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Interaction of Murine Cytomegalovirus with Separated Populations of Spleen Cells

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Mouse spleen cultures were infected with murine cytomegalovirus, either before or after separating the cells into various classes, and the growth of virus and production of infectious centers were measured in each cell class. The separation techniques, which were used in various combinations, comprised: adherence to plastic surfaces; adherence to nylon wool columns; gamma irradiation; and treatment with anti-immunoglobulin serum plus complement. The "macrophage population" took up most of the input virus, as shown by autoradiography of spleen cells infected with radioactive virus. These cells formed infectious centers but replicated the virus only transiently. In contrast, the "B-lymphocyte population" contained a minor cell fraction which was permissive for replication. The "T-lymphocyte population" appeared to be nonpermissive for replication and did not produce infectious centers.

Work in several laboratories has established the fact that murine cytomegalovirus (MCMV) can be recovered from lymphoid tissues of persistently infected mice, either by immunosuppressive treatment of the animals (1, 4, 12, 15) or by co-cultivating the tissue with embryonic fibroblast cultures in vitro (9, 10, 18). It is not known whether these manipulations work because they reactivate virus within lymphoid cells or because they suppress lymphoid cells involved in controlling the virus in other cells.

Our own studies have been directed toward identification of the cell types involved and the mechanisms of control and reactivation. For this purpose we used spleen cultures infected in vitro, to provide a system amenable to experimental manipulation and to quantitate the interactions between the virus and different cell types. Previous results (10) indicated that infectious centers (ICs; cells containing inducible MCMV genomes and genomes in the eclipse phase of a replication cycle) could be established in a small number of spleen cells, whereas an even smaller number of cells replicated the virus. Apart from the data of Olding and co-workers (18), which implicated B-lymphocytes as a possible source of reactivated virus, no information is available on the cell types involved in MCMV infections. The present study was therefore undertaken for the purpose of defining the cell populations responsible for replicating the virus and for forming ICs, making use of techniques for separating different classes of spleen cell.

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MATERIALS AND METHODS

Virus. The Smith strain of MCMV, propagated at low multiplicity (0.01 plaque-forming unit [PFU]/cell) in mouse embryo cultures, was used in these studies.

Mice. The strains of mice used were C3H/HeJ and SWR/J. No differences were observed between these strains regarding the results presented here.

Cells. Mouse embryo cells (derived from randomly bred Swiss white mice) were propagated in Dulbecco-modified minimal essential medium (GIBCO Laboratories, no. H-20), supplemented with 5% (vol/vol) fetal bovine serum, 20 μg of gentamicin per ml, and 0.25 μg of amphotericin B (Fungizone) per ml, in petri dishes or roller bottles and were used after one or two passages.

Spleen cells were obtained as described previously (10) and were cultivated in RPMI 1640 with 10% (vol/vol) fetal calf serum and the other supplements, in tubes or microtiter wells, at a concentration of 2 to 5 $\times 10^6$ cells per ml. Cell viability was determined by counting trypan blue-excluding cells in a hemacytometer.

Infectivity assays. MCMV was assayed by plaque formation on mouse embryo monolayers, usually using the standard method of assay (11, 19). Spleen cells were disrupted by three cycles of freezing and thawing before assaying for cell-associated MCMV.

ICs were assayed by pelleting the cells, washing them twice, and plating onto freshly confluent mouse embryo monolayers. An overlay of 2% (wt/vol) agarose was added 1 h after the spleen cells had been added, by which time the cells had settled onto the fibroblast monolayer. The final concentration of agarose was 0.5% (wt/vol). The presence of an overlay was shown not to inhibit IC information. Plaques were counted 4 to 7 days after adding the overlay. In the case of macrophages, the mouse embryo cells were added to the washed monolayers of macrophages, and after 6 h the overlay was added.

Assays were performed at least in duplicate and at two or more virus dilutions. Replicate values usually agreed to within 10%.

Infection of spleen cells in vitro. Cells were pelleted at low speed and resuspended in a small volume of medium containing MCMV. The cell concentration was 2×10^7 cells per ml. After 60 min of incubation at 37°C, the mixture was diluted tenfold and the cells were pelleted gently, followed by three washes to remove unadsorbed virus. Additional washes were found to be unnecessary. The cells were then resuspended in fresh medium at the initial concentration (5×10^6 cells per ml). Unless indicated otherwise, the input multiplicity of infection (MOI) was 1.0 PFU/cell. Separate classes of spleen cells were infected in the same manner, except for the macrophages, which were infected as monolayers and washed three times.

