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Rabies Virus Replication in Primary Murine Bone Marrow Macrophages and in Human and Murine Macrophage-Like Cell Lines: Implications for Viral Persistence

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To determine whether rabies viruses replicate in macrophage or macrophage-like cells, several human and murine macrophage-like cell lines, as well as primary cultures of murine bone marrow macrophages, were incubated with the Evelyn-Rokitnicki-Abelseth (ERA) virus and several different street rabies viruses (SRV). ERA rabies virus replicated well in human monocytic U937 and THP-1 cells and murine macrophage IC-21 cells, as well as primary cultures of murine macrophages. Minimal replication was detected in murine monocytic WEHI-3BD$^-$ and PU5-1R cells, and ERA virus did not replicate in murine monocytic P388D1 or J774A.1 cells. A tissue culture-adapted SRV of bat origin also replicated in IC-21 and U937 cells. Non-tissue culture-adapted SRV isolated from different animal species, particularly bats, replicated minimally in U937, THP-1, IC-21 cells and primary murine bone marrow macrophages. To determine whether rabies virus replication is dependent upon the state of differentiation of the macrophage-like cell, human promyelocytic HL-60 cells were differentiated with 12-*O*-tetradecanoylphorbol-13-acetate (TPA). ERA rabies virus replicated in the differentiated HL-60 cells but not in undifferentiated HL-60 cells. Persistent infections were established in macrophage-like U937 cells with ERA rabies virus and SRV, and infectious SRV was isolated from adherent bone marrow cells of mice that had been infected 96 days previously. Virus harvested from persistently infected U937 cells and the adherent bone marrow cells had specifically adapted to each cell. This specificity was shown by the inability of the viruses to infect macrophages other than U937 cells and primary bone marrow macrophages, respectively. Virus titers of the persistently infected U937 cells fluctuated with extended cell passage. After 30 passages, virus released from the cells had lost virulence as shown by its inability to kill intracranially inoculated mice. However, the avirulent virus released from the persistently infected cells was more efficient in infecting and replicating in naive U937 cells than the virus which was used to establish the persistent infection. These results suggest that macrophages may serve as reservoirs of infection in vivo, sequestering virus which may subsequently be activated from its persistent state, resulting in clinical infection and death.

Rabies viruses, of the family *Rhabdoviridae*, are highly neurotropic viruses which usually cause a fatal infection in all warm-blooded species, with virus replication primarily occurring in neurons. It also has been demonstrated that rabies viruses replicate in muscle cells prior to invasion of the peripheral and central nervous system (29). In vitro, rabies viruses replicate in many cell culture systems derived from various tissues, including nerve, kidney, muscle, and embryo. It has not been clearly established, however, whether replication occurs in macrophages or macrophage-like cells. King et al. determined that a two- to fourfold enhancement in the replication of the Evelyn-Rokitnicki-Abelseth (ERA) virus, challenge virus standard, and Flury LEP strains of rabies virus occurred in P388D1 murine monocytes when the cells were infected with virus that had been preincubated with anti-rabies virus antibody (16). The prototype virus of the family *Rhabdoviridae*, vesicular stomatitis virus (VSV) also has been shown to replicate in peritoneal macrophages derived from mice treated with anti-alpha/beta-interferon antibody (6). In contrast, Turner and Ballard determined that the challenge virus standard and Flury LEP strains of rabies virus did not replicate in cultures of primary murine peritoneal macrophages (39).

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Many viruses, including VSV (6), human immunodeficiency virus type 1 (HIV-1) (12), visna virus (10), Aleutian mink disease parvovirus (14), lymphocytic choriomeningitis virus (17), and human cytomegalovirus (13, 31, 41), infect macrophage or macrophage-like cells. Most of these viruses then productively replicate (6, 10, 11, 13, 17), and in some cases the state of differentiation of the target cell is important. For example, HIV-1 replication increased in a clone of chronically infected U937 cells that were differentiated with 12-*O*-tetradecanoylphorbol-13-acetate (TPA) (9), and human cytomegalovirus replication has been detected in differentiated THP-1 cells as well as differentiated primary macrophages (13, 31, 41). In terms of pathogenesis, in the case of HIV-1 infection, monocytes are believed to be a major reservoir for the virus, allowing it to persist and possibly transport virus to the brain (8). Lymphocytic choriomeningitis virus variants, which replicate more readily in macrophages, leading to virus persistence and chronic infection, have also been reported (17). In human cytomegalovirus infection, however, the macrophage does not appear to function as a reservoir of infection but may actually serve as a vector of virus dissemination as well as a source of viral amplification (13).

Experimentally, rabies virus has been found up to 18 days postinfection in hamster bone marrow cells which were identified as macrophages (29). In vivo, rabies virus persistence has been documented in dogs and in humans (2, 20), and apparent

incubation periods of up to 6 years have been recorded in cases in which humans were bitten by seemingly healthy dogs (34). Because of our interest in rabies pathogenesis and, in particular, resistance to and recovery from infection (21, 23), we became intrigued with the possibility that rabies virus may persist in vivo in macrophages which could serve as reservoirs of infection. Because there is a paucity of information concerning rabies virus replication in macrophages, our initial studies concentrated on infecting several human and murine macrophage-like tissue culture cell lines, as well as primary cultures of murine bone marrow macrophages, with tissue culture-adapted ERA rabies virus and several wild-type street rabies viruses (SRV) that had been isolated from different animal species. It was determined that rabies viruses replicated in several human and murine macrophage-like cells, as well as primary murine bone marrow macrophages. Replication also was detected in differentiated but not undifferentiated human HL-60 cells, suggesting, in conjunction with our data from the macrophage-like cell lines, that the state of differentiation of macrophage-like cells may be a factor permitting replication of rabies viruses. We also established ERA and SRV persistently infected U937 macrophage-like cells and isolated a wild-type SRV from adherent murine bone marrow cells of mice that had been infected up to 96 days previously. Viruses from these persistently infected cells and mice were studied in terms of virulence, adaptation, and ability to infect and replicate in macrophage or macrophage-like cells.

