group	Attenuated CMV (pfu)[b]	Virulent CMV (pfu)	Log_2 hemagglutinating titer
1	–	–	7.8
2	–	2×10^5	3.5*
3	10^4	–	7.6
4	10^4	2×10^5	12.3*

[a] Four-week-old Swiss-Webster mice were inoculated with attenuated CMV (group 3). Two weeks later this group and group 2 were given virulent CMV. Six days later all mice were immunized with SRBC. Ten days later mice were bled for antibody determination.

[b] Plaque-forming units.

* $P < 0.01$ compared to control animals (group 1).

TABLE III. SKIN GRAFT SURVIVAL IN MICE INFECTED WITH CYTOMEGALOVIRUS (CMV).[a]

Group	Attenuated CMV (pfu)[b]	Virulent CMV (pfu)	Median graft survival (days)
1	–	–	15.5
2	–	2×10^5	60.5*
3	10^4	2×10^5	9.3**

[a] Four-week-old C57BL/6 female mice were inoculated with attenuated CMV (group 3). Two weeks later these mice and group 2 were infected with virulent CMV. Seven days after infection all mice were grafted with C57BL/6 male skin.

[b] Plaque-forming units.

* $P < 0.001$ compared to control animals (group 1).

** $P < 0.01$ compared to control animals (group 1).

Summary. Virulent (wild) murine cytomegalovirus results in morbidity, weight loss, immunosuppression, and death in infected mice. An attenuated, tissue culture-passaged strain of CMV protected mice from the morbid, immunosuppressive, and lethal effects of subsequent challenge with virulent virus. In addition, vaccination with attenuated CMV led to an enhanced humoral antibody response to SRBC and decreased mean skin graft survival time.

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