Radioactive MCMV. Virus was labeled with [^{3}H]deoxythymidine and purified as described previously (17). Briefly, the labeled extracellular virus was freed from traces of cellular debris by low-speed centrifugation and pelleted at high speed. The pellets were resuspended in a small volume and treated with electrophoretically purified deoxyribonuclease (50 μg/ml) and pancreatic ribonuclease (100 μg/ml) at 37°C for 30 min. The virus was pelleted twice more and resuspended in phosphate-buffered saline or medium, after rinsing to remove traces of soluble radioactivity.

Cell separation by nylon wool columns. A 0.6-g amount of nylon wool from an LP-1 Leuko Pak (Fenwal Laboratories) was soaked in distilled water for 1 week with daily water changes. Then the nylon wool was dried, packed into a 10-ml plastic syringe up to the 8-ml mark, and sterilized by autoclaving.

Before the spleen cells were added, the column was filled with RPMI 1640 medium plus 10% (vol/vol) fetal calf serum and incubated for 30 min at 37°C. Then a maximum of 10^8 cells in 1 ml of medium were added to the column, and incubation was continued for 45 min at 37°C. The nonadherent cells were eluted with about 15 ml of prewarmed (37°C) culture medium at a rate of one drop every 2 s. The adherent cells could be removed by adding fresh 37°C medium to the column, loosening them by squeezing the nylon wool with a pair of sterile forceps, and collecting the eluted cells. Both cell fractions retained more than 90% cell viability, determined by trypan blue exclusion. Approximately 50% of SWR spleen cells and 60 to 65% of C3H spleen cells adhered to these columns. The number of surface immunoglobulin-bearing cells in the adherent cell fraction was enriched at least twofold.

Cell separation by adherence to plastic tissue culture plates. Spleen cells, in RPMI 1640 medium plus 10% (vol/vol) fetal calf serum, at a concentration of 5×10^6 cells per ml, were added to 50-mm Falcon tissue culture plates (2 ml/plate) or Linbro microtiter plates (0.2 ml/well) and incubated in a CO_2 incubator for 2 h at 37°C. Nonadherent cells were removed by washing the plates vigorously three times with fresh culture medium, pelleted by centrifugation, and resuspended in culture medium for further incubation. RPMI 1640 medium plus 10% (vol/vol) fetal calf serum was also added to the plates if further incubation of the adherent cells was desired.

According to the staining method of Yam et al. (24) for nonspecific esterase, more than 50% of the adherent cells were macrophages, and in fact more than 90% of the flat spread-out cells had typical macrophage morphology (2) and stained positively.

Gamma irradiation of spleen cells. A ^{60}Co gamma ray source (courtesy D. C. Walker, Department of Chemistry) was used. Spleen cells in polypropylene tubes were given 2,000 R of irradiation by lowering the gamma ray source into the shielded chamber, where the cells were placed for 62.5 s.

Treatment of spleen cells with antiserum plus complement. Spleen cells were pelleted by centrifugation, washed once, and resuspended in an equal-volume mixture of guinea pig complement and rabbit anti-mouse immunoglobulin G at dilutions previously found to give maximum cell killing. The mixture was incubated at 37°C in a CO_2 incubator for 60 min. The spleen cells were then pelleted again by centrifugation, washed, and resuspended in culture medium for further incubation. One anti-immunoglobulin G preparation was prepared in our laboratory; the other was obtained from Cappel Laboratories.

Fluorescent-antibody labeling of spleen cells. Spleen cells were pelleted by centrifugation, washed once, and incubated with rabbit anti-mouse immunoglobulin G serum (1:32 dilution) at 4°C for 30 min. The cells were then washed three times with phosphate-buffered saline and incubated with fluorescein isothiocyanate-conjugated goat anti-rabbit immunoglobulin G (1:16 dilution, Cappel Laboratories or Miles Laboratories) at room temperature for 30 min. Finally, after thoroughly washing with phosphate-buffered saline, the cells were resuspended in a 1:1 (vol/vol) mixture of glycerol and phosphate-buffered saline and mounted on glass slides for observation under a Leitz fluorescence microscope.

