Mouse Cytomegalovirus: Isolation from Spleen and Lymph Nodes of Chronically Infected Mice[1] (36556)

DONALD HENSON, ALFONSO J. STRANO, MARGERY SLOTNIK, AND CLYDE GOODHEART
(Introduced by A. S. Rabson)

Laboratory of Pathology, National Cancer Institute, Armed Forces Institute of Pathology, Washington, D.C. 20012; and Department of Microbiology, Presbyterian St. Lukes Hospital, Chicago, Illinois 60612

The human cytomegalovirus (HCMV) (1–7), in addition to other members of the herpesvirus family (8–13), has been recovered from cells of the leukocyte series. One of the original laboratory strains of HCMV, the Ad-169 strain, was isolated from cultures of human adenoids obtained from healthy children (1). More recently, HCMV has been recovered by co-cultivation with susceptible human cells from peripheral leukocytes of patients with suspected or proven cytomegalovirus infection and from healthy blood donors (2–7). Histologically, distinctive intranuclear inclusions, characteristic of HCMV infection, have been found in spleens and in lymph nodes of patients with a variety of disorders (14). The purpose of the study was, therefore, to find out if the mouse cytomegalovirus (MCMV) could be recovered from lymphoid tissues of mice, chronically infected with this virus.

Materials and Methods. Viruses. A mouse-passaged strain of MCMV originally isolated by Dr. Margaret Smith in 1954 was used. Rauscher leukemia virus (RLV) was obtained through the courtesy of Dr. Alan Rabson.

Mice. Six- to 8-week-old male C_3H/He and Balb/c mice free of chronic MCMV infection were used.

Infection of mice. Mice were infected intraperitoneally (ip) with MCMV according to procedures already published (15). Rauscher leukemia virus was injected ip into Balb/c mice 3 weeks after MCMV infection. Mice were killed 2 weeks after RLV infection for MCMV isolation. Mice were considered to be chronically infected with MCMV if virus could be isolated from any tissue more than 2 weeks after ip inoculation.

Cell cultures. Mouse embryo tissue culture (METC) cells and rat embryo tissue culture (RETC) cells were prepared from embryos removed from late term pregnant animals. METC cells were grown in small Falcon plastic flasks using RPMI 1640 medium with 2% fetal calf serum. RETC cells were grown in 32 oz bottles using Eagle's medium with 10% fetal calf serum.

Preparation of spleen or abdominal lymph node cell suspensions. Spleens or abdominal nodes were removed from one to three mice, minced with scissors in Hanks' solution, then repeatedly aspirated through a 5.0 ml syringe until the cells had largely dissociated. The cell suspension was added to a centrifuge tube for several minutes, to allow the large nondissociated clumps to settle, poured into another tube, centrifuged at 1500 rpm for 15 min, then resuspended in 20 to 40 ml of RPMI medium. Cell counts performed with trypan blue indicated that 8.0×10^5 to 8.0

[1] This investigation was supported in part by a research contract, Project No. 3A061102B71Q, from the Medical Research and Development Command, U.S. Army, Washington, DC. In conducting the research described in this report, the investigators adhered to the "Guide for Laboratory Animal Facilities and Care," as promulgated by the Committee on the Guide for Laboratory Animal Facilities and Care of the Institute of Laboratory Animal Resources, National Academy of Sciences-National Research Council. The opinions or assertions contained herein are the private views of the authors and are not to be construed as official or as reflecting the views of the Department of the Army or the Department of Defense.

10^6 lymphocytes/ml were present. In some experiments, the spleen was first bisected, and one-half was fixed for either light or electron microscopy.

Viral isolation procedures. Five milliliters of medium containing the resuspended spleen or lymph node cells were added to confluent METC monolayers in small flasks. Generally, spleen cells from one mouse were added to four or five monolayer cultures. Cultures were incubated at 37° and the medium changed once or twice a week, depending upon the acidity. Cultures were examined 2 or 3 times a week for a cytopathic effect (CPE). Attempts were made to hold cultures for at least 3 to 4 weeks before being considered negative for virus.