MATERIALS AND METHODS

Primary murine bone marrow macrophage cultures. Primary cultures of murine bone marrow macrophages were established with cells harvested from hindlimb femurs and tibias of BALB/cByJ mice. The marrow cells were flushed from the bones with phosphate-buffered saline (PBS) contained in a 1-cc^3 tuberculin syringe fitted with a 26-gauge needle. Following centrifugation, the cells were resuspended in RPMI 1640 medium (Gibco Laboratories, Grand Island, N.Y.) supplemented with 20% heat-inactivated fetal bovine serum (Hyclone Laboratories, Logan, Utah) (RPMI-20) and counted. Cells (10^6) in 2 ml of medium were seeded in 35-mm-diameter plastic tissue culture dishes (Corning, Corning, N.Y.) coated with 2% gelatin and allowed to adhere for 2 h at 37°C. Nonadherent cells were then removed by washing the adherent cells four times with RPMI-20. After the final wash, 2 ml of RPMI-20 containing 50 U of human recombinant macrophage colony-stimulating factor (M-CSF) (Sigma Chemical Co., St. Louis, Mo.) per ml was added to each dish. Prior to infection, adherent cells were incubated with M-CSF for 72 h to promote macrophage differentiation and colony formation (26). Bone marrow macrophages were used instead of peritoneal macrophages since >95% pure macrophage cultures which could be maintained for several weeks without fibroblast overgrowth were readily attained.

Cell lines and propagation. All cell lines were tested for mycoplasma (Myco Tect Kit; Gibco Laboratories) and were determined to be mycoplasma free. Human monocytic cell lines THP-1 and U937, obtained from Hiro Kanno (Rocky Mountain Laboratories, Hamilton, Mont.) and the American Type Culture Collection (ATCC), respectively, were maintained in RPMI medium supplemented with 10% heat-inactivated fetal bovine serum (RPMI-10). Human promyelocytic HL-60 cells also were acquired from the ATCC and were maintained in RPMI-10 in the absence of a differentiating agent.

The murine myelomonocytic cell line WEHI-3BD$^-$ obtained from Gerald Spangrude (Rocky Mountain Laboratories) and the murine macrophage cell line, IC-21, acquired from the ATCC, were maintained in RPMI-10. The murine monocytic cell line P388D1, also obtained from the ATCC, was maintained in RPMI-10 containing 0.01 M β-mercaptoethanol.

Chicken embryo-related (CER) cells were received from Abigail Smith (Yale University, New Haven, Conn.) and were maintained in minimal essential medium with Earle's salts (MEM) (Gibco Laboratories) supplemented with 10% heat-inactivated fetal bovine serum (MEM-10). Mouse embryo cells used in interferon assays were prepared from Swiss Webster mice (24) and also were maintained in MEM-10, as were murine monocytic J774A.1 and PU5-1R cells, which were obtained from the ATCC.

Differentiation of HL-60 cells. Undifferentiated, nonadherent, nonphagocytic, and nonspecific esterase-negative promyelocytic HL-60 cells were differentiated by incubating the cultures for 48 h with TPA (Calbiochem, San Diego, Calif.) at a final concentration of 10^{-6} M. At this time, the cells were adherent, phagocytic, and nonspecific esterase-positive and were considered to be differentiated macrophage-like cells.

Viruses and infection of cells. ERA rabies virus (SAD strain) was obtained from the ATCC (22) and passaged in CER cells. The virus had a titer of 2×10^8 focus forming units (FFU)/ml in CER cells. SRV used in the majority of our studies was of bat origin (*Eptesicus fuscus*) (21) and had been either passaged in murine neuroblastoma cells (SRV-MNB) or intracranially in Swiss-Webster mice. Additional SRV tested were isolated from several different animal species including human, bat, fox, skunk, raccoon, and dog. Each virus belonged to a unique genetic group of rabies virus (35, 36) and was obtained from Jean Smith (Centers for Disease Control and Prevention, Atlanta, Ga.). To produce enough virus for use in our studies, the SRV isolates were passaged intracranially one time in A/WySnJ mice. VSV (Indiana strain) used in the interferon assays was obtained from the ATCC and had a titer in mouse embryo cells of 7.6×10^5 PFU/ml.

All virus replication experiments were done in Linbro TC-24 plates (Flow Laboratories, McLean, VA.), except bone marrow macrophage and HL-60 cell replication experiments, which were done in 35-mm-diameter dishes (Corning). Rabies viruses diluted in the appropriate media were used to infect 10^6 cells at multiplicities of infections (MOIs) ranging from 0.002 to 1. Cells and media were harvested at 0, 24, 48, 72, 96, 120, 144, and 168 h postinfection and either frozen for subsequent fluorescent focus assays or used immediately in infectious center assays. Cells harvested at 0 h were washed, incubated with 0.25% trypsin at 25°C for 15 min, and tested in infectious center assays to quantitate the number of cells which were initially susceptible to infection. Previous experiments using similar conditions determined that 10^8 FFU of extracellular virus was completely inactivated following incubation with 0.25% trypsin. Minimal replication is defined as the detection of infectious virus in culture supernatant fluids 96 h postinfection, a time at which infectious ERA and SRV were undetectable following incubation in medium without cells.

Primary cultures of adherent bone marrow cells were infected in the absence of M-CSF at an MOI of 0.002, whereas differentiated and undifferentiated HL-60 cells were infected at an MOI of either 0.5 or 1. Cells and media were harvested at 24-h intervals.

To determine whether all cells in a culture, in contrast to a small, unrepresentative subpopulation of cells, were uniformly infected and replicating virus, 10^6 cells were infected with ERA virus at an MOI of 10, and the percentage of positive cells was determined by immunofluorescence at 24-h intervals.

Persistently infected cultures of U937 cells were established by infection at an MOI of 0.5 with either ERA virus or SRV-MNB. Incubation with SRV-MNB was repeated once to attain infection of at least 50% of the cells. Cultures were then passaged weekly, and aliquots were stored at −70°C. The cells subsequently were assayed for fluorescent antigen, while culture fluids were quantified in vitro for infectious virus, and 0.03 ml of undiluted cell culture medium was inoculated intracranially into 21-day-old mice to determine viral virulence.

Rabies virus-specific immunofluorescent assays. Infectious virus was quantified with a fluorescent focus assay (33). CER cell monolayers in Linbro TC-24 plates were incubated for 1 h with serial 10-fold dilutions of rabies virus-infected cell cultures (total infectious virus) or supernatant fluids (extracellular virus). The inocula were aspirated, and the monolayers were washed with PBS and incubated at 37°C (5% CO_2) for 96 h with a methocel overlay. The monolayers were then washed with PBS, fixed with 4% formalin, stained with equine/bovine anti-rabies fluorescein isothiocyanate-conjugated antibody (Becton Dickinson Microbiology Systems, Cockeysville, Md.), and observed for fluorescent foci with a Leitz Orthoplan microscope.

