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Pathogenesis of Murine Cytomegalovirus Infection: Identification of Infected Cells in the Spleen during Acute and Latent Infections

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Spleen cells which replicate murine cytomegalovirus (MCMV) during acute infection in vivo were identified by electron microscopy and combined immunocytochemical staining and in situ cytohybridization. Most infected cells, as defined by in situ hybridization for viral RNA with MCMV-specific probes, were shown to be positive for factor VIII-related antigen and negative for Ia, Thy-1, and F4/80 antigens. Electron microscopic ultrastructural observations indicated that the infected cells in the spleen are predominantly sinusoidal-lining cells. We also studied reactivation of MCMV from latently infected mice by cocultivation of spleen cells with mouse embryo fibroblasts. Virus was only recovered from cells in preparations of stromal (or reticular) fragments, and not from spleen cell suspensions. Neither removal of immunoglobulin-bearing cells from the stromal fragments by panning nor depletion of Thy-1- and Ia-bearing stromal cells by treatment with monoclonal antibodies and complement reduced the frequency of reactivation of MCMV. These data suggest that T lymphocytes, mature B lymphocytes, and other Ia-bearing cells are not predominant reservoirs of latent MCMV.

Human cytomegalovirus (HCMV) is an important human pathogen (for a review, see reference 47). Most HCMV infections are asymptomatic; however, in fetuses, neonates, and immunosuppressed adults, including patients with acquired immunodeficiency syndrome, HCMV infection may be much more serious. Cytomegalic inclusion disease results in birth defects affecting the nervous system, including blindness, deafness, and microcephaly. HCMV has also been associated with the reticuloendothelial tumor Kaposi's sarcoma (reviewed in reference 57). Although the pathogenic effects of this virus at the macroscopic level have been defined, the molecular events remain to be elucidated.

Infection of mice by murine cytomegalovirus (MCMV) resembles in many ways its human counterpart with respect to pathogenesis during acute infection, establishment of latency, and reactivation after immunosuppression, transfusion, and transplantation (for reviews, see references 27, 30, and 49). For this reason, we chose the murine system as a model to unite studies on the molecular biology and in vivo pathogenesis of the virus. As with HCMV, MCMV infects a variety of organs and cell types. The spleen, liver, adrenal gland, salivary gland, kidney, lung, ovary, and testis have been shown to be infected (1, 2, 8, 9, 23, 39, 42, 44), and in most cases, the infected cells appeared to be epithelial, interstitial, or parenchymal cells. Neurons are also infected by MCMV (13), and occasional infected endothelial cells have been observed (39, 44). During in vivo infection, MCMV also appears to be associated with peripheral blood leukocytes, as shown by isolation of virus (62) and by transfusion studies (11).

Recovery of both MCMV and HCMV from peripheral blood leukocyte fractions and transmission of the virus by transfusions have focused attention on the potential role of leukocytes in active viral replication and latency. In HCMV, leukocytes can be infected in vitro, but the infection is abortive or only a very low level of viral replication is observed (6, 16, 18, 51, 52, 60, 61). By in situ hybridization, Schrier et al. (53) have also been able to detect HCMV nucleic acid in peripheral blood leukocytes from infected patients. However, in another study, in which HCMV pathogenesis was investigated by in situ hybridization in bone marrow transplant patients with disseminated infection, Myerson et al. (46) found HCMV nucleic acid in epithelial, endothelial, stromal, and interstitial cells, but not in lymphocytes.

In mice, many studies have focused on the spleen, a large reservoir of leukocytes as well as an active site of MCMV replication during acute infection and a source of latent virus. The precise identity of the cells involved, however, remains obscure. In vivo and in vitro experiments indicate that cells that fractionate as B cells, T cells, and macrophages may all be acutely infected and contain MCMV in a latent state (4, 7, 28, 31, 35, 36, 43, 48, 54, 55, 59, 62). The interpretation of all of these experiments, however, is complicated by the varied methods used for infection and for separating and identifying B cells, T cells, and cells of the monocyte-macrophage series. In addition, only a small percentage of any cell fraction seems to be involved in CMV infection.

In a previous study (41), we showed by in situ hybridization that the primary site of MCMV replication in the spleens of susceptible and resistant mice involved the red pulp. The perifollicular areas within the red pulp hybridized most strongly, whereas the follicular areas showed no hybridization at any time in the infection. In those studies, however, we were not able to identify precisely the cell types involved in the infection. Recently, a powerful technique for the simultaneous detection of antigens and RNA in situ has been developed (5, 20). In this study, we used this technique with cloned subgenomic MCMV DNA fragments (40) as hybridization probes and well-characterized antibodies to cell-

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type-specific markers, along with electron microscopy, to identify the primary cells involved in acute MCMV infection of the spleen in vivo.

We also demonstrate that, with simple isolation of stromal fragments from the spleens of latently infected mice, we achieved 20- to 50-fold enrichment for latently infected cells. Further fractionation of stromal cells showed that the predominant population of latently infected cells did not include T lymphocytes, mature B lymphocytes, and other Ia-bearing cells.

MATERIALS AND METHODS

Virus, mice, and cells. MCMV (Smith strain) was obtained from Frank Dutko, Sterling-Winthrop Research Institute. BALB/c and C3H/HeN mice were obtained from Simonsen Laboratories. Mouse embryo cells (MEC) were prepared from 15- to 19-day-old BALB/c embryos as described by Mercer et al. (40). Salivary-gland-passaged MCMV was prepared as a 10% homogenate of BALB/c salivary gland in Dulbecco minimal essential medium containing 10% calf serum plus 10% dimethyl sulfoxide.

Restriction endonuclease digestions. Restriction endonucleases were obtained from one of the following sources: Bethesda Research Laboratories, New England BioLabs, Boehringer Mannheim Biochemicals, or Pharmacia P-L Biochemicals. Restriction digests were performed as recommended by the manufacturer.

Labeling of DNA with ^{35}S. Labeling of DNA by nick translation was performed as described by Tamashiro et al. (58), except that the incubation time with DNA polymerase I was lengthened to 7 to 8 h for labeling with [^{35}S]dCTP (Amersham Corp.).

Purification of DNA from agarose gels. DNA to be recovered was electrophoresed on low-melting-point agarose from Marine Colloids. Melted agarose containing DNA was loaded onto a benzoyl-naphthol-DEAE (BND)-cellulose (Accurate Chemical Co.) column and washed and eluted as described by Tamashiro et al. (58).