Electron microscopy. Fragments of spleen (1 mm^3) were fixed in 4% glutaraldehyde in phosphate buffer (pH 7.2) and postfixed with osmium tetroxide at pH 7.2. Fixed tissue was dehydrated in ethanol and embedded in Epon for electron microscopy.

Histology. Spleens and salivary glands were fixed in Bouin's solution and paraffin-embedded sections were stained with hematoxylin and eosin.

Production of MCMV antibody. After the fifth passage in RETC cells, the medium and cell detritus was freeze–thawed three times, centrifuged at 1500 rpm for 15 min, and the supernatant medium frozen at —70°. Rabbits were injected weekly with 10 ml aliquots of the supernatant medium using ip, subcutaneous, and intramuscular routes. Ten days after the fifth injection, the rabbits were bled. The antibody titer was 1:8 as determined on Ouchterlony plates against MCMV grown in METC cells and concentrated by ultrafiltration.

Neutralization test. Approximately 100 to 200 plaque-forming units (pfu) of virus (isolated from spleen cell cultures) was mixed with phosphate-buffered saline (PBS), normal rabbit serum, and rabbit antiserum diluted in PBS. After 30 min at 37°, the virus was added to METC monolayers, covered with methylcellose, and incubated for 5 to 7 days.

Results. Virus was regularly isolated from spleen cell suspensions of chronically infected C_3H mice which showed no clinical evidence of infection. However, a prolonged period of co-cultivation from 9 to 21 days was re-

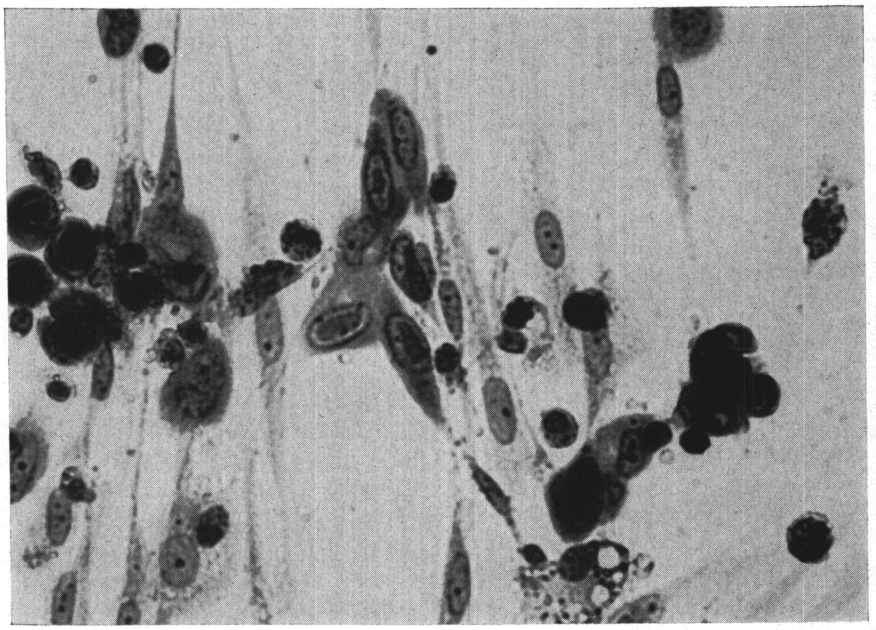

FIG. 1. Inclusion bearing cells in partly degenerated monolayer of mouse embryo tissue culture cells. Giemsa stain. ×380.

quired before a CPE appeared. Evidence of virus isolation was first manifested by the appearance of one or several foci of large rounded cells, typical of a MCMV-induced CPE. These foci subsequently enlarged to become plaques and progressively involved the entire monolayer (Fig. 1). Virus was isolated from mice 14 to 55 days after ip infection, the maximal time interval tested. In all, virus was recovered from spleen cells in 14 of 18 experiments.

Virus was also recovered from spleen cells of chronically infected Balb/c mice. However, for the same ip inoculum the interval between inoculation of monolayers and time of appearance of CPE was often shorter with spleen cells obtained from Balb/c mice than with C_3H mice.

