Rabies Virus Infects Mouse and Human Lymphocytes and Induces Apoptosis

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Attenuated and highly neurovirulent rabies virus strains have distinct cellular tropisms. Highly neurovirulent strains such as the challenge virus standard (CVS) are highly neurotropic, whereas the attenuated strain ERA also infects nonneuronal cells. We report that both rabies virus strains infect activated murine lymphocytes and the human lymphoblastoid Jurkat T-cell line in vitro. The lymphocytes are more permissive to the attenuated ERA rabies virus strain than to the CVS strain in both cases. We also report that in contrast to that of the CVS strain, ERA viral replication induces apoptosis of infected Jurkat T cells, and cell death is concomitant with viral glycoprotein expression, suggesting that this protein has a role in the induction of apoptosis. Our data indicate that (i) rabies virus infects lymphocytes, (ii) lymphocyte infection with the attenuated rabies virus strain causes apoptosis, and (iii) apoptosis does not hinder rabies virus production. In contrast to CVS, ERA rabies virus and other attenuated rabies virus vaccines stimulate a strong immune response and are efficient live vaccines. The paradoxical finding that a rabies virus triggers a strong immune response despite the fact that it infects lymphocytes and induces apoptosis is discussed in terms of the function of apoptosis in the immune response.

The rabies virus is an enveloped bullet-shaped virus of the *Rhabdoviridae* family, genus *Lyssavirus*. It consists of a helical nucleocapsid (NC) surrounded by a viral membrane composed of host lipids and two proteins, the glycoprotein G and the matrix protein M. Most rabies virus strains are highly neurotropic. Evidence that the virus multiplies at the site of inoculation in the periphery is controversial (14, 17, 27, 39), since peripheral multiplication of rabies virus depends on the strain of the rabies virus. Highly neurovirulent strains, such as the challenge virus standard (CVS) strain, have a narrow cellular tropism restricted to neurons and replicate poorly in nonneuronal cells, whereas attenuated strains such as the Evelyn Rotnycki Abelseth (ERA) rabies virus strain or avirulent laboratory mutants (RV194-2 and AVO-1) have a less restricted tropism and infect nonneuronal cells (5, 17, 46).

Attenuated virus strains are efficient live vaccines because they trigger a much longer-lasting and more efficient immune response than inactivated (killed) rabies virus vaccines (8, 40). The great efficacy of live vaccines may be due to their capacity to replicate at the periphery (1), so identification of the peripheral targets is of critical importance in understanding the protective mechanism of this type of vaccine. The nature of the nonneuronal target cells is obscure. It has been reported that skunk rabies virus infects myocytes in vivo (26) and that the attenuated rabies virus strain ERA infects primary bone marrow macrophages and macrophage-like cell lines in vitro (32). Immunohistochemistry and electron microscopy studies have

detected large numbers of lymphocytes at the site of inoculation as well as macrophages (5, 44), suggesting that activated lymphocytes may be the main rabies virus target.

The goal of the work was to determine whether lymphocytes are peripheral targets of the rabies virus. We compared ERA and CVS infection in spleen lymphocyte cultures and in several lymphocyte cell lines. We report that some lymphocyte cell lines and a fraction of spleen lymphocytes are susceptible to infection, and more so to the attenuated rabies virus strain ERA than to CVS. Infections by lymphotropic viruses such as human immunodeficiency virus, feline leukemia virus, avian influenza virus, and chicken anemia virus are known to trigger lymphocyte apoptosis (15, 20, 25, 30, 33). We investigated whether rabies virus infection induced lymphocyte apoptosis. The induction of apoptosis would be expected to limit the capacity of lymphocytes to launch an efficient virus-specific immune response. The paradoxical result that a rabies virus triggers a strong immune response despite the fact that it infects lymphocytes and induces apoptosis is discussed in terms of the function of apoptosis in antigenic presentation.