Infection of mice. All mice were infected intraperitoneally at 5 to 6 weeks of age with 10^5 PFU of salivary-gland-passaged MCMV.

Combined immunoperoxidase labeling and in situ cytohybridization. Cell suspensions or frozen sections were prepared as described by Haase et al. (22), fixed in 0.067 M lysine monohydrochloride–0.33 M Na_2HPO_4 (pH 7.4)–0.1% glutaraldehyde–0.05% paraformaldehyde–0.01 M *m*-periodate sodium (a modification of PLPG; 19) for 20 min, and washed three times in distilled water. Immunoperoxidase labeling was performed with biotinylated rabbit anti-rat immunoglobulin G (IgG; adsorbed with mouse serum and immunoglobulin; Vector Laboratories, catalog no. BA-4001) or biotinylated goat anti-rabbit IgG with avidin-biotinylated horseradish peroxidase complex (supplied as Vectastain ABC kits by Vector Laboratories) under the conditions described by the manufacturer. The substrate for peroxidase was 0.5 mg of 3,3'-diaminobenzidine tetrahydrochloride (DAB) per ml plus 0.01% hydrogen peroxide in phosphate-buffered saline (PBS). After DAB-peroxide treatment, slides were washed twice in distilled water and postfixed in ethanol-acetic acid (3:1) for 15 min, followed by 95% ethanol for 5 min. Prehybridization for in situ detection of RNA was performed as described by Brahic et al. (5). Hybridization and washing conditions were as described by Gendelman et al. (20), with the hybridization buffer and all washes containing 0.1% Triton X-100. Slides were dehydrated, dipped in emulsion, exposed, developed, and counterstained with methyl green as described by Brahic et al. (5).

Isolation and cultivation of stromal fragments from spleens. Mice were killed by CO_2 inhalation. Spleens were removed aseptically and placed in balanced salt solution (BSS; 45) or RPMI 1640 plus 15% fetal bovine serum. A hole was made in each end of the spleen capsule, and the contents of the spleen were extruded through these holes with bent 18-gauge needles. The empty capsule was discarded after preliminary experiments had shown that no virus could be recovered from this portion of the spleen (data not shown). The contents of the spleen were suspended in 10 ml of medium and placed in a 15-ml conical centrifuge tube. The stromal fragments are defined as those fragments which settle to the bottom of the tube in 10 to 20 min at $1 \times g$. C3H spleens typically yielded 50 to 100 μl of fragments, and collagenase-dispase digestion of these fragments yielded 5×10^6 to 8×10^6 cells. Stromal fragments or cells were cultured alone or with monolayers of MEC in RPMI 1640 medium containing 15% heat-inactivated fetal bovine serum and 8×10^{-5} M 2-mercaptoethanol. Cultures were fed by gentle aspiration of approximately 1.0 ml of the 1.5 ml in each well and replacement with fresh medium. Cultures were assayed for recovery of MCMV by direct observation of plaques in the MEC monolayer for cocultivation experiments. When stromal fragments were cultured without MEC, culture supernatants were transferred to MEC monolayers, which were scored for plaques 4 to 6 days later. Cultures were maintained for a minimum of 6 weeks in all experiments.

Collagenase-dispase digestion of stromal fragments. After lysis of erythrocytes by NH_4Cl, stromal fragments were suspended in 1 mg of collagenase-dispase (Boehringer-Mannheim) per ml–50 μg of DNase I (Worthington Diagnostics) per ml in PBS. After 30 to 45 min, cells were pelleted and resuspended in the same solution. This procedure was repeated until no visible clumps remained. The total time of digestion was usually 100 to 150 min.

Antibodies and complement. Monoclonal antibodies to Thy-1.2 and Ia^k were obtained from Becton Dickinson. Monoclonal antibody to Ia was obtained from Boehringer Mannheim. Monoclonal antibody F4/80 was a gift from Carol Cowing, Medical Biology Institute. Monoclonal antibody (IgM) to Ia^k was obtained from Litton Bionetics. Monoclonal antibodies HO and F7D5 to Thy-1 were a gift from Susan Swain, University of California, San Diego. Polyclonal rabbit anti-human factor VIII-related antigen was obtained from Dako. Rabbit complement was obtained from Pel-Freez and Litton Bionetics. Cytotoxicity was determined as follows. After collagenase-dispase digestion, cells were washed three times and suspended in BSS at 1×10^7 to 1.5×10^7 cells per ml. The suspension was divided in half; one half was treated with antibody plus complement, and the other half was treated with complement only. The two halves were subsequently cultured in equal numbers of wells. Antibodies HO and F7D5 (anti-Thy-1) were added at dilutions of 1:100 and 1:125, respectively. Antibodies to Ia or Ia^k were used at dilutions of 2 μg/ml and 1:500, respectively. After incubation with antibody for 30 min, cells were washed three times in BSS, suspended in a 1:10 dilution of complement in BSS, incubated for 20 to 30 min at room temperature, and washed three times in BSS.

Depletion of immunoglobulin-bearing cells by panning. Goat anti-mouse immunoglobulin was obtained from Cappel Laboratories. Panning was performed as described by Lewis and Kamin (34).

