Replication of Murine Cytomegalovirus in Reproductive Tissues

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Murine cytomegalovirus (MCMV) was found in reproductive tissues of newborn mice acutely infected with this virus. Using ^{3}H-labeled complementary RNA (cRNA) probe made from MCMV DNA, viral genetic material clearly occupied ovarian stromal cells surrounding the follicular region but not cells of the follicle or cells in the outer tunic layers. In the testes, squamous epithelial cells external to the tunica albuginea harbored viral genetic material. The immaturity of germ line cells in testes of one-week-old mice precluded identification of these cells or examination of their involvement. The presence of viral DNA in reproductive tissue during acute infection raised the possibility that the ovary and/or testes might act as a reservoir for latent virus later in life of adult mice. Hybridization kinetics analysis of DNA isolated from ovaries and testes of 5- and 6-month-old mice latently infected with MCMV suggested that the viral genome was present in both organs. (Am J Pathol 98:213-224, 1980)

NEONATAL INFECTION by cytomegalovirus (CMV) is responsible for a substantial portion of developmental defects in humans.[1,2] Perinatal acquisition of CMV occurs during the passage of the fetus through the birth canal.[3] However, there is evidence that CMV may be transmitted transplacentally as well [4,5] and that CMV may be carried in or on sperm carried in seminal vesical fluids.[6,7,8,9] Further support for possible vertical transmission are experiments showing that female mice infected with murine cytomegalovirus (MCMV) prior to conception produced fetuses containing the genetic information from the same virus,[10] other experiments showing MCMV DNA in mature sperm obtained from adult athymic mice,[11] and *in vitro* evidence that MCMV affects fertilization.[12]

We have now studied whether MCMV can replicate in reproductive tissues of mice inoculated with virus at birth and then examined whether the virus remains latent within the reproductive organs of these mice during adult life.

Materials and Methods

Mice

Pregnant 2–3-month-old C3H/St mice (major H-2 alleles:kk) were purchased from L. C. Strong Research Foundation, Del Mar, California.

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Supported by USPHS grants AI-07007 and GM-02179, Biomedical Research Support Program 1 S07 RR-05514, and National Foundation March of Dimes Grant 1-364.

Accepted for publication August 10, 1979.

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0002-9040/80/0109-213$01.00

Virus

MCMV was obtained and passaged as previously described.[13,14] Newborn mice became acutely infected after intraperitoneal (IP) inoculation with 100 $TCID_{50}$ of tissue culture passaged MCMV within 24 hours of birth and were nursed by their own mothers. Survivors of this acute infection were considered latently infected (by published criteria [14,15]). In this model both the route of inoculation (IP) and use of tissue-culture–passed virus are necessary to achieve a latent state.[16,17] Latency was determined by the inability to detect infectious virus on tissue explants alone or tissue explants cocultivated with syngeneic mouse embryo cells.

Infectivity Titers

Reproductive organs were removed from 8–12 mice killed at the ages of 1, 2, 3, or 4 weeks. After one wash with phosphate-buffered saline, separate tissues were immediately dounce homogenized in 2 ml of Dulbecco's minimal essential medium containing 10% fetal calf serum (DMEM). This solution was clarified by low-speed centrifugation, and the supernatant was removed, diluted in DMEM, and plated in duplicate at 1 ml/well on 75–85% confluent secondary mouse embryo fibroblast monolayers grown in Linbro plates containing 24 wells. The fluid in these wells was aspirated, and the cells were fed with 1.5 ml DMEM the following morning. For the next 7 days, the cytopathic effects were evaluated and the $TCID_{50}$ calculated.