The amount of virus released from spleen cells was small. Often only one or two foci appeared in the METC monolayers, even after prolonged incubation, and not all cultures in every experiment developed a CPE.

We were unable to develop a satisfactory infectious center assay system for spleen cells under a 1% methylcellulose overlay. Reasons for this were the relatively long incubation period before a CPE appeared and, often, the methylcellulose overlay caused the spleen cells to aggregate in large clumps around the periphery of the flask.

MCMV isolation from lymph nodes. Using co-cultivation, MCMV was also isolated from cell suspensions of abdominal lymph nodes of chronically infected mice. As with spleen cell suspensions, a 2 or 3 week incubation period was required before a CPE developed in the METC cells.

Isolation of CMV from cultured spleen cells. In these experiments dissociated spleen cells were cultured in flasks without METC monolayers. Periodically, 1.0 ml medium, which contained some spleen cells, was removed from the flask and added to METC cultures. A CPE was observed in most METC cells after a 3 to 7 day incubation period. Results from one experiment are shown in Table I. These data demonstrated that MCMV persists *in vitro* in spleen cell cultures for at least 3 weeks and that virus cannot be recovered from all spleen cell cultures.

TABLE I. Isolation of Mouse Cytomegalovirus from Spleen Cells Cultured in Flasks and Periodically Sampled for Virus.[a]

Days in culture	Flask no.					
	1	2	3	4	5	6
7	—	—	—	+[b]	—[c]	—
13	+	+	—	+	—	+
19	+	+	—	+	—	+
24	+	+	—	+	—	+

[a] Spleen cells were obtained from two C_3H mice, 27 days after infection.

[b] Indicates culture from which virus was isolated.

[c] Indicates no virus isolated.

Identification of isolates as MCMV. Viruses recovered from spleen cells were identified by comparing them to *in vitro* passaged laboratory strains of MCMV. The CPE, rate of plaque formation, and appearance of intranuclear inclusion bodies after Giemsa staining of the spleen cell isolates were identical to laboratory strains which originated from the submaxillary glands. Furthermore, a 1:16 dilution of rabbit MCMV antiserum, but not control serum, neutralized 100 to 200 pfu of the spleen cell isolates.

The isolates were not considered to be laboratory contaminants because unused METC cells carried along with co-cultivated cultures never developed a CPE and many of the isolations were made at a time when laboratory strains of MCMV were not being passed.

Light and electron microscopic studies. Inclusion bodies were not seen histologically and herpes type viral particles were not found under the electron microscope in spleens of 14 chronically infected mice.

Failure to demonstrate an inhibitor. In these studies, spleen cell suspensions from infected and noninfected mice were incubated for 6 days. Then, the cells were sedimented at 2000 rpm and the supernatant medium added to METC monolayers immediately after infection with approximately 100 pfu of MCMV. In both groups, that is, the METC cultures with medium from spleen cells of infected mice or noninfected mice, the time for the infection to involve the entire mono-

layer was identical. These results indicate that an inhibitor was not present in the medium from spleen cell cultures from infected mice. This does not, however, exclude the existence of an inhibitor intracellularly or within concentrations below detection by these methods.

Isolation of MCMV from mice infected with Rauscher leukemia virus. Virus was also recovered from the enlarged spleens of Balb/c mice infected with RLV. As with normal lymphoid organs, a 2 to 3 week incubation period was required before a CPE appeared.

Transfer of infection by spleen cells. C_3H and Balb/c mice were inoculated ip with suspensions of spleen cells prepared from chronically infected C_3H mice. Some of the C_3H recipient mice were injected with cortisone acetate after inoculation to decrease resistance to infection. Generally, spleen cells from one infected mouse were used to inject two uninfected recipients. After 3 weeks, intranuclear inclusion bodies were found in the submaxillary glands of some of the cortisone-conditioned mice, but not in the noncortisone conditioned ones. In other experiments, recipient mice were challenged with lethal doses of MCMV. All recipient C_3H and Balb/c mice survived while control mice not injected with spleen cells died. This indicates that recipient mice had previous exposure to the virus.