Rabies virus antigen was detected with a fluorescent antigen assay, in which cells were fixed with 4% formalin, stained with anti-rabies antibody, and loaded into multichambered slides. At least 200 cells were observed with the Leitz Orthoplan microscope, and the percentage of fluorescent antigen-positive cells was determined.

Infectious center assay. Infectious center assays were done to quantitate the number of cells which were infected at given intervals. Cells were washed and counted, and serial 10-fold dilutions of the cells were mixed with methocel (0.2 ml of cells per 0.5 ml of methocel). The cell-methocel mixture was placed on CER monolayers and incubated at 37°C for 96 h. Monolayers were observed for fluorescent foci as described above.

Identification of cells as macrophages. (i) Phagocytosis. Latex and fluorescent (Fluorescebrite) beads, approximately 1 μm in diameter, were purchased from Sigma Chemical Co. and Polysciences Inc. (Warrington, Pa.), respectively. Each stock solution of beads was diluted 1:25 in heat-inactivated fetal bovine serum (Hyclone Laboratories), added at a final concentration of either 1 or 10% to cell cultures, and incubated with the cells for 2 to 3 h at 37°C. The cells were washed four times with PBS, and the number of cells that had phagocytized at least 10 beads was determined.

(ii) Nonspecific esterase staining. Nonspecific esterase staining was performed as described by Koski et al. (19) with 1% methyl green counterstain.

(iii) DIL labeling of acetylated low-density lipoprotein. Live murine adherent bone marrow cells were incubated for 1 h with 1 μl of the macrophage marker acetylated low-density lipoprotein fluorescently labeled with 1,1-dioctodecyl-3,3,3,3-tetramethylindocarbocyaninate (DIL) (Molecular Probes, Eugene, Oreg.) per ml.

(iv) Mac-1 staining. Fixed murine adherent bone marrow cells were incubated for 30 min at 37°C with monoclonal rat anti-Mac-1 (M1/70.15.11.5HL) antibody (obtained from Jerry Spangrude, Rocky Mountain Laboratories) and were subsequently stained with horseradish peroxidase as described by Czub et al. (7) or

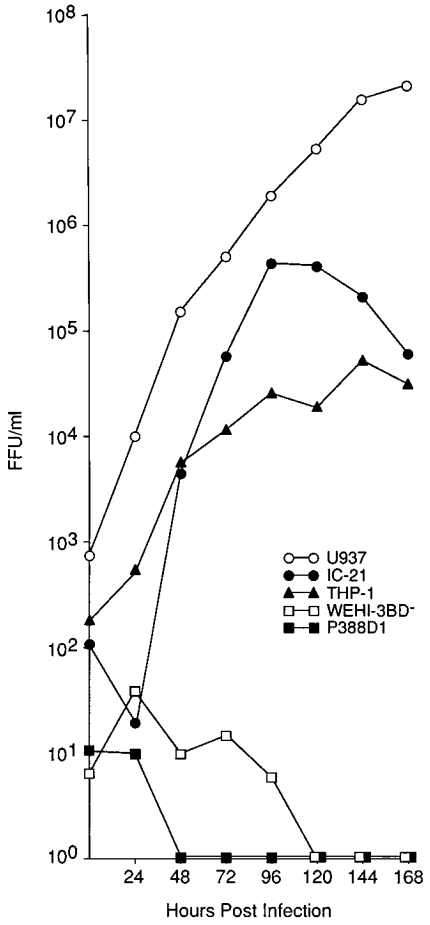

FIG. 1. ERA rabies virus replication in macrophage-like cell lines. Cells were infected at an MOI of 0.002. Data for each cell line are representative of three independent experiments.

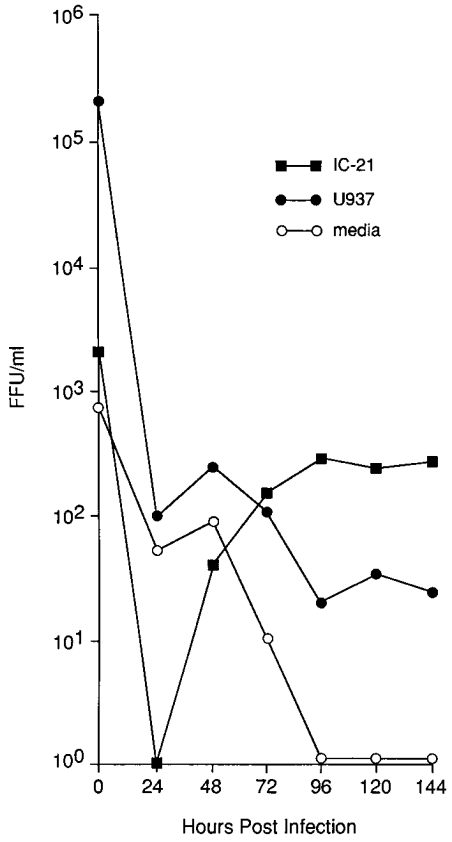

FIG. 2. Tissue-culture-adapted SRV (SRV-MNB) replication in murine IC-21 cells and human U937 cells. Virus titers were compared with the half-life of SRV-MNB in media alone. The zero hour concentration of virus represents the amount of virus that was added to the media or used to infect the cells. Data are representative of three independent experiments.

incubated with biotinylated anti-rat immunoglobulin G antibody (Vector Laboratories, Burlingame, Calif.) followed by rhodamine-labeled avidin D (Vector Laboratories) for immunofluorescence staining.

Interferon assay. Supernatant fluids were harvested from murine WEHI-3BD$^-$, P388D1, and IC-21 cells which were uninfected or had been infected for 2, 6, 24, and 48 h with ERA virus at an MOI of 0.002. The fluids were then assayed for antiviral activity on outbred Swiss Webster mouse embryo cells by using a standard cytopathic effect bioassay (4) and VSV challenge (24). Standard mouse interferon (National Institutes of Health international reference mouse interferon [G002904511]) was used as a positive control.