Hybridization Kinetics Analysis

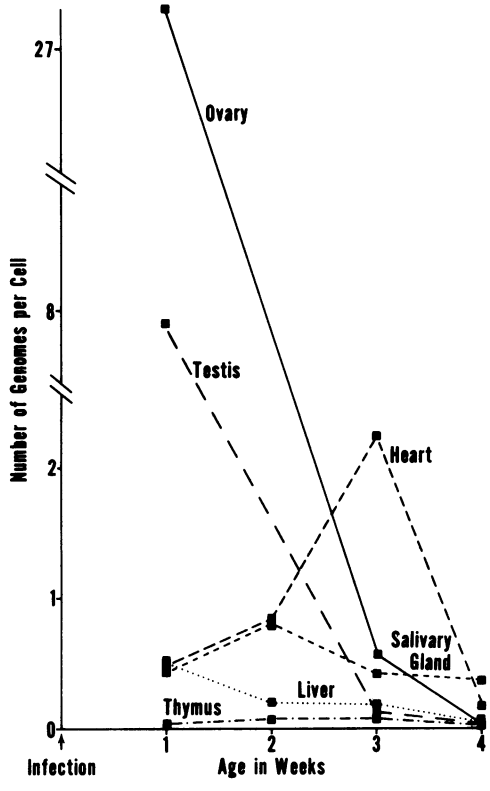
The methods used to prepare, purify, and label ^{3}H-thymidine MCMV DNA have been published.[13,15] DNA was extracted from pools of each type of organ taken from groups of ten MCMV-infected mice sacrificed at appropriate ages. Pilot experiments indicated difficulty in consistently recovering enough DNA from 1–2-week-old individual mice for all assays. Hence, all studies were done on tissues harvested from groups. As controls, DNA was obtained from the pooled organs of 2-month-old uninfected C3H/St mice containing no antibodies to MCMV, which was determined by either complement fixation or immunofluorescence assays. Cellular DNAs were extracted, sonicated, and hybridized in solution (0.48 M phosphate buffer at 65 C) at a concentration of 5 mg/ml with a trace amount of ^{3}H-thymidine–labeled MCMV DNA as described by Brautigam et al.[13] ^{3}H-labeled MCMV DNA had a specific activity of 5×10^6 cpm/μg and was present at a ratio of 1 viral genome/12 cellular haploid genome equivalents. Reactions were carried to a Cot of 30,000 mole-sec/l, or if not, to at least half of completion, and the reaction rate was analyzed at intervals by hydroxyapatite column chromatography.

In Situ Hybridization

Methods involved in transcription of complementary RNA (cRNA) with purified MCMV DNA as a template and use of this cRNA for cytohybridization reactions have been published.[13,18,19] Reactions were performed on 8-μ-thick cryostat-cut sections of ovaries and testes taken at 1 week from mice infected with MCMV at birth. A single reaction was performed on each of sequentially cut sections placed side by side on the same slide. Controls run concurrently consisted of reactions performed on sectioned tissues from uninfected ovaries and testes and from uninfected and MCMV-infected secondary mouse embryo fibroblast cells.

Stimulation of Follicular Development With Pregnant Mare Serum Gonadotropin (PMSG)

Fifteen female mice infected with MCMV at birth were inoculated intraperitoneally with 5 IU PMSG as follows: 2 mice were inoculated on the fifth day after birth and killed 4 days later, 4 mice were inoculated on the sixth day after birth and killed 4 days later, 7



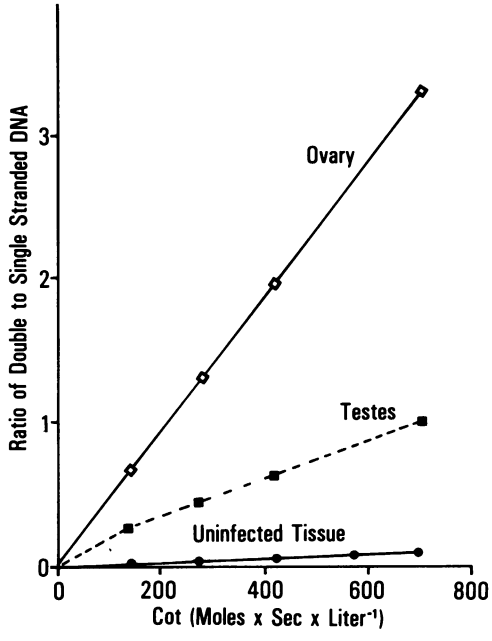
TEXT-FIGURE 1—Quantitation by MCMV DNA: murine DNA hybridization kinetics. The average number of viral DNA copies per cellular DNA equivalent in DNA extracted weekly from organs of mice infected at birth with MCMV is shown. Curves for spleen, kidney, and brain were similar to that for the thymus and are not shown.