Discussion. These results demonstrate that, in addition to submaxillary glands, urine and conjunctiva (15, 16), MCMV can be recovered from abdominal lymph nodes and spleens of chronically infected mice, and from the enlarged spleens of chronically infected mice secondarily infected with Rauscher leukemia virus. Virus was isolated by co-cultivation with METC cells and often required a 9 to 21 day incubation period before a CPE appeared. In contrast, it takes only 2 or 3 days before a CPE appears when infectious virus is added directly to METC cells. Virus was recovered from spleens for as long as 55 days after infection, the maximal time tested.

Intranuclear inclusion bodies were not demonstrated histologically in spleens from infected mice, nor were herpes-type viruses visualized under the electron microscope. This suggests that either very few cells were infected, or that virus, existing in a suppressed or quiescent form, was released from a small number of cells after *in vitro* cultivation. The cell type or types with which MCMV was associated was not determined. Recently, HCMV was isolated by co-cultivation with WI-38 cells from lymphoblastoid cells which grew out of a cultured paraganglioma (17).

Viruses isolated from spleen cells resembled laboratory strains of MCMV serologically and by their behavior in tissue culture, therefore, we have reason to believe that the isolates are indeed MCMV. Association with leukocytes or lymphoid tissue may be a common phenomenon among many of the cytomegaloviruses. Further, leukocytes should be considered a potential provenance of virus in disseminated cytomegalovirus infections. Infection could be transfered to mice by inoculation of spleen cells prepared from chronically infected mice.

Summary. MCMV was recovered from abdominal lymph nodes and spleens of chronically infected C_3H and Balb/c mice by prolonged co-cultivation with mouse embryonic fibroblasts. Intranuclear inclusions were not found histologically in spleens from infected mice nor were herpes-type viruses seen in the electron microscope. Virus was isolated from mice for as long as 55 days after infection, the maximum time tested. MCMV could be passed to uninfected mice by injection of spleen cells from chronically infected mice.

1. Rowe, W. P., Hartley, J. W., Waterman, S., Turner, H. C., and Huebner, R. J., Proc. Soc. Exp. Biol. Med. **92**, 418 (1956).

2. Harnden, D. G., Elsdab, T. R., Young, D. E., and Ross, A., Blood **30**, 120 (1967).

3. Stulberg, C. S., Zuelzer, W. W., Page, R. H., Taylor, P. E., and Brough, A. J., Proc. Soc. Exp. Biol. Med. **123**, 976 (1966).

4. Lang, D. J., and Hanshaw, J. B., N. Engl. J. Med. **280**, 1145 (1969).

5. Foster, K. M., and Jack, I., N. Engl. J. Med. **280**, 1311 (1969).

6. Diosi, P., and Roth, L., Pathol. Microbiol. **33**, 146 (1969).

7. Diosi, P., Moldovan, E., and Tomescu, N., Brit.

Med. J. 4, 660 (1969).

8. Epstein, M. A., Achong, B. G., and Barr, Y. M., Lancet 1, 702 (1964).

9. Hsiung, G. D., Fong, C. K. Y., and Lam, K. M., J. Immunol. 106, 1686 (1971).

10. Churchill, A. E., J. Nat. Cancer Inst. 41, 939 (1968).

11. Carmichael, L. E., J. Amer. Vet. Med Ass. 156, 1714 (1970).

12. McFerran, J. B., and Dow, C., Amer. J. Vet. Res. 26, 631 (1965).

13. Appel, M. J. G., Menegus, M., Parsonson, I. M., and Carmichael, L. E., Amer. J. Vet. Res. 30, 2067 (1969).

14. Wong, T. W., and Warner, N. E., Arch. Pathol. 74, 403 (1962).

15. Henson, D., and Neapolitan, C., Amer. J. Pathol. 58, 255 (1970).

16. Medearis, D. N., Jr., Amer. J. Hyg. 80, 103 (1964).

17. Heine, U., Kondratick, J., Ablashi, D. V., Armstrong, G., and Dalton, A. J., Cancer Res. 31, 542 (1971).

Received Mar. 14, 1972. P.S.E.B.M., 1972, Vol. 140.