RESULTS

Virus replication in macrophage-like cell lines. Several macrophage-like cell lines of murine and human origins were studied to determine whether rabies viruses replicate in different macrophage cell types. Figure 1 illustrates that ERA virus replicated well in the human cell line U937, the murine cell line IC-21, and the human cell line THP-1 (an increase in virus titer of $>10^4$, $>10^3$, and $>10^2$, respectively). Minimal replication was detected in the murine cell lines WEHI-3BD$^-$ (Fig. 1) and PU5-1R (data not shown), and ERA did not replicate in murine P388D1 (Fig. 1) or J774A.1 cells (data not shown). In contrast, SRV-MNB replicated well in IC-21 cells (an increase in the virus titer of $>10^2$) but only a steady level of minimal replication was detected in U937 cells (Fig. 2). SRV-MNB did not replicate in THP-1, WEHI-3BD$^-$, or P388D1 cells (data not shown).

To approximate natural infection more closely, macrophage-like cell lines were infected with an SRV of bat origin which had been passaged intracranially in mice but had not been adapted to MNB tissue culture cells. Seventy-two hours after the cells had been infected at an MOI of 1, immunofluorescence staining revealed that 0.1% of the U937 cells and 0.001% of the IC-21 cells were positive for rabies antigens (data not shown). Several wild-type SRV from human, bat, skunk, raccoon, and dog origins that had not been previously adapted to tissue culture cells were also tested with the macrophage-like cell lines. It was determined that a virus isolated from a vampire bat replicated to low levels in THP-1, U937, and IC-21 cells, whereas a virus isolated from a skunk replicated minimally in the IC-21 cells but not in THP-1 or U937 cells. The remaining viruses failed to replicate in the three cell lines (data not shown). The cell lines that were used for these replication experiments were positive for phagocytosis and had Fc and C3 receptors; some cells that replicated virus were adherent and some were nonadherent. Thus, no obvious markers or characteristics distinguished cell lines that were permissive for rabies replication from those that were not.

HL-60 cells were utilized to determine whether the state of differentiation of the macrophage-like cells is an important factor permitting replication of rabies viruses. After a 48-h incubation with TPA, 90% of the HL-60 cells became adherent and were characterized as macrophages by nonspecific esterase staining and phagocytosis of latex beads. Differentiated and

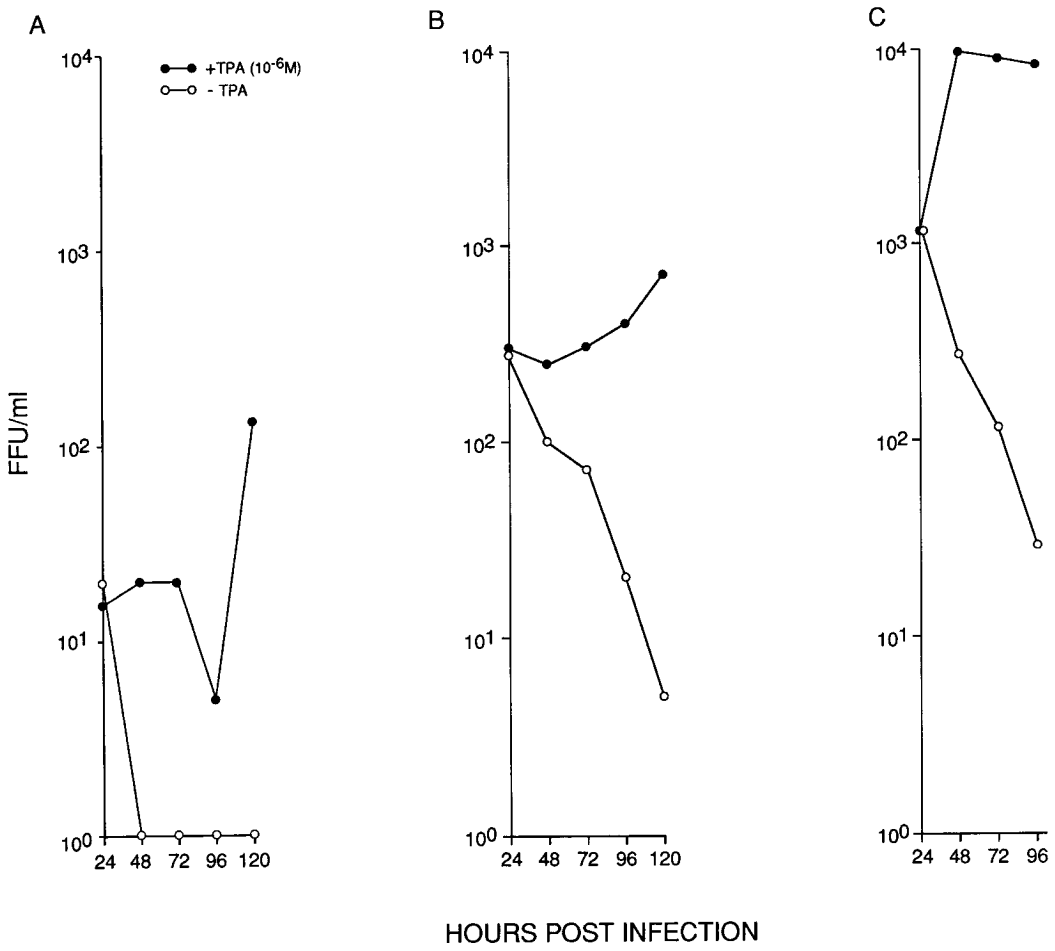

FIG. 3. ERA rabies virus replication in differentiated but not undifferentiated human HL-60 cells infected at an MOI of 0.002 (A), 0.5 (B), and 1 (C).

undifferentiated cells, initially infected at a low MOI, were also infected with higher concentrations of virus since differentiated cells aggregate in the presence of TPA, making viral cell-to-cell spread difficult. The data in Fig. 3 show that in all instances, differentiated HL-60 cells replicated ERA virus, whereas replication was not detected in undifferentiated cells. Undifferentiated HL-60 cells that were incubated with TPA 24 and 48 h postinfection also failed to replicate virus. This result suggests either that the cells must be differentiated prior to infection to insure virus replication or that the initial infecting dose of virus had diminished through inactivation to such a minimal level that none was available in the culture media to infect the newly differentiated cells.