mice were inoculated on the seventh day after birth and killed 2 days later, and 2 mice were inoculated on the 13th day after birth and killed 2 days later. Additionally, 3 mice were inoculated with 10 IU PMSG on the fifth day after birth and killed 3 days later. (PMSG was provided by Dr. B. Lasley, of the San Diego Zoo Research Division, San Diego, California.)

Results

Viral DNA Levels in Neonatal Organs After Acute MCMV Infection

At increasing time periods after birth, the relative amounts of MCMV DNA in different tissues harvested from mice infected at birth with MCMV were studied by hybridization kinetics analysis. To obtain enough tissue DNA for analysis, tissues from 10 mice were pooled. Examination of the levels of MCMV DNA per cell in a variety of tissues from 1-week-old mice showed that ovaries and testes harbored significantly more MCMV DNA than did hearts, salivary glands, thymuses, livers (Text-figure 1), brains, kidneys, and spleens (not shown). In repeated experiments with tissues from 1-week-old mice, the ovaries harbored more than 27 viral DNA



TEXT-FIGURE 2—Hybridization kinetics analysis of the rate of reassociation of ^{3}H-labeled MCMV DNA in the presence of DNA extracted from pooled ovaries or testes from 1-week-old MCMV-infected mice. Numbers of genome copies per cell were calculated from the half-Cot by comparison with an assumed half-Cot of 3000 for the mouse unique-sequence DNA, and were corrected for the reassociation in the presence of uninfected (negative control) mouse DNA.

genomes per cell, the testes 5–8 viral genomes per cell; but the other tissues had no more than 1 viral DNA copy per cell. In tissue harvested from 3-week-old mice, the number of genomes of MCMV DNA found per ovarian or testicular cell had decreased markedly. At this time the number of MCMV DNA copies per reproductive cell was equivalent to amounts found per cell in the spleen, liver, and kidney but less than that in the salivary gland and heart and slightly more than that in the thymus (Text-figure 1). Text-figure 2 shows the raw data for Cot analysis on reproductive tissues from 1-week-old MCMV-infected mice. Data from this and similar experiments were used to plot Text-figure 1.

Infectivity Titers in Reproductive Tissues Closely Paralleled the Results Obtained From MCMV DNA: Mouse DNA Hybridization Assays

Measuring infectivity in gonads from individual mice, we found the highest viral titers in 1-week-old mice and a rapid decline thereafter. Thus, the geometric mean infectivity titer of 1-week-old mice was $10^{7.4}$ $TCID_{50}$ per gram of tissue in ovaries and $10^{6.2}$ in testes. In contrast, in ovaries and testes harvested from 3-week-old mice, no infectious virus was detectable (<10 $TCID_{50}$).

Localization of MCMV Replication to Specific Cell Types in Reproductive Tissues Harvested From the Neonatal Mouse During Acute Infection

The finding of approximately 27 copies of MCMV DNA per ovarian cell and 8 copies of MCMV DNA per testicular cell (Text-figure 1) by DNA:DNA hybridization indicated the possibility of utilizing *in situ* hybridization to localize the specific cells harboring MCMV DNA, although viral inclusions were not observed in cells by histologic assay. With ^{3}H-labeled cRNA transcribed from purified MCMV DNA, *in situ* cytohybridization was performed on ovarian sections from 45 1-week-old mice and testes from 5 1-week-old mice infected at birth with MCMV.