High-speed scintillation autoradiography. The method of Durie and Salmon (3), with slight modifications, was used for scintillation autoradiography.

Mock-infected or MCMV-infected spleen cells were washed with phosphate-buffered saline four times and resuspended in fetal calf serum. Cell smears were made by spreading the cell suspensions onto clean glass coverslips with fine paint brushes. The cover slips were subsequently air dried, fixed in methanol for 30 min, rinsed in distilled water, and dried before the emulsion coating procedure.

In the dark room, the cover slips were dipped into Kodak NTB_2 nuclear track emulsion for 10 s at 42°C. After drying for 1 h at room temperature (22°C), the cover slips were dipped for 10 s into the scintillator solution (22°C). The scintillator consisted of 35 g of 2,5-diphenyloxazole (PPO) and 100 mg of 1,4-bis-[2]-(4-methyl-5-phenyloxazolyl)benzene (dimethyl POPOP) dissolved in 500 ml of dioxane. The scintillator-impregnated emulsion was exposed in the dark for 18 days at −20°C before development and fixation. The cells were then stained with Wright stain and observed under a light microscope. Cells containing more than five grains were registered as positive.

RESULTS

The following definitions of cell types will apply to the data presented: spleen cells which adhere to plastic culture dishes are referred to

as "macrophages"; the cells which adhere only to nylon wool columns are designated as "B-cells"; those cells which adhere neither to plastic nor to nylon wool are designated "T-cells." These are operational definitions only and are not intended to specify pure populations of cells.

ICs denote intact viable cells containing no infectious virions but from which infectious virus can be obtained by cultivating with mouse embryo monolayers. Thus, they represent cells with inducible viral genomes as well as cells in which a viral replication cycle is passing through the eclipse phase. The number of ICs was corrected for the presence of cell-associated infectious virions by simply subtracting the number of PFU obtained in disrupted cells from the same sample.

Cell separation by adherence to nylon wool. Nylon wool columns provide a simple and effective method for separating T- and B-lymphocytes (7, 13, 22). Those cells which adhere to nylon wool are enriched in B-cells, whereas the non-adherent fraction is enriched in T-cells.

Spleen cells were infected with MCMV just before nylon column fractionation, and the progress of virus replication was followed in the separated cell populations. Data from one such experiment are presented in Fig. 1. It is evident that the B-cell fraction contained most of the cells supporting virus replication, and in fact it is likely that the low level of replication seen in the T-cell fraction was due to contaminating B-lymphocytes (7).

To ensure that the results could not be affected by an alteration in column-binding properties of certain cells after infection, the above experiment was repeated with spleen cells that were infected after column fractionation. This result also indicated that the cells supporting MCMV replication are enriched in the B-cell fraction.

Cell separation by adherence to plastic culture dishes. The cells which adhered to nylon wool probably contained macrophages, and other cell types, in addition to B-lymphocytes. Therefore, to compare B-cell and macrophages it was necessary to use an alternative technique for cell separation. The ability of mac-