To determine whether a majority of cells in the cultures were susceptible to ERA virus infection or whether a small, unrepresentative population of cells was infected and responsible for the increasing virus titers, susceptible U937, IC-21, and THP-1 cells and differentiated HL-60 cells were infected at an MOI of 10 and assayed at 24-h intervals thereafter. Immunofluorescence assays indicated that >80% of the U937, IC-21, and THP-1 cells and >50% of the differentiated HL-60 cells were infected (data not shown). Thus, the majority of cells in these macrophage-like cell lines were permissive to infection.

Infectious center assays performed immediately after the virus and cells had been incubated together suggested that ERA virus, which did not productively replicate in P388D1 and undifferentiated HL-60 cells, did infect these cells (Table 1). In fact, the number of cells initially infected was quite similar for cell lines that did replicate virus (IC-21) and that failed to replicate virus (P388D1) (Table 1). Low amounts of viral antigen also were detected by immunofluorescence in the P388D1 and HL-60 cells up to 24 h postinfection; however, no viral antigen was detected after 24 h (data not shown). Although interferon has been determined to block the productive infection of macrophages or macrophage-like cells with HIV-1 (25) and the rhabdovirus VSV (6), interferon was not detected

TABLE 1. Number of cells that initially scored positive for rabies virus infection by infectious center assay[a]

Cell	No. of cells incubated with virus	MOI	No. of cells infected (0 h)
U937	5×10^5	0.002	715
THP-1	5×10^5	0.002	175
P388D1	5×10^5	0.002	105
WEHI-3BD$^-$	5×10^5	0.002	65
IC-21	7×10^4	0.002	110
HL-60[b]	5×10^5	1.0	130
HL-60[c]	5×10^5	1.0	16,000

[a] Cells were incubated with ERA for 1 h (0-h time point), treated with trypsin, washed, and incubated on CER monolayers (see Materials and Methods).
[b] Undifferentiated cells.
[c] Differentiated cells.

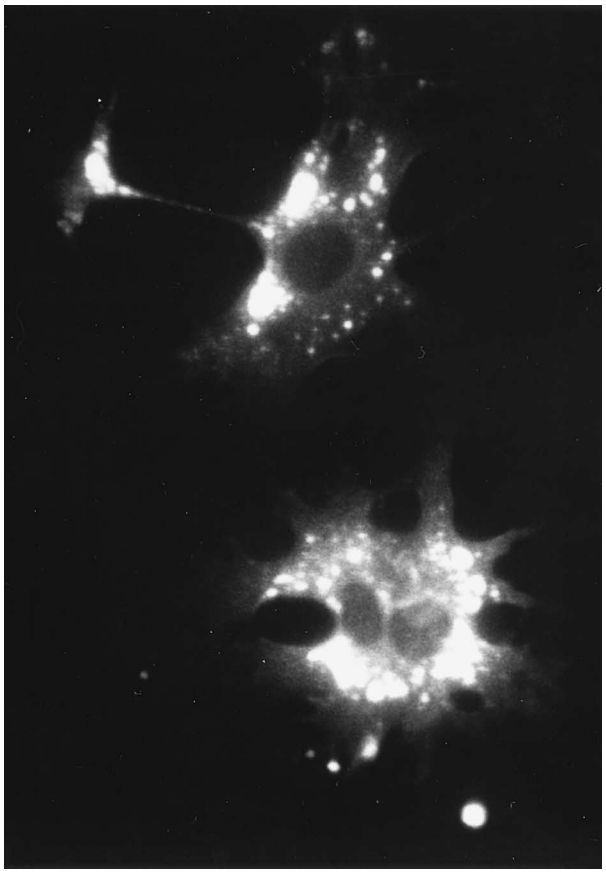

FIG. 4. ERA rabies virus-infected bone marrow macrophages, formalin fixed and stained with fluorescein isothiocyanate-labeled anti-rabies antibody 72 h postinfection.

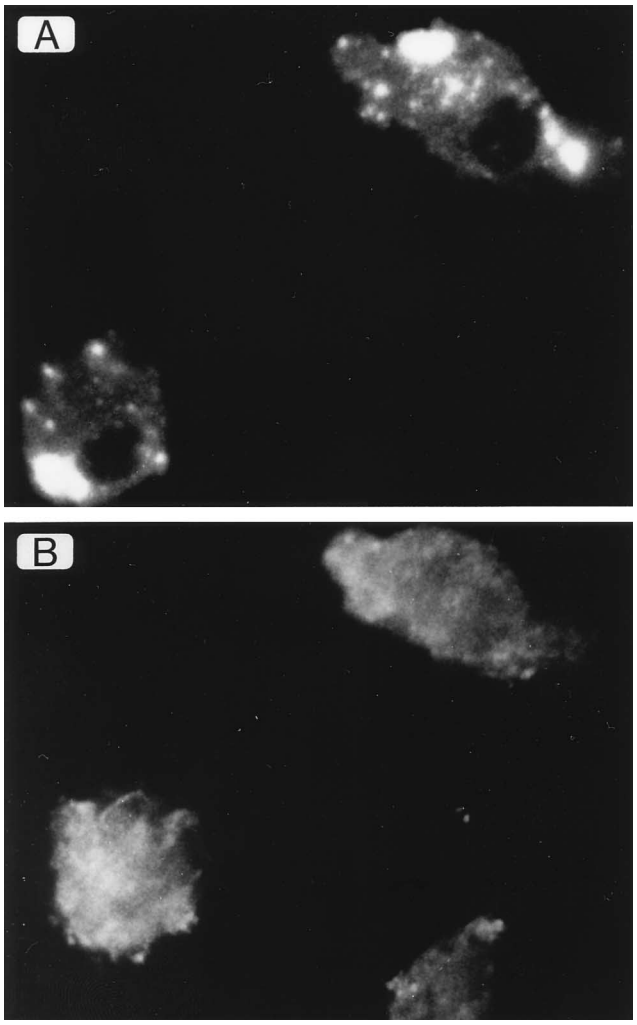

FIG. 5. Double fluorescent labeling with anti-rabies virus (A) and anti-Mac-1 (B) antibodies of ERA rabies virus-infected primary murine bone marrow macrophages. Cultures were infected for 72 h, fixed with 95% ethanol, and permeabilized with 0.1% Nonidet P-40 after anti-Mac-1 staining and before incubating with anti-rabies antibody.

in culture fluids prior to or following infection of P388D1 or WEHI-3BD$^-$ cells (data not shown), which failed to replicate rabies viruses.