Examination of ovarian sections from infected mice consistently revealed numerous grains over individual stromal cells and small groups of such cells in the interstitial perifollicular region of the ovary, beneath the tunica albuginea. These labeled cells were located just outside the central follicular region composed of the oocyte-containing follicles (Figure 1). When serial cryostat sections from each of a number of individual ovaries were examined, we observed that labeled cells repeatedly appeared in the same location from section to adjacent section (Figure 2), thus certifying that the clustering of grains was not a matter of mere chance. Further, grains were not detected over uninfected ovaries or uninfected mouse embryo fibroblast cells but did occur over mouse embryo fibroblasts infected with MCMV. The ovarian cells containing MCMV DNA were located in the interstitial cell area of the ovary. Neither granulosa cells of the follicle nor oocytes were labeled with the cRNA probe.

We then attempted to stimulate follicular development by administering high doses of gonadotropin (PMSG). Even after several courses of PMSG injections, we found no cRNA MCMV grains over developing follicles or oocytes.

Cells in testes from 1-week-old mice were not differentiated sufficiently to allow definitive identification of germ line cells among the other supporting cells. Consequently only a small number of testes were examined by *in situ* hybridization. We were able to find grains over epithelioid-like cells external to the tunica albuginea (Figure 3).

Detection of MCMV Genome in Reproductive Tissues of Adult Mice Latently Infected With MCMV

The finding that MCMV replicated in cells of the ovaries and testes of young mice suggested that the virus might possibly remain latent for prolonged periods in these tissues. To examine this possibility, we removed ovaries and testes from 5–6-month-old C3H/St mice that had been infected with MCMV since birth. These mice were latently infected and not

shedding detectable MCMV as determined by criteria of Olding et al.[14,15] Hybridization kinetic analysis was performed with a ^{3}H-thymidine–labeled MCMV DNA probe, and DNA was isolated from pools of 10 ovaries or 10 testes. In all three experiments done the MCMV genome was found above reassociation background levels and detected at 2–5 viral genome equivalents per 100 gonadal cells from infected mice. Hybridization to DNA from infected ovarian and testicular tissues was 15% above background controls. These levels of DNA approximate the levels previously observed in lymphoid tissues [15] that harbor virus in a latent state. In contrast, the viral genome was not detected in DNA obtained from ovaries or testes from uninfected mice or DNA obtained from brain or thymus from MCMV-infected mice.

Discussion

This study demonstrates that MCMV replicates at significantly higher levels in the reproductive tissues of newborn C3H/St mice after acute infection than in cells harvested from hearts, salivary glands, livers, thymuses, brains, spleens, or kidneys. Thus, 1 week after infection, approximately 27-fold more viral DNA copies were found in ovarian cells and 8-fold more viral DNA copies in testicular cells than in cells harvested from the other organs. The amount of virus carried in reproductive tissues sharply declined thereafter, so that by 4 weeks after infection fewer DNA viral copies per cell were found in reproductive tissues than in salivary gland, heart, or liver tissues. Further, ovaries and testes harvested from adult mice that had survived the acute infection apparently harbored low levels of MCMV genome. The amount of viral genome carried per ovarian and testicular cell varied from 2–5 MCMV gene equivalents per 100 cells in these adults. This amount was 15% above background control levels and was found in three separate experiments.

Next, to determine whether germ line cells found within the reproductive tissues contained MCMV DNA, we used *in situ* hybridization with a MCMV cRNA probe. In ovaries of 1-week-old infected mice, we found MCMV DNA localized within investing stromal cells at a distinct zone peripheral to the central region containing the follicles but interior to the tunica albuginea. In multiple sections studied from numerous ovaries, we were unable to detect viral DNA in oocytes. Even after gonadotropin was injected to induce follicular development, no evidence of the viral genome was apparent in female germ line cells. With our present technology we are unable to determine whether some cells that did not become labeled contained MCMV DNA at amounts below the limit of detection. Owing to the immaturity of the testicular cells in 1-week-old mice, we

were unable to determine whether MCMV replicated in male germ line cells.