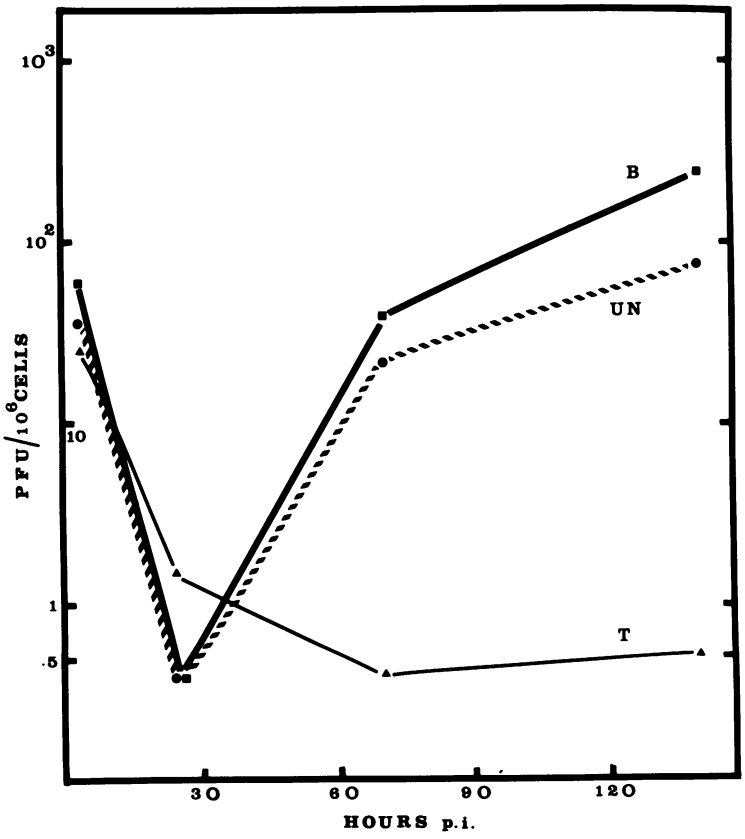

FIG. 1. *Replication of MCMV in C3H/He spleen cells fractionated on a nylon wool column after infection (p.i.). PFU refers to disrupted cell titers. Symbols: ●, unfractionated cells (UN); ■, cells adherent to nylon wool (B); ▲, cells not adherent to nylon wool (T).*

rophages to adhere to plastic tissue culture dishes has been utilized by many workers for selectively isolating these cells. We therefore decided to test the ability of plastic-adherent and nonadherent cells for their ability to support MCMV replication. Data are presented in Fig. 2, from which it is evident that the nonadherent cells produced virus at a rate similar to that of unfractionated spleen cells, whereas the adherent cells, the macrophage population (M), supported replication only transiently.

The nonadherent cells (B + T) were apparently capable of supporting several cycles of infection, indicating that not all of the susceptible cells were infected initially, whereas the macrophage fraction, which contained only about 10% of the total spleen cells, ran out of susceptible cells much quicker. Alternatively the B + T fraction may simply have required a longer time to synthesize and release virus.

In several experiments, infected cultures were assayed separately for intracellular and extracellular PFU. At all times, and in all cell populations (macrophages, B-cells, T-cells, and unfractionated cells), the majority of the infectious MCMV was found to be extracellular.

ICs were also measured (after subtracting residual PFU due to whole virus), and these data are presented in Table 1. The nonadherent cells (mostly lymphocytes) showed an early decrease in IC as virus passed through the eclipse phase of a replicative cycle, followed by a gradual increase again, presumably due to further infection of susceptible cells (cf. with PFU in Fig. 2).

In contrast, the adherent macrophages, which initially contained relatively more ICs, showed a different response. In this cell population, the number of ICs decreased initially, but there was no subsequent increase, which implies that the macrophages were not susceptible to further infection. This correlates with the PFU data in Fig. 2.

Additional experiments were done in which the nonadherent lymphocytes were further separated by nylon wool into B-cell-enriched and T-cell-enriched fractions. In accordance with the above results, it was found that the B-cell fraction contained most of the virus-replicating cells and the ICs.

Effects of gamma irradiation. Macrophages are known to be more resistant to gamma rays than are lymphocytes. Preliminary tests indicated that spleen cells in vitro lost their viability much more rapidly after exposure to