Virus replication in primary macrophages. Our first set of experiments showed that ERA rabies virus and several SRV replicated in several different macrophage-like cell lines. To determine whether ERA virus replicated in primary macrophages, primary murine bone marrow adherent cell cultures were established and differentiated in the presence of M-CSF. The cells were shown to be >95% macrophages by the nonspecific esterase stain, DIL-labeled acetylated low-density liprotein uptake, Mac-1 antibody staining, and the phagocytosis of latex beads. At 72 h after infection, at an MOI of 10, 10% of the cells stained positive for rabies antigens with fluorescein isothiocyanate-labeled anti-rabies antibody (Fig. 4). The infected cells were identified as macrophages by their uptake of DIL-labeled acetylated low-density lipoprotein and staining with anti-Mac-1 antibody (Fig. 5). Furthermore, virus replicated in these cells as shown by the >90% increase in virus titer, which occurred between 24 and 144 h postinfection (Fig. 6). In contrast to ERA virus, SRV-MNB did not replicate in primary murine macrophages (data not shown). An SRV of bat origin that had not been adapted to MNB tissue culture cells was incubated with the primary murine macrophages to determine whether a wild-type rabies virus could infect these cells. Immunofluorescence staining revealed that 0.001% of the macrophages was positive for rabies virus antigens 72 h postinfection (data not shown). This minimal but positive infection of primary macrophages, as well as the replication of other wild-type isolates in the macrophage-like cell lines, suggests that rabies viruses replicate to low levels in macrophages in vivo.

Virus persistence. It is well-known that rabies viruses can establish persistent infections in nonmacrophage cell lines (3, 15). To determine whether persistent infections could be established in a macrophage-like cell line, U937 cells were infected with either ERA virus or SRV-MNB. Persistent infections were established with both viruses. Through 60 passages the SRV-MNB-infected cells were 50 to 80% positive and the ERA virus-infected cells were 80 to 95% positive for rabies antigens as determined by immunofluorescence (data not shown). Quantification of infectious virus in supernatant fluids of these persistently infected cultures showed that the virus titers initially decreased but stabilized at the 20th passage (Fig. 7). Furthermore, the viruses released from these cells through the 30th passage were virulent, as determined by their ability to kill intracranially inoculated mice (Fig. 7). Thereafter, virus released from subsequent cell passages lost virulence, as shown by the inability of the virus to kill mice. The failure to kill mice was not associated with the concentration of virus released into

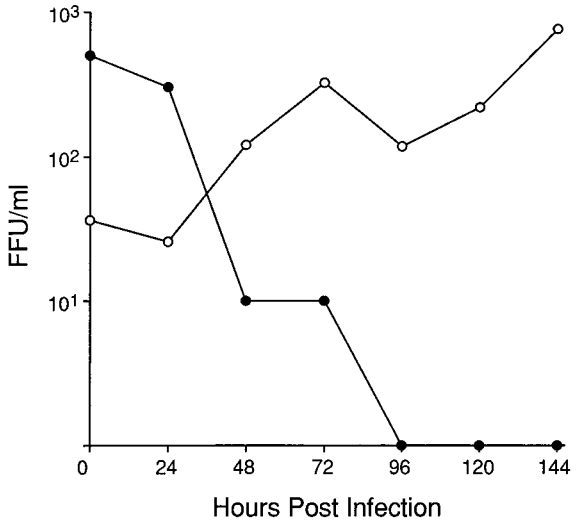

FIG. 6. ERA virus replication in primary murine bone marrow macrophages. After a 72-h preincubation with M-CSF, cells were infected at an MOI of 0.002. Virus titers (○) were compared with the half-life of ERA in media alone (●). Data represent the geometric means of three independent experiments.

the culture media since there was minimal variability in the amount of virus that was released from the cells at the different passages. Phagocytosis of similar amounts of fluorescenated latex beads by uninfected U937 cells and the persistently infected U937 cells, as detected by anti-rabies virus fluorescenated antibody, indicated that this macrophage function was not altered by the persistent rabies virus infection (data not shown).

Our initial data have shown that SRV-MNB replicated to minimal titers in U937 cells following an acute infection (Fig. 2). In contrast, virus harvested from SRV-MNB persistently infected U937 cells (passage 10) replicated to high titers when used to acutely infect naive U937 cells ($>10^5$ increase in virus titer) (Fig. 8). Interestingly, virus harvested from this 10th passage of persistently infected U937 cells did not infect or replicate in primary murine macrophages, WEHI-3BD$^-$ or P388D1 cells, suggesting that it had specifically adapted to only U937 cells and not to macrophages or macrophage-like cells in general (data not shown). Additional studies with virus harvested from the SRV-U937 persistently infected cells determined that virus released from cells at the 45th and 60th cell passages, which were essentially identical in titer (Fig. 7), rep-