Labeling of cells for fluorescence-activated cell sorter anal-

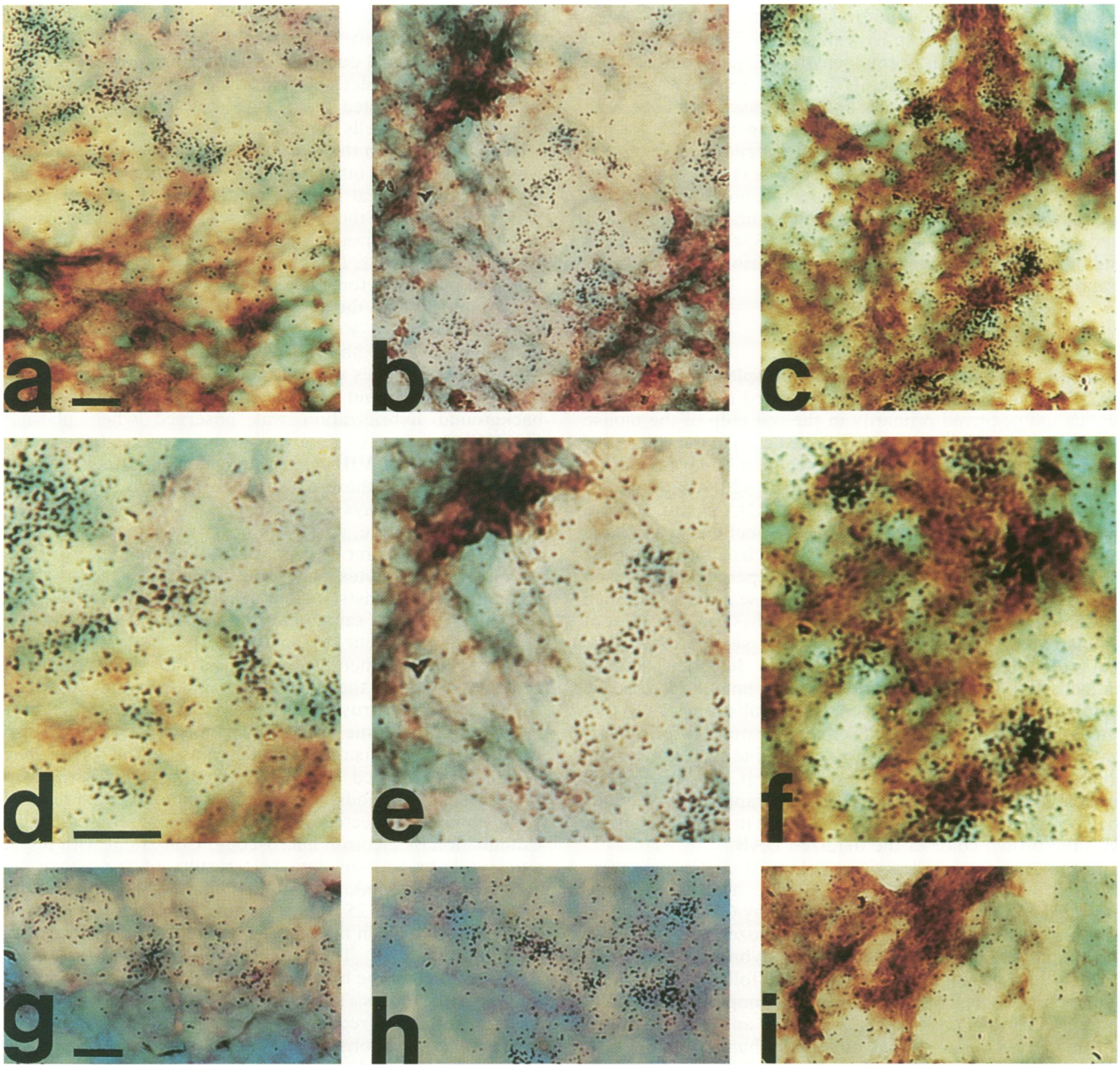

FIG. 1. Combined detection of cell-type-specific antigens and MCMV RNA. Spleen sections from BALB/c mice sacrificed at 2 days p.i. were fixed and reacted with the following: rat anti-mouse Ia (0.7 μg/ml; panels a and d), rat anti-mouse F4/80 (0.5 μg/ml; panels b and e), or rabbit anti-human factor VIII-related antigen (1:800; panels c, f, and i); biotinylated rabbit anti-rat IgG (3.3 μg/ml; panels a, b, d, e, and g) or biotinylated goat anti-rabbit IgG (10 μg/ml; panels c, f, and h); avidin-biotin-horseradish peroxidase complex; and DAB-H_2O_2 in PBS. After postfixation, the sections (panels a to h) were pretreated and hybridization for MCMV RNA was performed with ^{35}S-labeled MCMV *Eco*RI fragments E, P, and V as probes. The section in panel i was hybridized with ^{35}S-labeled plasmid pACYC184 as a probe. Panels a to i, methyl green counterstain. The autoradiographic exposure time was 20 h (panels a, b, d, and e) or 72 h (panels c, f, and i). Panels d, e, and f are higher magnifications of the fields shown in a, b, and c, respectively. Bars, 20 μm.

ysis. Cells were pelleted and suspended in 2 to 3 ml of 0.144 M NH_4Cl–0.017 M Tris hydrochloride, pH 7.65, for 5 min at room temperature to lyse erythrocytes. The remaining cells were washed three times in cold PBS and suspended at 5×10^5 cells per ml in PBS. Aliquots of 0.1 ml were labeled with primary antibody (rat anti-mouse Thy-1.2 or mouse anti-mouse Ia^k; Becton-Dickinson) at 2.5 μg/ml for 20 min. Cells were washed three times in 1 ml of PBS, suspended in 0.1 ml of PBS, and labeled for 20 min with 2.5 to 7.5 μg of fluoresceinated secondary antibody (goat anti-rat immunoglobulin [Cappel] or goat anti-mouse IgG [Becton-Dickinson]) per ml. The cells were washed with PBS–5% fetal bovine serum and again with PBS and suspended in 1 ml of PBS–1% formaldehyde. All steps were performed at 0 to 4°C. PBS contained 0.1% sodium azide. Negative controls were performed by omitting primary antibody treatment. Fluorescence was analyzed with an Ortho Cytofluorograf System 50-H.

Electron microscopy. One BALB/c mouse was euthanized at 3 days postinfection (p.i.) with sodium pentobarbital and perfused with 2% paraformaldehyde–3% glutaraldehyde in 0.1 M phosphate buffer at pH 7.4. The spleen was diced, fixed at 4°C for 1 h in fresh fixative, rinsed in buffer, and postfixed in 1% osmium tetroxide in phosphate buffer for 1 h. Tissue was rinsed, dehydrated, and embedded in Epon and Araldite (in equal proportions). Sections (1 μm thick) were cut, and regions containing red and white pulp were selected for thin sectioning and poststaining in uranyl acetate and lead citrate. Sections were viewed at ×8,000 magnification, and all cells containing discernible virions were photographed.

RESULTS

Identification of infected cells in the spleen during acute MCMV infection. We previously reported (41) that MCMV replication occurred primarily in the red pulp of the mouse spleen. To identify further the infected cells in the spleens of susceptible mice, we used the technique developed by Brahic et al. (5) and Gendelman et al. (20) which allows simultaneous detection of antigens and RNA in situ. Briefly, the technique involves fixation of tissue sections in a fixative compatible with both immunoperoxidase labeling of antigens and in situ cytohybridization. For our experiments, we used frozen rather than paraffin-embedded sections. Although paraffin-embedded sections provide the best preservation of cellular and tissue morphology, frozen sections allow for markedly increased sensitivity for the detection of both antigens and nucleic acid. After fixation, immunoperoxidase staining is performed with a peroxidase substrate (DAB) that remains stable during the subsequent treatments for hybridization. The sections are then pretreated to increase permeability and hybridized with ^{35}S-labeled MCMV subgenomic probes. After washing and autoradiography, the sections may be counterstained with methyl green to provide contrast with the brown color of the oxidized DAB.