MATERIALS AND METHODS

Cells. The human lymphoblastoid Jurkat T-cell line, the CCRF-CEM cell line (a pre-T-lymphoblastoid cell line lacking the T-cell receptor and CD3), and the human Burkitt lymphoblastoid B-cell lines Raji and Daudi were grown in RPMI 1640 medium supplemented with 2 mM L-glutamine, 100 U of gentamicin per ml, 10 mM HEPES, and 10% fetal calf serum (FCS) at 37°C with 5% CO₂. Chicken embryo-related cells were grown in minimal essential medium supplemented with phosphate tryptose broth, 2 mM L-glutamine, 8% FCS, and gentamicin. The rabies virus laboratory strains CVS and ERA were obtained from the American Type Cell Collection, Rockville, Md. (vr959 and vr332, respectively). Rabies virus was propagated on BSR cells (36), a baby hamster kidney (BHK-21)-derived cell line, and cell culture supernatant was used as the inoculum. In some cases supernatants were also purified on a sucrose gradient and concentrated as previously described (50) before being UV inactivated. Cell lines and viruses were mycoplasma free as determined by the plaque assay technique.

MAb and reagents. Concanavalin A (ConA) was purchased from Sigma (distributed by Cogem, Paris, France). Texas red-conjugated streptavidin was obtained from Gibco BRL, and fluorescein isothiocyanate (FITC)-conjugated

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streptavidin was obtained from Amersham. FITC-conjugated affinity-purified F(ab')₂ fragment anti-mouse immunoglobulin G goat antibodies (Ab) were purchased from Jackson ImmunoResearch (distributed by Immuntect, Marseille Luminy, France). Phycoerythrin-conjugated anti-CD4 and anti-CD8 monoclonal Ab (MAb) were obtained from Caltag Laboratories (San Francisco, Calif.). FITC-conjugated NC-specific rabbit Ab were obtained from Sanofi Diagnostics (Marnes la Coquette, France). Agarose (SeaKem) was purchased from FMC Bioproducts (Rockland, Maine), and TUNEL (terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end labeling) reagents were purchased from Boehringer Biochemical (Mannheim, Germany).

Infection of spleen lymphocytes and lymphocyte cell lines. Spleen lymphocytes were prepared from teased spleens from 6- to 8-week-old female BALB/c mice (Janvier, Labresle, France). After treatment with NH₄Cl to remove erythrocytes, spleen lymphocytes were seeded in 24-well tissue culture plates (10⁶ cells/ml) in RPMI 1640–10% FCS–10⁻² mM β -mercaptoethanol in the presence (activated cells) or absence (nonactivated cells) of ConA (1 μ g/ml). After 24 h at 37°C with 7% CO₂, activated and nonactivated splenocytes were washed and infected with rabies virus (multiplicity of infection [MOI] = 1) or mock infected (control) and plated at 37°C with 5% CO₂ for further analysis. This time is day 0 for virus production kinetics. A similar infection protocol was used for T- and B-cell lines, but these cells were infected on the first day of culture.

Titration of virus. Infectious rabies virus particles from cell culture were titrated by the plaque-forming assay with chicken embryo-related cells as described elsewhere (19). Results are expressed as PFU per milliliter.

Immunostaining. Infected or mock-infected cells were washed with phosphate-buffered saline (PBS), fixed in 4% paraformaldehyde for 20 min at 4°C, and incubated for 30 min at 4°C with FITC-conjugated NC-specific Ab diluted in PBS containing 1% heat-inactivated FCS, 0.1% (wt/vol) sodium azide, and 0.1% (wt/vol) saponin (permeabilization buffer). The cells were then washed in permeabilization buffer and then in PBS. For double staining, cells were incubated for a further 30 min at 4°C with phycoerythrin-conjugated anti-CD4 and anti-CD8 MAb diluted in staining buffer (PBS containing 1% heat-inactivated FCS and 0.1% [wt/vol] sodium azide). The cells were then analyzed by flow cytometry with a FACScan cytofluorimeter and PC-Lysis II software (Becton Dickinson Co., Mountain View, Calif.).

Measure of viability by light scatter analysis. Morphological changes detected by cell side light scatter (SSC) and forward light scatter (FSC) analysis were used to identify apoptotic cells as described previously (16). The relative increase in cell death was expressed by the following formula: (percentage of dead cells in infected culture – percentage of dead cells in noninfected culture)/(100 – percentage of dead cells in noninfected culture).