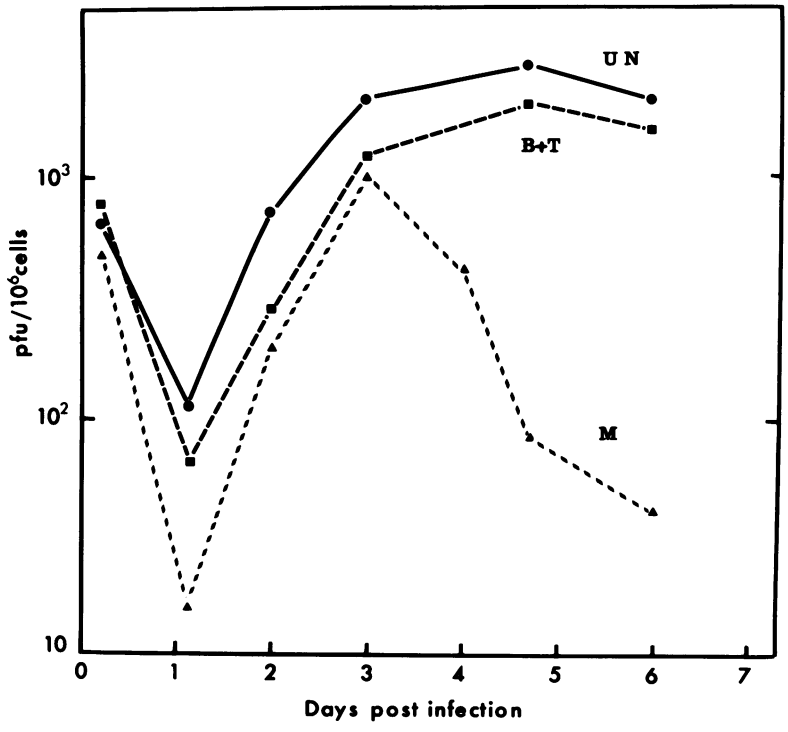

FIG. 2. *Replication of MCMV in SWR spleen cells fractionated by adherence to tissue culture dishes after infection. PFU refers to disrupted cell titers. Symbols: ●, unfractionated cells (UN); ▲, cells adherent to plastic (M); ■, cells not adherent to plastic (B+T).*

2,000 R of gamma irradiation and could no longer respond to mitogenic stimulation by concanavalin A or lipopolysaccharide (data not shown). Experiments were therefore performed in which spleen cultures were exposed to 2,000 R followed by MCMV infection and cultivation in plastic tissue culture dishes. The adherent, nonadherent, and unfractionated cells were then assayed for PFU and ICs in comparison with similar fractions of untreated infected cultures. Figure 3 shows the virus replication curves for the unfractionated (UN) cultures, the B + T fraction, and the macrophage (M) fraction. Virus replication was reduced in all cell populations derived from the gamma-irradiated cultures, especially in the B + T fraction, which contains the greatest proportion of gamma-sensitive cells. The corresponding data for ICs are shown in Fig. 4. Irradiation effectively abrogated the IC formation in the B + T fraction but had relatively little effect on ICs in the macrophage fraction.

TABLE 1. *IC assays on MCMV-infected SWR spleen cells separated by adherence to plastic*

Time post-infection (h)	ICs per 10^6 cells		
	Unfractionated cells	Adherent cells[a]	Nonadherent cells
5	8.9×10^2	4.4×10^3	1.2×10^3
27	1.3×10^2	3.8×10^2	7.6×10^1
48	2.4×10^2	5.0×10^2	2.1×10^2
72	8.1×10^2	3.8×10^2	5.3×10^2
113	1.3×10^3	1.4×10^2	6.8×10^2
144	1.3×10^3	5.5×10^1	8.0×10^2

[a] Cells which adhered to plastic tissue culture dishes within 2 h at 37°C.

The kinetics of ICs and PFU in the macrophage fraction were slightly different in this experiment, compared with the one shown in Fig. 2 and Table 1. However this difference was less obvious after gamma irradiation, which suggests that it was simply a reflection of different degrees of lymphocyte contamination.

Additional experiments were done in which spleen cells were treated with anti-immunoglobulin serum plus complement, to kill cells bearing surface immunoglobulin G, followed by cell separation by adherence to plastic. These and untreated fractions were then infected with MCMV and assayed for PFU and ICs. There was no significant change in either parameter resulting from the anti-immunoglobulin treatment. The small differences observed were within the range of experimental variation. However, the proportion of cells stainable with fluorescein-anti-immunoglobulin only decreased from about 50 to 20% after the treatment, and the treated cultures still responded to some extent to lipopolysaccharide indicating that some functional B-cells remained.

Autoradiography of spleen cells infected with [^{3}H]deoxythymidine-labeled MCMV. Previous results (10) have shown that the amount of virus taken up by spleen cells, and the number of ICs found, were proportional to the MOI, although even at an MOI of 25 PFU/cell less than 3% of the cells registered as ICs. We did not know whether the number of ICs represented all of the cells capable of taking up the virus. Experiments with radioactive virus confirmed that even at high MOI relatively little virus became cell associated (see Table 2). This