Recently Dutko and Oldstone [11] used *in situ* hybridization assays on testicular sections and solution hybridization of purified sperm from testes of adult nude athymic CBA mice and found evidence of MCMV replication in male germ line cells. This finding, in association with our data that MCMV viral DNA lingers in reproductive organs, marks these tissues, as well as splenic B lymphocytes,[14] peritoneal macrophages,[13] and salivary glands,[20] as another potential area for the harboring of latent virus. Further, the MCMV in germ line cells of the male reproductive system [11] could account for the possible maintenance or spread of virus by vertical transmission, although there is no information yet as to whether such sperm are lytically infected.

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Acknowledgments

The authors thank Kurt Benirschke, Clark Huang, and David T. Kingsbury for their interest and support and Laura Taxel for secretarial help.

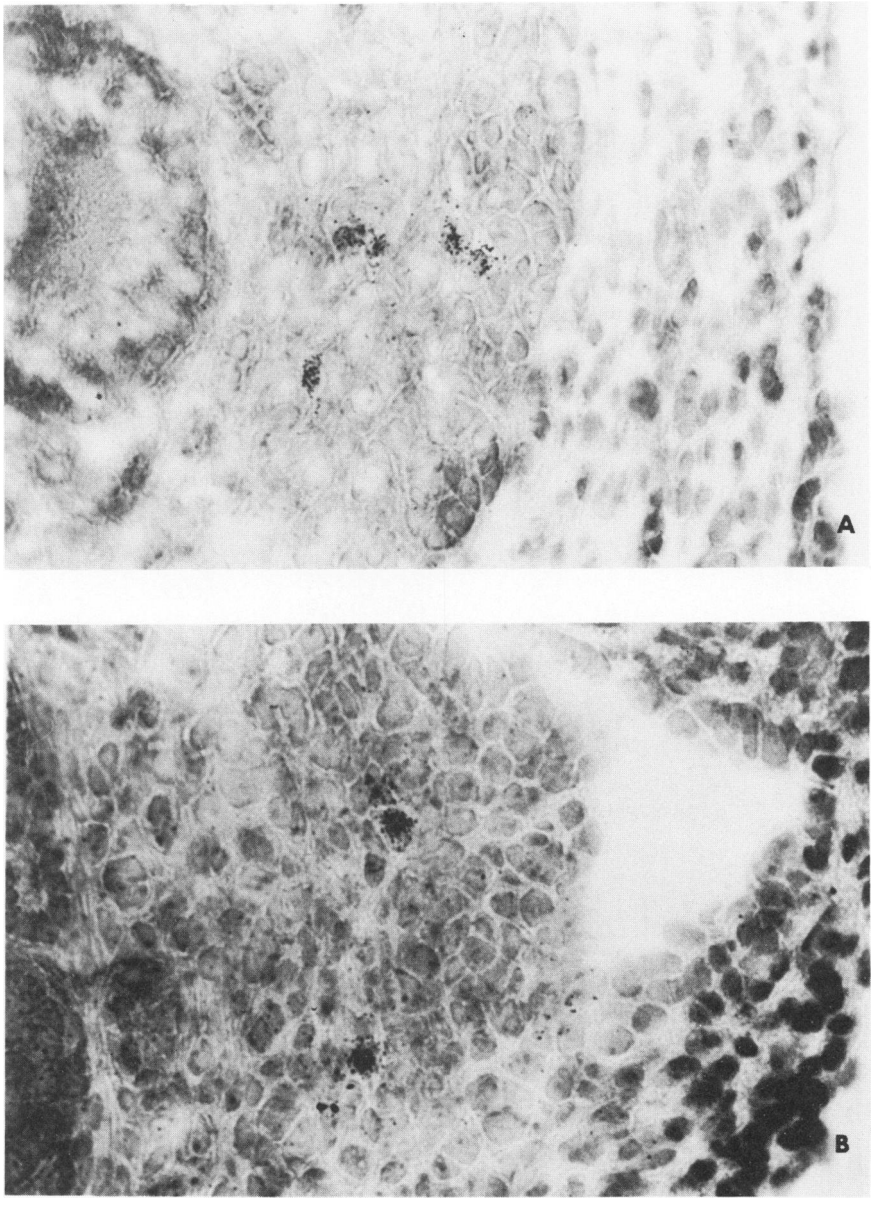