Detection of apoptosis by DNA fragmentation assays. (i) **DNA electrophoresis.** Infected and mock-infected T cells (5 $\times$ 10⁵ cells/well in a final volume of 1 ml) were cultivated in 24-well culture dishes for 48 or 72 h. The cells were centrifuged and stored as pellets at –80°C until required. DNA was extracted as described elsewhere (35). Briefly, the pellet was incubated in 0.5 ml of lysis buffer (1 M Tris, 500 mM EDTA, 10% sodium dodecyl sulfate) and treated with RNase. The DNA was extracted twice with phenol-chloroform-isoamyl alcohol, resuspended in Tris-EDTA buffer (10 mM Tris, 1 mM EDTA), loaded (3 μ g of DNA per lane) onto a 1.5% agarose gel containing ethidium bromide, and observed after electrophoresis at 50 V for 3 h at 4°C.

(ii) **TUNEL method.** Fragmentation of DNA was identified by the TUNEL technique of labeling the 3'OH DNA terminus as described previously (10). Cells (10⁶) were washed in PBS, resuspended in 3% paraformaldehyde, and incubated for 30 min at room temperature. They were then washed in PBS and placed on Dynatech 10-slot slides. Once dry, the slides were fixed in pure ethanol for 30 min at –20°C and dried. After rehydration, cells were incubated for 30 min at 37°C with (per slot) 25 μ l of the labeling preparation containing 12.5 U of terminal deoxynucleotidyltransferase, 0.05 nmol of biotin-16-dUTP, 2.5 mM CoCl₂, 0.2 M potassium cacodylate, 25 mM Tris-HCl, and 0.25 mg of bovine serum albumin per ml. The slides were then incubated for 15 min at room temperature in 2 $\times$ saline sodium citrate buffer (30 mM trisodium citrate and 0.3 M NaCl), rinsed in H₂O, saturated for 30 min in bovine serum albumin (20 mg/ml in PBS) at 37°C, and rinsed in H₂O again. Samples were then stained for 30 min at 37°C with 25 μ l of a 1:10 dilution of FITC-conjugated streptavidin or Texas red-conjugated streptavidin per slot and rinsed in H₂O. Finally, slides were mounted and observed with a Zeiss UV microscope. Results were expressed as the number of cells with fluorescent nuclei per 500 or 5,000 cells. Alternatively, slides which had been assayed for TUNEL terminated with Texas red-conjugated streptavidin were stained for rabies virus antigens. Following several PBS washes, cells were incubated in a blocking solution (0.2% Triton X-100 and 3% heat-inactivated fetal bovine serum in PBS) for at least 30 min at 37°C. Cells were incubated with anti-rabies virus G protein MAb PVE (22) in the blocking solution for 30 min at 37°C, washed, and then incubated with FITC-conjugated anti-mouse immunoglobulin G (heavy plus light chains) F(ab')₂ fragments under the same conditions. The cells were examined with a photomicroscope (Carl Zeiss, Inc., Thornwood, N.Y.). Confocal microscopy of samples labeled with two fluorochromes was performed with a confocal laser scanning microscope (Leica Instruments, Nussloch, Germany), which uses an argon-krypton laser operating in multiline mode. Cells labeled with both Texas red and fluorescein conjugates were sequentially analyzed at 567 and 488 nm, respectively, with a long-path RG590 filter for Texas red emission and a narrow-band filter centered on 535 nm for

FITC signal detection. For each observation, results for 12 optical sections taken at intervals of 0.5 μ m were recorded.

Detection of apoptosis by electron microscopy. Mock-infected and ERA virus-infected Jurkat cells (2 $\times$ 10⁶) were prefixed 2 days after infection with 1.6% glutaraldehyde in 100 mM phosphate buffer, pH 7.3. After centrifugation, cells were postfixed in 1% buffered OsO₄. The samples were dehydrated in acetone and embedded in epoxy resin. After preliminary examination of semithin sections under light microscope, ultrathin sections were counterstained with uranyl acetate and lead citrate and examined under a Jeol-JEM 100 Cx II electron microscope.