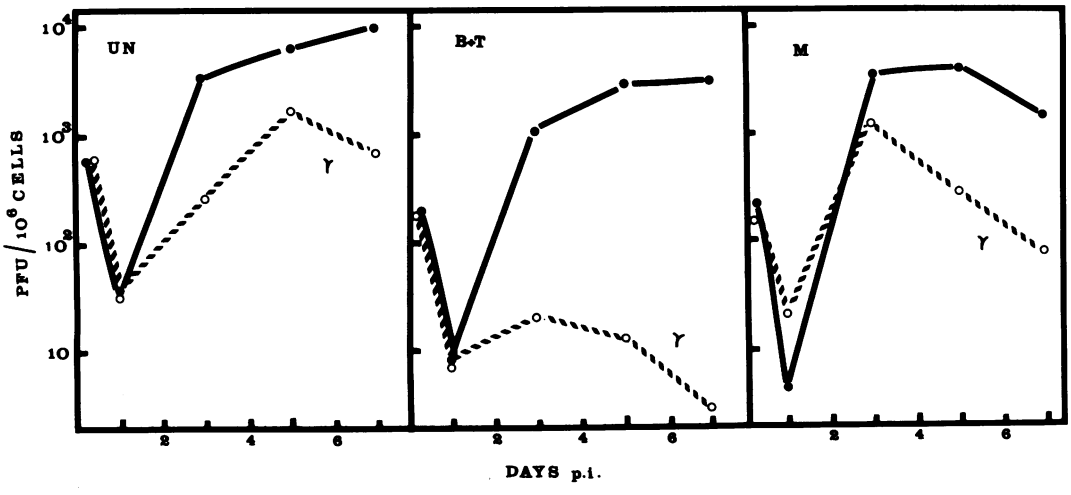

FIG. 3. *Replication of MCMV in gamma-irradiated SWR spleen cells, fractionated after infection (p.i.). Symbols: solid lines and circles, nonirradiated; broken lines and open circles, irradiated with 2,000 R (γ). UN, Unfractionated cells; B + T, cells not adherent to plastic; M, cells adherent to plastic.*

problem makes it difficult to be certain of the identity of the cell types interacting with MCMV.

We decided to resort to autoradiography to resolve this problem. Spleen cells were infected with [^{3}H]deoxythymidine-labeled MCMV, at various MOIs, and the cells were subjected to scintillation autoradiography (3) to obtain high sensitivity. Results are shown in Table 3. At each MOI three to five separate slides of fixed cells were used for cell counting. Even after infection with 100 PFU/cell, only 7.7% of the cells showed significant grain counts. In all cases most of the cells that could be counted definitely as positive were macrophages, recognizable by their flat spread-out appearance. Only rarely did a smaller round cell show any grains at all, and then considerably fewer in number than the macrophages. Thus, the macrophages, which constitute 10 to 15% of the spleen cells, appear to take up most of the virus, although an occasional lymphocyte must receive enough to initiate one or more cycles of replication.

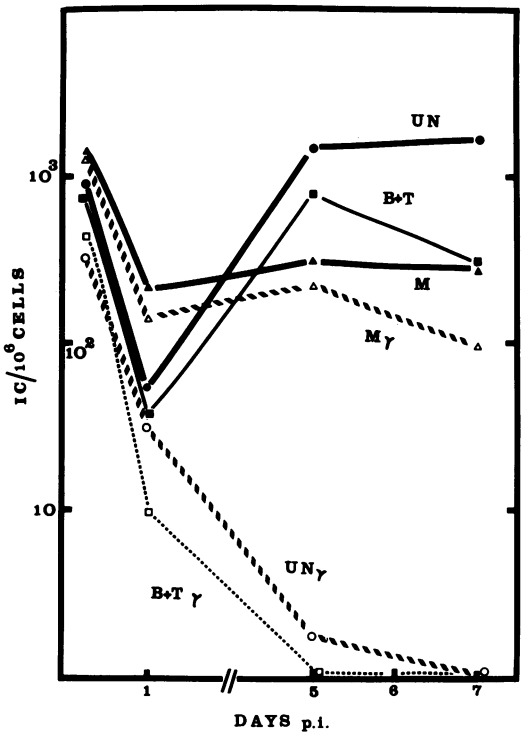

FIG. 4. *ICs in gamma-irradiated SWR spleen cells, fractionated after infection (p.i.). Symbols: ●, unfractionated (UN); ▲, adherent to plastic (M); ■, nonadherent (B+T); ○, unfractionated, gamma irradiated (UNγ); △, adherent to plastic, gamma irradiated (Mγ); □, nonadherent, gamma irradiated (B+Tγ).*