In the experiments described below, BALB/c mice were infected intraperitoneally with 10^5 PFU of salivary-gland-passaged MCMV. Mice were sacrificed by CO_2 inhalation at 2, 4, and 6 days p.i., and spleens were frozen and processed for immunoperoxidase staining and hybridization as described in Materials and Methods. The following cell-type-specific antibodies were used for immunoperoxidase staining: monoclonal anti-Thy-1.2, anti-Ia, and F4/80 (all rat anti-mouse) and polyclonal rabbit anti-human factor VIII-related antigen. Biotinylated rabbit anti-rat IgG and biotinylated goat anti-rabbit IgG were used as secondary antibodies. An equimolar mixture of cloned subgenomic MCMV *Eco*RI fragments E, V, and P (which represent regions of immediate-early, early, and late transcription, respectively) were used as hybridization probes (38, 40).

Figure 1 illustrates the results of combined immunoperoxidase staining and hybridization on spleen sections at 2 days p.i. In Fig. 1a and d, we show the results obtained with anti-Ia as the primary antibody, which reacts primarily with mature B lymphocytes, most macrophages, and dendritic cells (for a review, see reference 17). We found that most of the infected cells (>95%) were Ia negative, with a few cells scoring as slightly Ia positive. When the macrophage-specific antibody F4/80 (29) was used as the primary antibody, infected cells were not labeled (Fig. 1b and e), suggesting that the primary site of replication was not a macrophage.

The use of anti-factor VIII-related antigen, which stains endothelial cells, sinusoidal-lining cells, megakaryocytes, and platelets (3, 10, 14, 26, 50), as the primary antibody allowed us to identify the site of MCMV replication. Virtually all of the signal due to in situ hybridization was seen in the regions of the spleen labeled by this antibody (Fig. 1c and f). It should be noted that, in general, we observed more hybridization to cells which labeled less with anti-factor VIII-related antigen than to those which labeled more. This was due to general inhibition of hybridization by the dense immunocytochemical staining of the cells. Furthermore, it was not specific for the antibody used, since a similar loss of in situ hybridization signal, particularly noticeable in densely stained preparations, has been reported when other nucleic acid and immunocytochemical probes are used (20).

As controls for the experiments described above, we showed that there was no staining of the sections with biotinylated rabbit anti-rat IgG or biotinylated goat anti-rabbit IgG antibodies when primary antibody treatment was omitted (Fig. 1g and h, respectively). Furthermore, only background hybridization was observed when plasmid pACYC184 was used as a probe on sections stained with antibody to factor VIII-related antigen (Fig. 1i).

On the basis of these data, most of the MCMV-infected spleen cells at 2 days p.i. were factor VIII-related-antigen positive, Ia negative, and F4/80 negative. The infected cells also were negative for Thy-1 antigen, which appears primarily on T lymphocytes and some brain cells and in small amounts on fibroblasts and epithelial cells (for a review, see reference 33). This same pattern of labeling of the infected cells was also evident at 4 and 6 days p.i. (data not shown).

To evaluate further the identity of the MCMV-infected cells, we examined the ultrastructural morphology of spleen cells containing virus at 3 days p.i. To permit the best preservation of tissue and cellular morphology, the mouse was perfused with fixative before removal of the spleen. The spleen was prepared for light microscopy as described in Materials and Methods, and regions containing both red and white pulp were selected, thin sectioned, and poststained for examination by electron microscopy. Consistent with the in situ cytohybridization data (41; results of the above-described experiments), virus-containing cells were only found in the red pulp. Of 25 cells examined, 24 contained most of the virus particles in the nucleus and had similar ultrastructural morphologies. These cells (Fig. 2) did not appose a basement membrane but rather showed intimate association with reticular fibers. They had an overall stellate morphology and formed infrequent primitive desmosomal junctions with similar neighboring infected and uninfected cells. They had more electron-dense cytoplasm containing rare pinocytotic vesicles. On the basis of this morphology and the cell location within sinuses, they were classified as sinusoidal-lining cells. The one cell containing MCMV in both the nucleus and the cytoplasm (Fig. 3a and b) had microvillus processes and was apposed to a partially denuded basement membrane, suggesting that it had undergone retraction. The cytoplasm was of low density and contained several degenerative organelles. This morphology is consistent with an endothelial cell.

Identification of MCMV-infected cells in spleens of latently infected mice. The spleen is not only a site of active MCMV replication but also a noted reservoir of latent MCMV. As discussed below, however, because of the low numbers of latently infected cells per spleen, we were not able to use the technique of in situ cytohybridization to identify the site of latency. Therefore, we chose an approach which involved detection of reactivated virus after in vitro culture of fractionated spleen cells isolated from latently infected mice. In