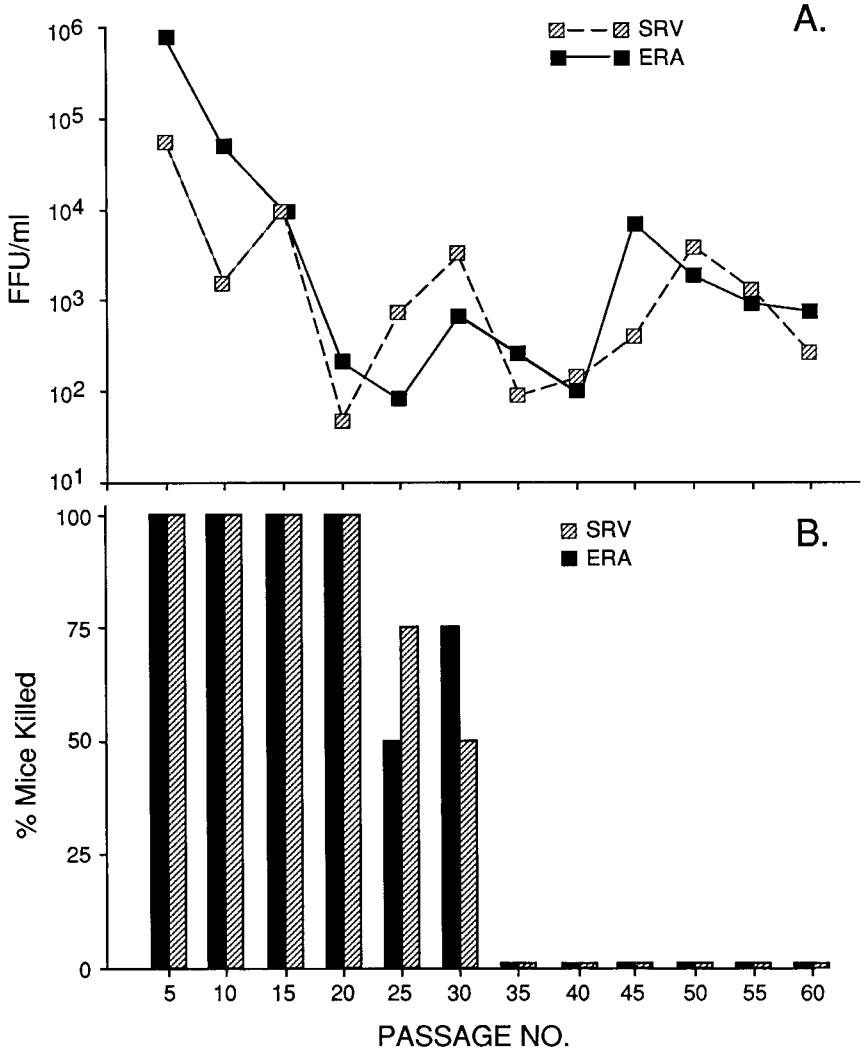

FIG. 7. Comparison of virus titers and virulence of viruses released from SRV and ERA rabies virus persistently infected U937 cells.

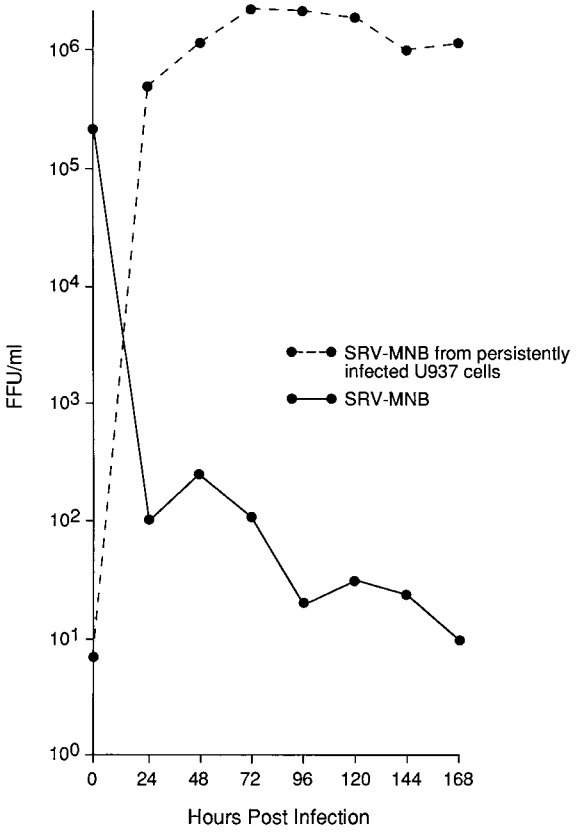

FIG. 8. Acute infection of naive U937 cells with SRV-MNB or SRV-MNB that had been harvested from the 10th passage of persistently infected U937 cultures. Data are representative of three independent experiments.

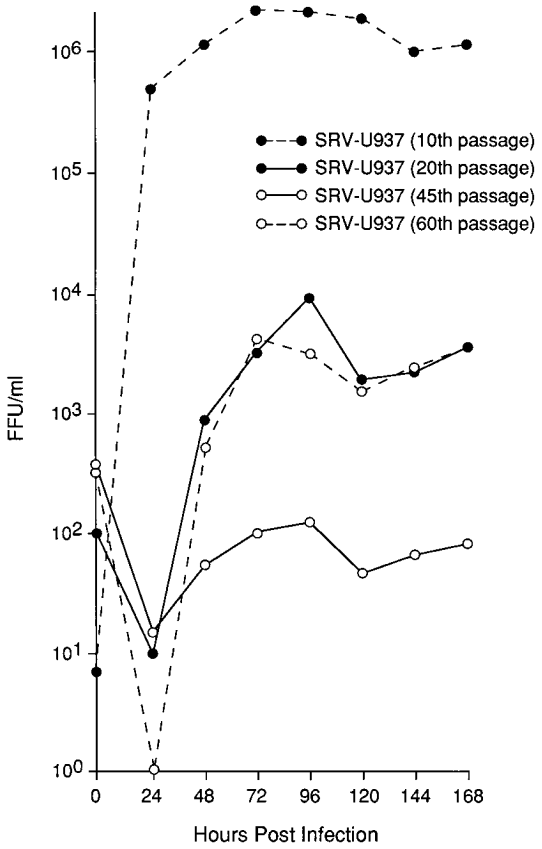

FIG. 9. Acute infection of naive U937 cells with virus harvested from various passages of SRV-MNB persistently infected U937 cultures.

licated quite differently when they were used to acutely infect naive U937 cells ($>10^{1.5}$ difference in virus titers at 168 h postinfection) (Fig. 9). Considering that viruses from both of these passages did not kill intracranially inoculated mice, there appeared to be no correlation with the loss of viral virulence and the ability of the viruses to adapt to and acutely infect naive U937 cells.

To determine whether rabies viruses persist in macrophages in vivo, adherent cell bone marrow cultures from mice that had been previously infected and recovered from a wild-type SRV infection were established (23). Infectious virus was isolated by in vitro amplification procedures in 5 of 46 mice that had been inoculated up to 96 days previously (data not shown). The virus which was used to infect the mice and the virus isolated from a mouse that had been inoculated 96 days previously were subsequently used at similar MOIs to infect cultures of primary bone marrow macrophages. It was determined that virus titers in the cultures infected with the bone marrow isolate, compared with the titer of virus that was originally used to infect the mice, were >90% higher (data not shown). This result suggests that the virus isolated from the adherent bone marrow cells had previously adapted to macrophages in vivo (as also shown in vitro with the U937 persistently infected cells) and therefore was more competent to infect macrophages in vitro. Thus, the possibility that adaptation occurred is strengthened by the dual observations that the virus used to infect the mice replicated minimally in the primary bone marrow macrophages and the bone marrow isolate failed to replicate in macrophage cell lines.