RESULTS

Rabies viruses infect mouse spleen lymphocytes in vitro. We compared the abilities of strains ERA and CVS to infect mouse lymphocytes in vitro in cultures of BALB/c mouse spleen lymphocytes either activated by a 24-h preexposure to ConA or nonactivated. Virus infections were studied under these two cell culture conditions because in vivo, most lymphocytes are in a resting state and frequently fail to support productive virus infection (9). After 3 or 7 days, both infected and mock-infected spleen lymphocyte cells grown under the two culture conditions were permeabilized. The cells were stained with FITC-conjugated Ab specific to the rabies virus NC, which accumulates in the cytoplasm of infected cells. The percentage of NC-positive cells was determined by cytofluorimetry analysis. Three days after exposure to the virus, 37% of activated lymphocytes were infected by ERA but only 14% were infected by CVS, as shown in Fig. 1A. During the course of infection, the number of CVS-infected cells remained constant, whereas 7 days postinfection, the percentage of live cells infected by the ERA virus fell by a factor of 5 (from 37 to 6.7%). The kinetics of infection were different in the absence of mitogen exposure; infection progressed more slowly than in activated cultures. By day 3, only 3.6% of lymphocytes were infected by ERA and 6.9% were infected by CVS. In contrast to the case for activated cultures, the percentages of NC-positive spleen lymphocytes infected by ERA and CVS were not significantly different, and a decrease in the number of infected cells during the time course of ERA virus infection was not recorded. In fact, the number of ERA-infected cells increased by a factor of 5 by day 7 (from 3.6 to 21.4%).

The type of cells infected was determined by double staining with anti-CD4 and anti-CD8 MAb. Two spleen cell populations were identified: T lymphocytes expressing either CD4 or CD8 molecules and non-T splenocytes (B lymphocytes and natural killer cells) which did not express either CD4 or CD8 molecules. The proportions of T and non-T cells in the infected-cell population are indicated in Fig. 1A, and the numbers of T cells and non-T cells infected are presented in Fig. 1B. T cells are the preferred target for rabies virus infection under mitogen exposure conditions. In ERA virus infection, 29% of infected cells at day 3 are T cells, and 41% of the T cells are infected. Half of the infected activated cells (56%) are CD4⁺ cells, and a quarter (21%) are CD8⁺ cells. By contrast, under nonactivated conditions, non-T cells are the preferred targets (at day 7, 21.5% of the total cells were infected and 14.5% of these were non-T cells).

The day 7 supernatants of infected and mock-infected, activated and nonactivated spleen cultures were assayed for viral production by the plaquing technique to determine whether NC-positive lymphocytes produce infectious rabies virus particles. The viral titer in the activated culture was higher than that in the nonactivated culture by a factor of 2 log units (4.9 versus 2.8 log PFU/ml) for ERA and by a factor of 1 log unit (3.9 versus 2.7 log PFU/ml) for CVS. This indicates that infected activated lymphocytes produce the virus and rules out the possibility that NC-positive cells engulf virus antigens. In

FIG. 1. Mouse spleen lymphocyte cultures are permissive to ERA rabies virus and less so to CVS. BALB/c mouse spleen cells were cultivated for 24 h with ConA (activated) or without ConA (nonactivated) and then infected with ERA or CVS at an MOI of 1. The number of NC-positive cells in 3-day-activated spleen cultures infected with ERA or CVS or in mock-infected cultures was determined by cytofluorimetry. (A) Among the infected cells, the proportion of T lymphocytes (dark bars) and non-T cells (gray bars) was determined by double staining with anti-NC Ab as an infection marker and anti-CD4, anti-CD8 MAb as a T lymphocyte marker. (B) Percent infection of spleen T lymphocytes (dark bars) compared to that of non-T cells (gray bars). With cellular activation, spleen cultures consisted of 89% T lymphocytes, whereas under nonactivated conditions, 43% of the total live cells were T lymphocytes.

activated cultures, ERA produces 10 times (1 log unit) more virus progeny than CVS (4.9 versus 3.9 log PFU/ml), suggesting that spleen lymphocytes are more permissive to ERA than to CVS.

Our data demonstrated that the ERA rabies virus, and to a much lesser extent CVS, infected mouse CD4⁺ and CD8⁺ T lymphocytes. ConA-activated lymphocytes were preferentially infected, and ERA virus infection of these activated lymphocytes yielded infectious virus progeny.