Figure 1—*In situ* hybridization of sections from ovaries of 2 1-week-old MCMV-infected mice. Grains appear over cells in the layer peripheral to the follicular region but inside the tunica albuginea. **A**—Single large follicle seen at left. The tunica albuginea is the light colored band of cells at right, clearly demarcated from the investing cell layer. No grains are noted over follicular area. **B**—Portions of several follicles are seen at far left; external to these is the zone of investing stromal cells (*center*) that surrounds the follicular region. The tunica albuginea is seen at the right and has separated from the investing cell layer in one area. The white region in the lower right corner is external to the ovary. Again, no grains are noted over follicular cells. (×566)

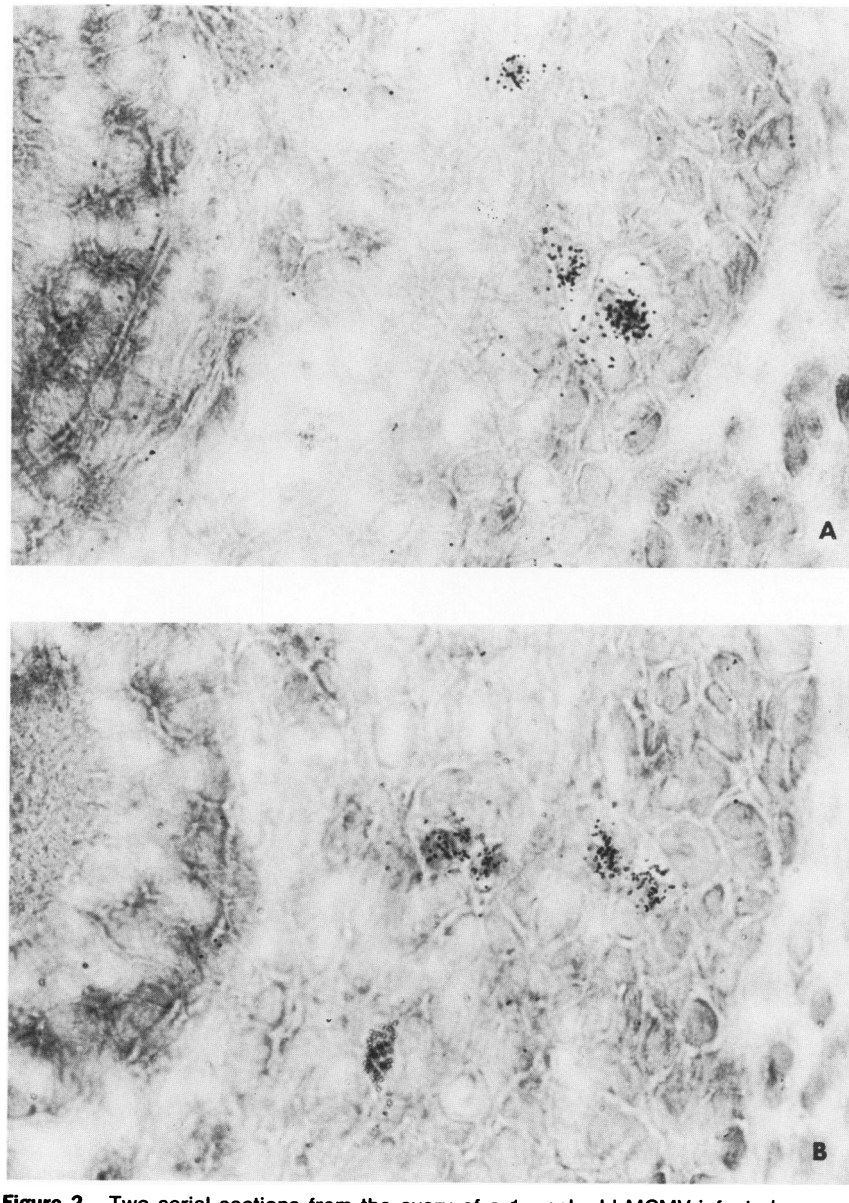

Figure 2—Two serial sections from the ovary of a 1-week-old MCMV-infected mouse were placed side by side on a slide and hybridized *in situ*. Both photos show the same region. At *left*, follicle. *Center*, investing cell layer with ^{3}H-thymidine label over cells. *Right*, tunica albuginea. (×893)