DISCUSSION

Two strains of rabies virus were extensively used in this study; tissue culture-adapted ERA virus and a bat SRV that had been either adapted to tissue culture cells or passaged in mice. It is shown that significant ERA virus replication occurred in two human macrophage-like cell lines, U937 and THP-1, and in the murine macrophage cell line IC-21, as well as in primary cultures of murine bone marrow macrophages. The tissue culture-adapted SRV also replicated in U937 and IC-21 cells. Non-tissue culture-adapted SRV, and in particular SRV isolated from bats, infected U937, THP-1, and IC-21 cells as well as primary bone marrow macrophages but did not replicate to high titers.

In contrast to the studies by King et al. (16), we failed to detect ERA virus replication in P388D1 cells. These differing results may be explained by the cell line that was used since two different P388D1 cell lines have been reported. The cells used in their study are adherent (18), while the cells used in this study are nonadherent. King et al. also detected rabies antibody-dependent enhanced virus replication in P388D1 cells. Although we did not use P388D1 cells in an antibody-dependent assay, we failed to detect rabies antibody-dependent enhanced virus replication in our primary murine bone marrow macrophage cultures (data not shown).

It is interesting that rabies virus replicated in U937 and THP-1 cells, which in certain conditions can differentiate (28, 38), and in IC-21 cells that are derived from murine peritoneal macrophages and are known to be more differentiated than P388D1 cells (40). In contrast, WEHI-3BD$^-$ cells do not dif-

ferentiate (27) and also did not support the replication of rabies virus. To determine if the state of differentiation of macrophage-like cells is an important determinant for rabies replication, differentiated and undifferentiated HL-60 cells were incubated with ERA virus. Replication was detected in differentiated but not undifferentiated cells. Although replication did occur in differentiated cells, high titers of ERA virus were not detected in the cultures, suggesting that virus was inactivated by the high concentrations of proteolytic enzymes which are known to be released by these cells (1). Thus, the HL-60 cell data, in addition to our other replication data for the different cell lines, suggest that rabies virus replication is dependent upon the degree of differentiation of macrophage-like cells. Differentiated macrophage-like cells also have been found to be more permissive than undifferentiated cells for the replication of viruses such as human cytomegalovirus (41), HIV-1 (9), and visna virus (10). The differentiated cells may be more permissive for virus replication because they are capable of producing host factors that are required for virus replication, such as receptors or transcription factors (42). Support for this possibility has been shown with U937 cells treated with TPA, in which expression of transcription factors Jun and Fos is upregulated. Jun and Fos in turn bind to a specific site on visna virus DNA, which causes upregulation of viral gene expression (32). Furthermore, significant levels of visna virus replication, as measured by viral RNA synthesis, do not occur until infected monocytes differentiate into macrophages (10).

With the infectious center assay, we were able to detect infectious ERA virus in P388D1 and WEHI-3BD$^-$ cells that were tested immediately after incubation with virus; subsequently, the cells failed to support ERA virus replication. These data, as well as the fact that we could not detect viral antigen 48 h postinfection, suggest that virus entered these cells but that productive infection was blocked early after entry, possibly through the absence of a factor(s) that may be required for genomic replication or transcription. Although the cells were treated with trypsin at a concentration sufficient to inactivate 10^8 FFU of extracellular virus, the possibility remains that some virus adhering to the cell surface may not have been inactivated and scored in the infectious center assay.

Although U937 cells were not readily infected with SRV-MNB, persistent infections were established. In contrast to the virus that was used to establish the persistently infected cells, virus harvested from these cells readily infected naive U937 cells and replicated to high titers. It did not, however, replicate in primary bone marrow macrophages or in P388D1 or WEHI-3BD$^-$ cells, suggesting that virus released from persistently infected U937 cells had not adapted to readily infect other types of macrophages or macrophage-like cells. It also was shown that virus released from U937 cells persistently infected with SRV or ERA virus lost virulence (ability to kill intracranially inoculated mice) after 30 cell passages. This loss of virulence (attenuation) may have been the result of the typical attenuation of rabies viruses which occurs following extended passages in tissue culture cells, or it may have resulted from an increase in the concentration of defective interfering particles. Thus, with an increase in defective interfering particle concentration, infectious virus titers decreased and subsequently fluctuated at levels of 10^2 to 10^3 FFU/ml. Similar increases in the concentration of defective interfering particles and concurrent fluctuating concentrations of infectious virus have been reported in nonmacrophage cells persistently infected with rabies viruses (3, 15). It also was noted in our study that the attenuated virus released from the persistently infected cells replicated to high titers when used to acutely infect naive U937 cells. The attenuated virus did not replicate in primary murine macrophages or other macrophage-like cell lines, suggesting that it also had specifically adapted to infect only U937 cells. That a similar adaptation may occur in vivo was shown by the failure of the virus isolated from adherent bone marrow cells to replicate in vitro in cells other than primary macrophage cultures. The mechanism(s) responsible for this adaptation is currently under study.

When considering these data in total, we suggest that rabies viruses may infect, replicate, and subsequently persist in vivo in macrophages. Evidence supporting infection of macrophages in vivo has been shown previously (29), and in this study, wild-type SRV were isolated from adherent bone marrow cells of several mice, one which had been infected 96 days previously. It may be that low virulent or attenuated rabies viruses persist in vivo in macrophages in a chronic or latent state. Then, during this persistent state a mutagenic or cytokine activation signal event occurs, eventuating conversion of the persistent attenuated virus to a virulent virus, which results in a productive infection. Since RNA viruses are known to have a high mutagenic frequency (37) and macrophages have been determined to have highly mutagenic intracellular environments, particularly when activated (5), the ideal combination of factors would be in place for this conversion to occur. Furthermore, it is known that cytokines can up-regulate expression of HIV-1 in chronically infected U937 cells (8). We have demonstrated similar cytokine activation with our persistently infected cells; following incubation of the cells with cytokine-enriched supernatant fluids, the virus released from the cells had converted from avirulent to virulent (30). The present studies and the persistently infected macrophage model may help to elucidate the complex series of events which account for the long incubation periods and subsequent onset of the clinical infections which are known to occur following some rabies virus infections (20, 34).

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