Rabies virus induces mouse spleen lymphocyte cell death. The reduction in the number of ERA rabies virus-infected cells, by 30%, in activated culture by day 7 (Fig. 1A) suggested that a number of activated T cells died during the course of ERA virus infection. This hypothesis was tested by comparing the viabilities of cells in mock-infected and ERA rabies virus-infected splenocytes, at days 3 and 7, by cytofluorimetry. Cell viability was determined from typical light scatter alterations in dying cells. Cell morphology was analyzed by measuring the FSC and SSC (Fig. 2). Under these conditions, two cell populations were detected: a low-FSC, high-SSC population (R1) containing dying cells and a high-FSC, low-SSC population (R2) of live cells. In the absence of infection, spleen lympho-

cyte cultures have a natural background level of cell death represented by a characteristic R1 value that increases with time (from 34% at day 3 to 45.3% by day 7). In ERA rabies virus-infected cultures, the R1 value was already higher at day 3 (51%) than that in the noninfected cultures, and by day 7 it was 64.4%. The relative increase in cell death due to infection, calculated as described in Materials and Methods, was 25% at day 3 and 34.4% at day 7.

The light scatter analysis strongly supports the hypothesis that ERA rabies virus infection causes an increase in cell mortality. Further analysis of cell death mediated by rabies virus in spleen culture is limited, since this type of culture is short lived and heterogeneous. We therefore used the well-characterized human T- and B-lymphocyte cell lines Jurkat, CCR/CEM, Daudi, and Raji as a model system to study rabies virus-induced cell death and the possibility that the rabies virus induces apoptosis.

Rabies viruses infect human T-cell lines and not B lymphocytes. We tested the susceptibilities of the Jurkat and CCR/CEM T-cell lines and the Raji and Daudi Burkitt lymphoma B-cell lines to rabies virus. Infection of the four cell lines with ERA and CVS was monitored by determining the percentage of NC-positive cells. Jurkat T cells were susceptible to ERA virus infection, and the culture was totally infected 2 days after initial contact with the virus, as shown in Fig. 3A. By contrast, the CCR/CEM cell line was refractory to rabies virus infection, with only 3% of cells infected at day 3. The B-lymphoma cell lines were totally resistant to infection, with less than 0.2% of cells positively stained 3 days after the initial virus contact (Fig. 3A). The resistance of the CCR/CEM and B-lymphoma cell lines to ERA virus infection did not depend on the MOI. MOIs of 0.1, 5, and 10 yielded similar results (data not shown). The Jurkat T-cell line was also the only cell line susceptible to CVS; however, CVS infection was slower and less spread than ERA infection. At day 2, 35% of the cells were infected with CVS compared to 100% with ERA (Fig. 3A). Susceptibility of the four cell lines to CVS infection was also tested at an MOI of 0.1, since CVS infection of fibroblasts is more successful with an MOI of 0.1 than with an MOI of 1. In this case however, the percentage of cells infected did not increase with an MOI of 0.1, as shown in Fig. 3B, suggesting that the CVS cycle differs in fibroblasts and lymphocytes. At an MOI of 0.1, Jurkat T-cells infected with either CVS or ERA were productive (Fig. 3B), and infectious virus particles were titrated in the culture supernatants 3, 5, and 7 days postinfection. Cells infected with CVS were less productive. Five days postinfection, titration of the supernatants of the ERA-infected cultures yielded 6.4 log PFU/ml, compared to 5.2 log PFU/ml for the CVS-infected cultures.

Our data showed that Jurkat T cells were highly permissive for ERA virus replication but that CVS replicated less efficiently. Rabies virus infection of Jurkat T cells produced infection patterns similar to those reported for splenocytes. Therefore, we have used Jurkat T cells as a model system to study rabies virus-induced lymphocyte cell death.

ERA rabies virus-infected Jurkat T cells undergo apoptosis. Morphological changes in ERA virus-infected Jurkat T cells were monitored by electron microscopy and flow cytometry. Morphological changes typical of apoptosis, including chromatin compaction and margination along the inner nuclear membrane, intense vacuolization, membrane blebbing, and formation of apoptotic bodies, were observed in a 48-h culture, as shown in Fig. 4A. Cytometric analysis indicated that the low-FSC, high-SSC population (R1 value) at day 2 was similar to the R1 value of rabies virus-infected mouse splenocytes shown in Fig. 2 (data for Jurkat T cells not given). In addition, frag-

FIG. 2. ERA rabies virus infection induces cell death in activated spleen lymphocyte cultures. Morphological changes related to cell death were monitored by cytofluorimetry in 3- and 7-day-activated spleen lymphocyte cultures, either noninfected or ERA virus infected. FSC and SSC analyses indicated that spleen lymphocyte cultures were composed of distinct R1 and R2 populations. R1 consisted of cells with high SSC scores and low FSC scores, whereas the R2 population consisted of cells with low SSC values and high FSC values. The R1 population represents cells engaged in the death process, whereas R2 represents live cells. The R1 value for ERA virus-infected spleen cell cultures was always larger than the R1 value for noninfected cultures, indicating that rabies virus infection induces cell death.