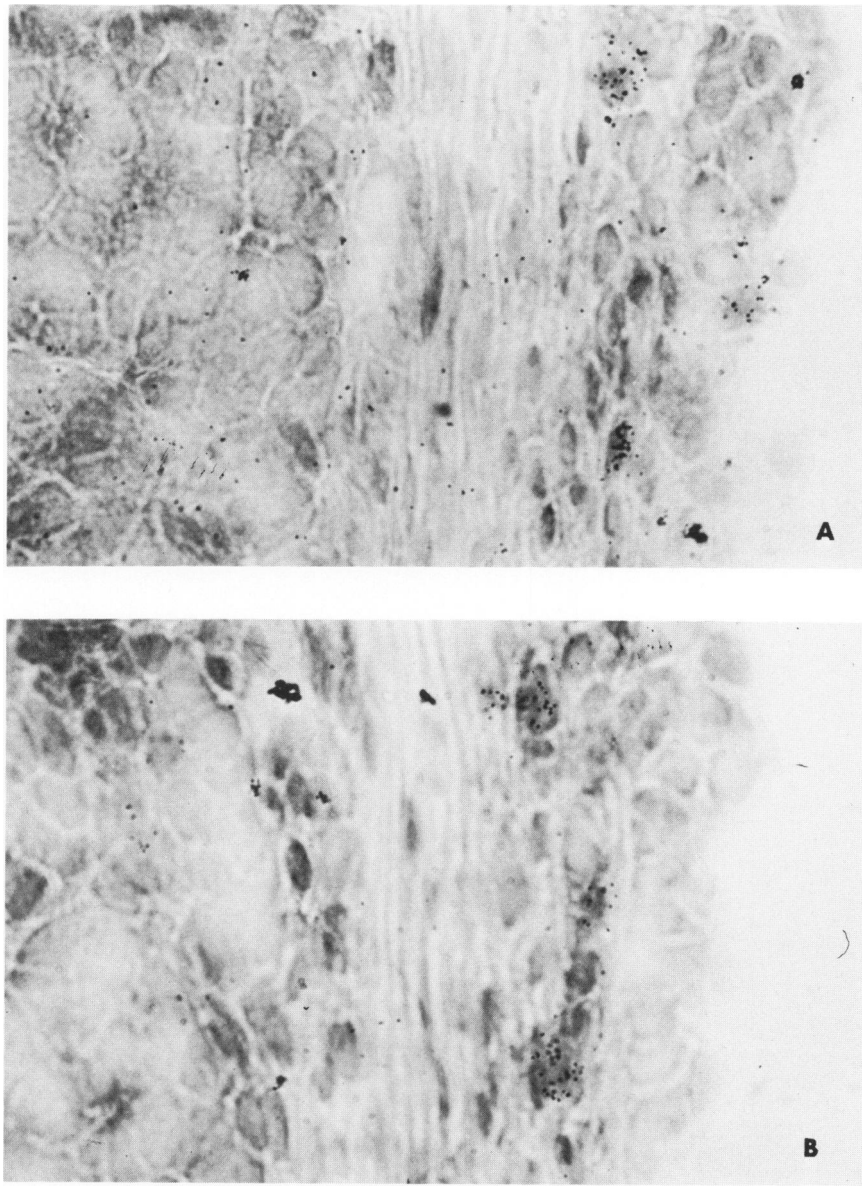

Figure 3—Adjacent serial sections from a testis of a 1-week-old MCMV-infected mouse. A single *in situ* hybridization reaction was performed on both sections. The same region is shown in both photos and grains are seen over corresponding cells at the right of center in both. *Left*, immature testicular cells. *Center*, tunica albuginea. *Right*, germinal epithelium. *Clear area at far right*, external to the testis. (×893)

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