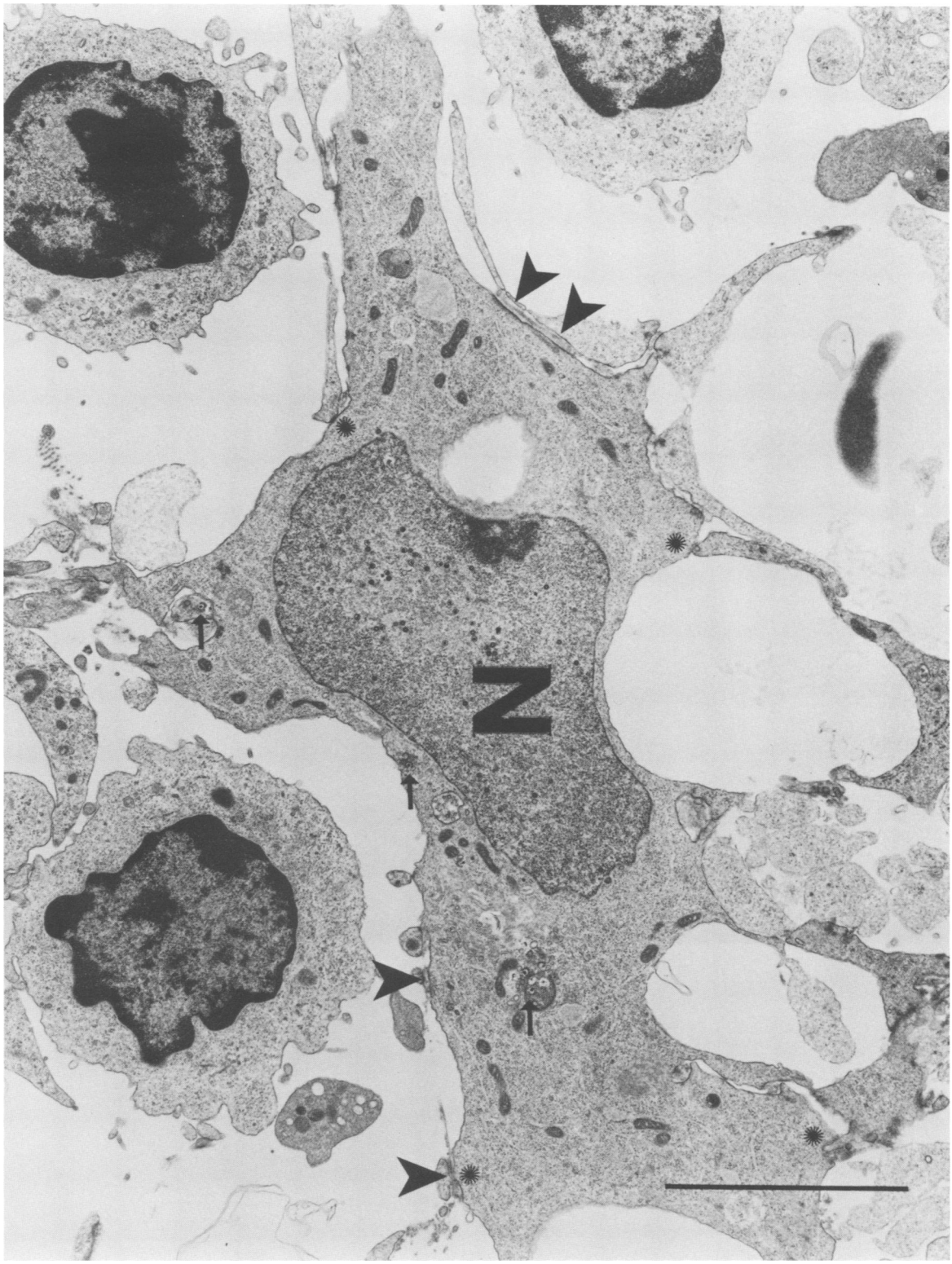

FIG. 2. Electron micrograph of an MCMV-infected cell at 3 days p.i. Spleen sections from a BALB/c mouse at 3 days p.i. were prepared for electron microscopy as described in Materials and Methods. A sinusoidal-lining cell infected with MCMV is shown. Numerous virions are noted in the nucleus (N), and occasional virions are seen in the cytoplasm (arrows), adjacent to degenerating organelles. The cell has a stellate morphology and forms desmosomal junctions with adjacent cells (asterisks). The cytoplasm is electron dense, and where extracellular reticular fibrils abut the cell, the cytoplasmic membrane shows a dense undercoating (arrowheads). Staining was with lead citrate and uranyl acetate. Magnification, ×8,000; bar, 5 μm.

these experiments, reactivated virus was detected by assaying culture supernatants or by directly placing susceptible indicator fibroblasts in culture with the spleen cells. In all of the experiments, cultures were maintained for at least 6 weeks.

C3H and BALB/c mice were infected intraperitoneally at 5 to 6 weeks of age with 10^5 PFU of salivary-gland-passaged MCMV. In all of the experiments (except where noted), mice were sacrificed more than 4 months p.i., and salivary gland homogenates were assayed for MCMV on mouse embryo fibroblasts to detect persistent MCMV replication. Assays for all mice, except where noted, were negative, with a sensitivity of 15 to 20 PFU per salivary gland.