TABLE 2. *Relationship between the MOI and MCMV uptake by SWR spleen cells*

MOI (PFU/cell)	% of cpm cell associated	Equivalent PFU (uptake/10^6 cells)
1.0	0.61	6.1×10^3
10	0.29	2.9×10^4
100	0.19	1.9×10^5

TABLE 3. *Autoradiography of SWR spleen cells infected with [^{3}H]deoxythymidine-labeled MCMV*

MOI (PFU/cell)	No. of cells with grains/no. of cells counted	% Positive cells[a]
1.0	133/7,411	1.85 ± 0.55
10	309/4,876	4.44 ± 1.81
100	68/729	7.75 ± 4.06

[a] Mean percent ± standard deviation for three to five individual slides.

DISCUSSION

On the basis of the results presented here and in the previous study (10), it is possible to consider the responses of the three major spleen cell populations to MCMV as follows.

The macrophage population (i.e., plastic adherent cells), which comprises approximately 10% of the total spleen cells, supports virus replication transiently and forms ICs, some of which can persist for more than 2 weeks in culture in the absence of infectious virions (10). This population takes up most of the virus added to the spleen culture. The transient nature of the replication could reflect a change in the cells, such that after 2 or 3 days in vitro they are no longer permissive for replication, or it could be due to exhaustion of a minor subpopulation of virus-permissive gamma-resistant cells in this fraction. A comparison of the number of ICs with the yield of PFU indicates that the majority of macrophages are nonpermissive. Other studies have indicated a limited amount of replication of MCMV in peritoneal exudate cells (20, 21).

In contrast, the B-cell fraction contained cells which could allow a typical virus growth cycle, although in view of the yield of virus, only a very small proportion ($\ll$1%) of these cells could have been permissive. The cells responsible were sensitive to gamma irradiation and adhered to nylon wool but not to plastic. Thus, they could be a specific subclass of B-lymphocytes or some other cell type such as a monocyte or immature macrophage. It is conceivable that B-lymphocytes are susceptible to MCMV only at a particular stage of differentiation. One is reminded in this context of the variety of interactions between B-

cells and Epstein-Barr virus, which are determined to a large extent by the host cell (5, 8). The presence of dividing cells is not necessarily conducive to MCMV replication, since lipopolysaccharide does not affect the yield of virus (10). This situation is quite different from herpes simplex virus replication in mouse spleen cells, which requires lipopolysaccharide stimulation (14).

The autoradiography results indicated that relatively few lymphocytes could take up the virus, and it is possible, therefore, that specific receptors are required which are only present on a small fraction of the B-cells. Such a phenomenon does appear to determine the ability of lymphocytes to take up Epstein-Barr virus and possibly other viruses (6).

The T-cell fraction, consisting of cells which did not adhere readily to plastic or nylon wool, did not contain virus-permissive cells, as indicated by the paucity of PFU and ICs in this fraction. These values were generally an order of magnitude lower than the corresponding values for the B-cell fraction and can be attributed to B-cell contamination of the T-cell fraction, since the cell separation techniques do not yield "pure" populations of cells. It was shown previously that concanavalin A does not affect the virus growth cycle in spleen cultures (10). Therefore, the nonpermissiveness of the T-cells was not due to a dearth of dividing cells.

The exact identity of the virus-permissive cells remains unknown. The results of the anti-immunoglobulin serum treatment were inconclusive, so we cannot say whether or not the cells bear surface immunoglobulin. Further studies will have to be done with more definitive markers for specific cell types.

The importance of the macrophage in MCMV infection has been emphasized recently by Mims and Gould (16), who showed that spleen macrophages were capable of restricting the spread of the virus to other spleen cells. Since these cells are important in many immune functions (23), further analysis of the effects of MCMV infection upon macrophage functions is warranted.

Our original intention was to develop an in vitro model for analyzing MCMV-spleen cell interactions in detail and to characterize the cell types involved. So far we have used mainly tissue culture-passaged virus because it is easily available in large quantities, although preliminary experiments indicate that salivary gland virus behaves in a similar fashion qualitatively. In additional studies we plan to compare the results derived from this system with those obtained from infection in vivo.

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