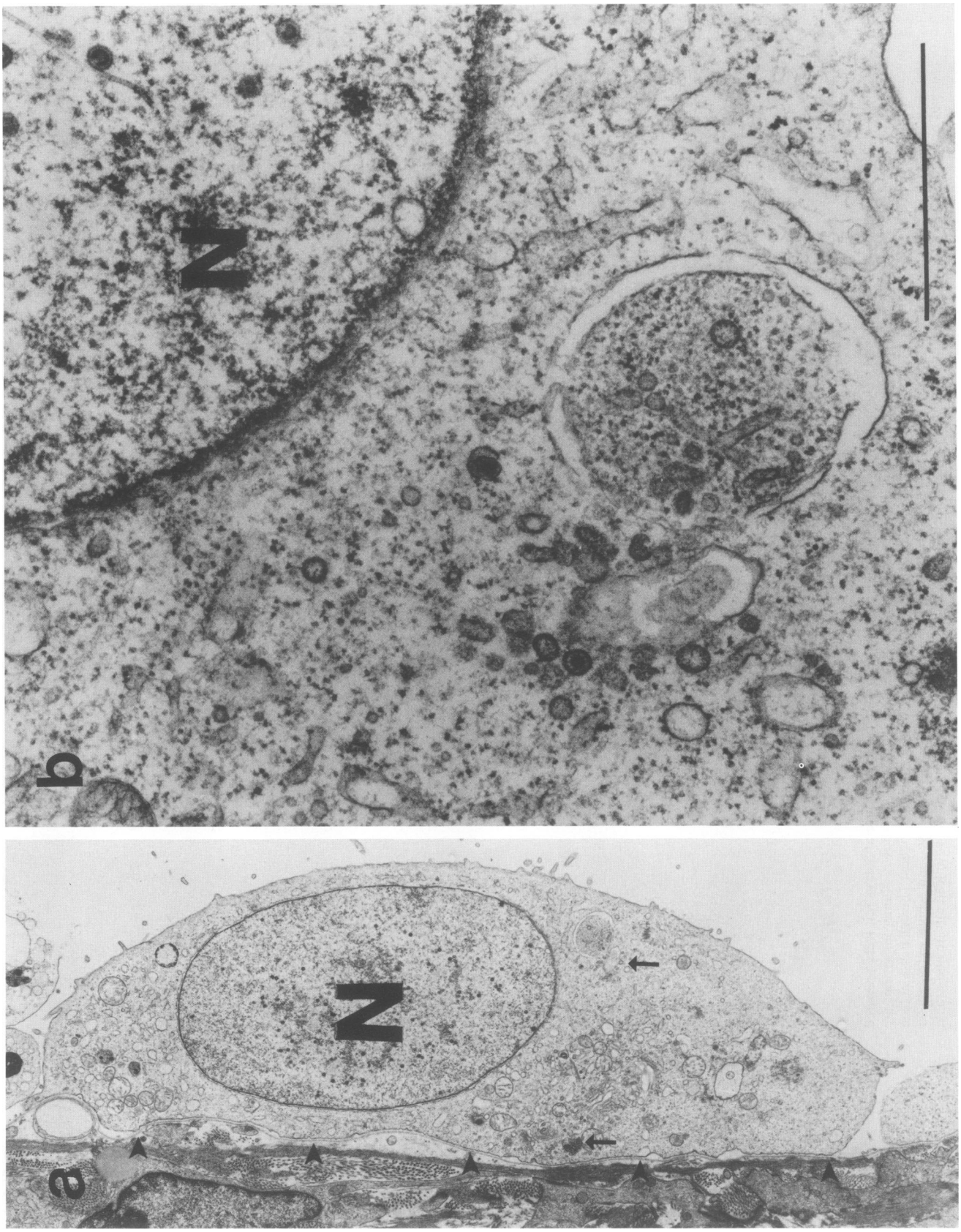
N
b
N
a

TABLE 1. Recovery of MCMV after reciprocal mixing of stromal and suspension fractions[a]

Stroma	Suspension	MCMV recovery from pairs of:			
		BALB/c mice		C3H mice	
		1	2	3	4
Uninfected	Uninfected	−	−	−	−
Uninfected	Infected	−	−	−	−
Infected	Uninfected	−	+	+	+
Infected	Infected	+	+	+	+

[a] For each pair (infected and uninfected) of mice, splenic stromal fragments and cell suspension fractions were separated as described in Materials and Methods. The fractions were cocultivated as indicated above, and supernatants were assayed for virus on BALB/c MEC in 24-well plates.

In a pilot experiment to determine the frequency of latently infected cells in the spleen, we performed a limiting dilution assay with one C3H and one BALB/c mouse. Spleens were removed, and with the use of two bent hypodermic needles, the contents of each spleen were forced through holes made at the ends of the capsule and suspended in 10 ml of medium. In this report, stromal fragments are splenic fragments which settled through 10 ml of medium to the bottom of a 15-ml conical centrifuge tube at $1 \times g$ in 10 to 20 min. Each spleen typically yielded 50 to 100 μl of stromal fragments containing 5×10^6 to 8×10^6 cells. The stromal fragments and cells remaining in suspension were then tested separately for reactivation of latent virus. We cultured 10^7, 10^6, and 10^5 of the suspension cells in eight separate wells for each dilution. The stromal fragments were also aliquoted and cultured in eight separate wells. Supernatants were assayed on mouse embryo fibroblasts, and cultures were fed with fresh medium every third day. Surprisingly, virus was only recovered from wells containing the stromal fragments, with two of eight wells positive for the BALB/c mouse and four of eight wells positive for the C3H mouse. No virus was recovered from the suspensions, even at a concentration of 10^7 cells per well. In addition, no virus could be recovered from the empty capsule of the spleen.

One possible interpretation of these results was that the stromal fragments were not necessarily the only source of virus but that the fragments were enriched in permissive cell types so that recovery of virus was enhanced. To determine whether the stromal fragments were the only source of MCMV, we performed a reciprocal mixing experiment with suspension and stromal fragments from additional infected and uninfected C3H and BALB/c mice. The results (Table 1) indicate that virus cannot be recovered from cell suspensions, even with the addition of stromal fragments from uninfected mice. Thus, with the simple isolation of stromal fragments from the spleen, we were able to achieve 20- to 50-fold enrichment for latently infected cells. In subsequent experiments, only C3H stromal fragments were used, and splenocyte suspensions were discarded.

TABLE 2. Effect of collagenase-dispase digestion of stromal fragments on frequency of MCMV recovery[a]

Treatment	No. of positive wells for mouse no.:			
	1	2	3	4
Collagenase-dispase	3	3	4	4
No digestion	2	1	5	0

[a] Stromal fragments from latently infected C3H mice were treated with collagenase and dispase and cocultivated with BALB/c MEC in 24-well plates. For each treatment and each mouse, 10 wells were used.

Before further fractionation of the stromal cells, it was necessary to disperse the stromal fragments into a single-cell suspension by treatment of the fragments with collagenase and dispase. There was no decrease in the frequency of recovery of MCMV when fragments were digested with collagenase-dispase and cocultivated with BALB/c fibroblasts (Table 2).

By removal of the splenocyte suspension cells, most of the splenic B and T lymphocytes and Ia-bearing cells were removed. However, it was still possible that a minor fraction, copurifying with the stromal fragments, harbored latent MCMV. To address this question, we first used the panning method (34) with plastic flasks coated with anti-mouse immunoglobulin to deplete any immunoglobulin-bearing B cells present in the dispersed stromal cells. The panning procedure reduced the fraction of immunoglobulin-positive cells approximately sixfold, as measured by fluorescence microscopy, but had no significant effect on the frequency of recovery of virus after cocultivation of the effluent cells with BALB/c fibroblasts (Table 3).

In the above-described experiment, as well as in some subsequent experiments, recovery of virus was noted at 5 to 7 days in some wells. Although this might have been due to low levels of free virus, most sonic extracts of spleen cells were negative, and all salivary gland homogenates (except for that in one experiment noted in Table 4) were negative for MCMV. In reconstruction experiments, in which free virus was mixed with stromal cell suspensions from uninfected mice, it could be detected within 4 to 9 days of culture (data not shown). However, because of the small number of latently infected cells, we could not be certain that virus appearing after only a few days in culture was due to free virus or reactivated latent virus. Therefore, in Tables 3, 4, and 5, the data are subdivided into groups of wells positive before 10 days of culture, possibly as a result of free virus, and wells positive at 10 or more days of culture, more likely as a result of reactivation of latent virus. The length of time required for reactivation of MCMV from latently infected cells was variable and ranged from 5 to 48 days.

We next determined whether Thy-1-bearing stromal cells were a reservoir of latent MCMV. In this experiment, stromal cell suspensions were treated with anti-Thy-1 monoclonal antibodies (HO and F7D5) and complement before cocultivation with BALB/c fibroblasts. Although the per-

FIG. 3. (a) Electron micrograph of an MCMV-infected spleen cell from a BALB/c mouse at 3 days p.i., which contains abundant MCMV virions in both the nucleus (N) and cytoplasm. Despite the rounded appearance and absence of intercellular junctions, the following morphologic features suggest that it is a degenerating endothelial cell. The cell apposes a partially denuded basement membrane (arrowheads) overlying collagen fibrils. A few cytoplasmic invaginations are seen along the basement membrane surface, and cytoplasmic vesicles consistent with pinocytosis are noted within the cell. The cytoplasm has electron-lucent cytoplasm and microvillus processes. Several degenerating organelles are seen in the cytoplasm adjacent to virions (arrows), but no definitive lysosomes can be discerned. Staining was with lead citrate and uranyl acetate. Magnification, ×6,250; bar, 5 μm. (b) Higher magnification of the same cell, showing cytoplasmic and nuclear (N) virus. Staining was with lead citrate and uranyl acetate. Magnification, ×50,000; bar, 1 μm.

TABLE 3. Recovery of MCMV after removal of immunoglobulin-bearing cells by panning[a]

Expt no.	Mo p.i.	No. of positive wells after treatment with:			
		Anti-immunoglobulin		No antibody	
		<10 days	≥10 days	<10 days	≥10 days
1[b]	2	8	6	11	5
2	8	7	11	6	3
3	11	0	0	2	1

[a] Stromal cell suspensions from latently infected C3H mice were incubated in anti-immunoglobulin-treated or untreated flasks. Nonadherent cells were removed from the flasks and cocultivated with BALB/c MEC in 24-well plates. For each experiment, 6 mice and 24 wells per treatment were used.

[b] In this experiment, assay of salivary gland homogenates for persistent MCMV infection was positive. In experiments 2 and 3, salivary glands were negative for MCMV.

TABLE 5. Recovery of MCMV after treatment with anti-Ia and complement[a]

Expt no. (no. of mice)	No. of MCMV-positive wells/total no. of wells after treatment with:			
	Anti-Ia + complement		Complement only	
	<10 days	≥10 days	<10 days	≥10 days
1 (5)	3/120	3/120	0/120	3/120
2 (6)	9/72	6/72	0/72	6/72
3 (5)	0/48	2/48	0/48	0/48
4 (4)	0/24	1/24	0/24	0/24
5 (6)	0/48	2/48	1/48	3/48
6 (5)	0/48	2/48	0/48	2/48

[a] Stromal cell suspensions from latently infected C3H mice were treated with anti-Ia or anti-Ia^k monoclonal antibodies and complement or complement only and cocultivated with BALB/c MEC in 24-well plates.

centage of Thy-1-positive cells was reduced approximately 100-fold (as assayed by fluorescence-activated cell sorter analysis), there was no statistically significant difference in the frequency of recovery of MCMV from the stromal cells (Table 4).

Table 5 shows the results of a separate series of experiments in which stromal cell suspensions were treated with anti-Ia (experiment 1) or anti-Ia^k (experiments 2 to 6) monoclonal antibodies and complement before cocultivation with BALB/c fibroblasts. Anti-Ia^k treatment reduced the frequency of Ia-positive cells (measured by fluorescence-activated cell sorter analysis) approximately 20-fold. Anti-Ia treatment (experiment 1 only) was less effective, giving only a fivefold reduction in the frequency of Ia-positive cells. The results showed no significant difference in the frequency of recovery of MCMV when Ia-positive cells were depleted and thus provide evidence that T lymphocytes, mature B lymphocytes, and other Ia-bearing cells are not the predominant reservoirs of latent MCMV.

DISCUSSION

In this report, we present an identification of the MCMV-infected cells in the spleens of acutely infected mice on the basis of cell-type-specific antigen markers. The recent development of the technique for simultaneous detection of antigens and RNA in situ has permitted us to demonstrate that most acute MCMV replication in the spleens of BALB/c mice occurs in cells which are Thy-1 negative, Ia negative, F4/80 negative, and factor VIII-related antigen positive. At the ultrastructural level, it appeared that most of the MCMV-infected cells were not surrounded by basement membrane but were intimately associated with reticular fibers. These results are consistent with the sinusoidal-lining cell, within the red pulp, being the predominantly infected cell in the spleen during acute infection. We have noted, however, that MCMV replication can occur, albeit to a lesser extent, in cells with morphologic features of endothelial cells.

TABLE 4. Recovery of MCMV after treatment with anti-Thy-1 and complement[a]

Expt no. (no. of mice)	No. of positive wells/total no. of wells after treatment with:			
	Anti-Thy-1 + complement		Complement only	
	<10 days	≥10 days	<10 days	≥10 days
1 (2)	0/12	3/12	0/12	3/12
2 (5)	6/60	8/60	2/60	2/60

[a] Stromal cell suspensions from latently infected C3H mice were treated with anti-Thy-1 monoclonal antibodies (HO and F7D5) and complement or complement only and cocultivated with BALB/c MEC in 24-well plates.

The nature of splenic sinusoidal-lining cells is enigmatic. Studies on human splenic sinusoidal-lining cells by Buckley et al. (10) have shown that they express a unique combination of antigens associated with monocytes, macrophages, endothelial cells, and T lymphocytes. In particular, like macrophages and monocytes, they display membrane antigens HLA-DR and OKM5 and express lysozyme and nonspecific esterase. Sinusoidal-lining cells share with endothelial cells the ability to synthesize factor VIII-related antigen. Furthermore, these cells express some antigens found on helper-inducer (OKT4, Leu-3a,b) and suppressor-cytotoxic (OKT8, Leu-2a) T-lymphocyte subsets. Of interest is the recent observation that human sinusoidal-lining cells also contain smooth-muscle myosin (50). Thus, sinusoidal-lining cells appear to be unique, exhibiting features of a variety of different cell types.

In earlier studies on the pathogenesis of MCMV in vivo, McCordock and Smith (39) and Henson et al. (23) described infection and hypertrophy of "large mononuclear reticular" cells in the spleen. Mims and Gould (42, 43), using antibodies to MCMV antigens and immunofluorescence, noted that the infected cells in the red pulp were medium or large and mononuclear but only slightly phagocytic, as defined by uptake of India ink. They further noted that treatment of spleen cells with complement plus theta antiserum or goat antiserum to mouse immunoglobulin had no effect on the number or type of infected cells seen. On the basis of these results, they concluded that the infected cells did not carry theta or immunoglobulin surface markers and also were not macrophages. Our results support their conclusions. In contrast, however, Katzenstein et al. (32), by electron microscopy, saw most of the virus particles in large mononuclear cells that had phagocytized latex particles and concluded that the mononuclear cells were probably macrophages. The apparent discrepancy between these studies can be reconciled by the observation that the sinusoidal-lining cells may be phagocytic (24, 37, 56). In other studies in which splenic macrophages were implicated in MCMV infection, it should be noted that this association was based on

the adherence properties of cells susceptible to MCMV infection in vitro (31, 35) or fractionation with infectious virus after in vivo infection (36). Our results indicate that spleen macrophages, as defined by the F4/80 antigenic marker and electron microscopy, are not infected in vivo, even when in close proximity to infected cells.

A major issue raised by these studies concerns the general role of sinusoidal-lining cells and endothelial cells in acute and, possibly, latent infections. Although our data only address replication of MCMV in splenic sinusoidal-lining and endothelial cells, there is evidence from other laboratories that both HCMV and MCMV replicate in endothelial cells at other sites. In vivo studies have shown the presence of viral inclusions in the endothelial cells of a variety of tissues isolated from patients with HCMV infections (12, 15, 21, 46). Myerson et al. (46), by in situ cytohybridization, also demonstrated that some of the endothelial cells containing HCMV nucleic acid exhibited neither cytomegaly nor inclusion bodies. Furthermore, HCMV productively infects human umbilical vein endothelial cells grown in culture (25). In the case of MCMV-infected mice, viral inclusions have been noted in capillary endothelial cells (39), and viral antigens have been detected by immunofluorescence in endothelial cells in the endocardium and venular endothelium. Whereas sinusoidal-lining cells and endothelial cells are not the only sites of CMV replication in vivo, they likely play an important role in the transmission and pathogenic manifestations of this virus. An important question, yet to be answered, concerns the relationship of HCMV with Kaposi's sarcoma. This human tumor, commonly seen in patients with acquired immunodeficiency syndrome, is of endothelial cell origin and has been associated with HCMV infections (for a review, see reference 57).

After the acute phase of CMV infection, the virus usually enters a latent state. However, an acute infection can also lapse into a chronic or persistent infection, during which time low levels of virus can be isolated from body fluids, particularly urine and saliva, and salivary glands (reviewed in reference 30). When discussing latent and chronic CMV infections, it is important to recognize that the best definition of latency is the lack of demonstrable chronic infection. Thus, the separation of latent and persistent infections is determined by the sensitivity of detection of active CMV replication.

In all of the latency experiments presented in this paper, we tested the mice for persistent infection by assaying salivary gland homogenates for MCMV. Only in one experiment (experiment 1 of Table 3), in which mice were tested at 2 months p.i., was there evidence of persistent infection. In all other cases, in which the mice were sacrificed later than 4 months p.i., the salivary gland homogenates were negative for MCMV. In these assays, the sensitivity was such that 15 to 20 PFU per salivary gland would have been detected. We also determined, using reconstruction experiments, that free virus could be detected in our assays within 4 to 9 days of culture. Therefore, because of the low number of latently infected cells in the spleen and the possibility that a low-level persistent infection might have escaped detection, we presented the data in terms of reactivation detected before or after 10 days of culture. In all experiments, cultures were maintained for at least 6 weeks.

Leukocytes have been proposed as good candidates for harboring latent virus, since infection can be transmitted by blood transfusion and organ transplantation. In the mouse model, most studies of CMV latency have focused on the spleen, since virus can easily be reactivated from cultures of this lymphoid organ. Precise identification of the latently infected cell, however, has been elusive.

The early results of Olding et al. (48) showed that latent MCMV could be reactivated from a population of spleen cells which had been depleted of both theta-bearing cells and glass-adherent macrophages and thus provided support for B cells as the site of latency. In a subsequent study, Jordan and Mar (31) studied reactivation of MCMV from spleen explants of C3H mice infected intraperitoneally with salivary-gland-passaged virus and showed that pieces of spleen placed in culture produced detectable virus after 12 to 18 days. When they made suspensions of spleen cells by forcing the splenic fragments through a screen, the recovery of virus was greatly reduced and virus could only be recovered from the nonadherent fraction of the suspension. It should be noted, however, that their technique for preparing suspensions would not have eliminated the population we refer to as stromal cells. Treatment of nonadherent cells with anti-Thy-1 and complement did not prevent recovery of virus. Furthermore, when the cells were separated on nylon wool or anti-mouse IgG affinity bead columns, virus was only recovered from the column-adherent fraction. On the basis of these data, they also concluded that the latently infected cells were most likely B lymphocytes.

The results of our experiments are in agreement with the above-described studies with respect to the conclusion that the latently infected cells are likely not Thy-1 positive. However, we also show that removal of immunoglobulin-bearing cells by panning on plastic dishes and ablation of Ia-bearing cells with anti-Ia antibody plus complement does not reduce the frequency of reactivation of latent MCMV. Thus, if the latently infected cells are B lymphocytes, they do not appear to have a high concentration of immunoglobulin or Ia surface antigens. The results of Olding et al. (48) and Jordan and Mar (31) can be reconciled with ours if the latently infected cells adhere nonspecifically to nylon wool or anti-immunoglobulin columns. These adherent cells, however, are likely not macrophages or dendritic cells, most of which have surface Ia antigen.

The precise answer to the question of latency still remains unanswered. We have come closer to the solution in that we show that the stromal fragments of the spleen, containing approximately 5% of the total cell population, are the reservoir of latent MCMV and that these cells are likely not mature T cells, B cells, macrophages, or dendritic cells. Although it is tempting to speculate that sinusoidal-lining cells or endothelial cells, which are a prominent population in the stroma, contain latent MCMV, we have no direct evidence for this conclusion. Additional candidates which remain as potential sites of latent MCMV infection in the spleen include precursor B lymphocytes, granulocytes, granulocyte-monocyte colony-forming cells, Ia-negative macrophages, neurons, and fibroblasts. It should also be noted that the spleen is not the only site of CMV latency, and any hypothesis concerning the cell type(s) which contains CMV in a latent state has to take into consideration the broad tissue distribution of latent CMV as indicated by organ transplantation. Once the site of latency in the spleen is defined, it will be important to determine whether these results can be generalized to other organs in mice and to HCMV latency.

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