mentation of cellular DNA into oligonucleosome-sized fragments in infected Jurkat cells was detected by electrophoresis (Fig. 4B) and the TUNEL technique (Fig. 5). Internucleosomal DNA cleavage into fragments of between 180 and 200 bp in rabies virus-infected Jurkat T cells at 3 days postinfection was revealed by a characteristic DNA ladder (Fig. 4B). DNA fragmentation was confirmed by the TUNEL technique 18 h after the initial virus contact (Fig. 5). DNA fragmentation was linked to the rabies virus infection. It was not observed either in noninfected Jurkat T cells (Fig. 4B, lane 5) or in rabies virus-incubated CRR/CEM cells (Fig. 4B, lane 6). Moreover, the percentage of infected cells is proportional to the number of apoptotic cells detected by TUNEL, as shown in Fig. 5. For example, CVS infected fewer cells (Fig. 5B and C) and induced fewer apoptotic nuclei (Fig. 5E and F) than ERA virus.

A double staining analysis was performed by confocal microscopy to determine whether infection of T cells by rabies virus causes apoptosis or whether apoptosis also occurs in noninfected cells as an indirect effect of infection. Cells from mock-infected and ERA virus-infected cells were stained 2 and 3 days postinfection, first by the TUNEL technique and then with an anti-rabies virus G protein mouse MAbs and an FITC-conjugated antimouse Ab. As shown in Fig. 6, apoptotic cells (nuclei in red) indeed expressed viral glycoprotein (cytoplasm in green). The double-stained cells (apoptotic infected cells) represented 24% of the cells at day 3, by which time the majority (80%) of apoptotic cells were infected. However, most infected cells were not yet apoptotic, since only 31% of the infected cells were undergoing apoptosis at day 2. Apoptotic infected cells broke apart into apoptotic bodies containing both nucleus fragments and viral antigens (Fig. 6, left panel).

Our data indicated that ERA rabies virus infection of cultured lymphocytes induced apoptosis in the infected cells,

strongly suggesting that apoptosis is connected with the rabies virus infectious cycle.

Apoptosis requires replicative virus. We analyzed the effect of incubation of Jurkat cells with UV-inactivated ERA rabies virus to test whether lymphocyte apoptosis was initiated early in the virus life cycle by membrane contact, for example, or later in the life cycle. UV treatment is known to abrogate transcription and replication of the rabies virus. The percentages of cells undergoing apoptosis, as determined by the TUNEL method, in Jurkat T cells treated with UV-inactivated virus, in untreated cells (background negative control), and in Jurkat T cells cultivated in medium without serum (apoptotic positive control) were compared. After a 2-day incubation, no significant variation in the number of TUNEL-positive untreated cells (16%) and UV-inactivated virus-treated cells (15%) was observed, whereas serum deprivation induced apoptosis in 29% of cells, as shown in Table 1. This suggests that the contact between ERA virus particles and Jurkat cells is not sufficient to induce apoptosis but that apoptosis is initiated by subsequent steps in the virus life cycle.

Apoptosis correlates with expression of rabies virus G protein. The results presented above suggest that induction of apoptosis requires virus multiplication. We compared the kinetics of virus-mediated apoptosis with those of viral G and N protein accumulation to determine which events in the viral life cycle initiate apoptosis. We compared the percentage of TUNEL-positive cells with the percentage of cells expressing G or N protein in an infection time course with ERA virus-, CVS-, and mock-infected Jurkat T-cell cultures from 12 h to 6 days postinfection. In ERA virus-infected cultures the number of TUNEL-positive cells differed significantly from the background level of TUNEL-positive cells in mock-infected cultures from 18 h postinfection, as shown in Fig. 7 (top). The

FIG. 3. Jurkat T cells, but not CCR/CEM T cells or B-lymphoma cells, are permissive to rabies virus infection. (A) Kinetics of ERA and CVS infection (MOI = 1) expressed as percentages of rabies virus NC-positive cells in Jurkat, CCR/CEM, and Raji cells. Only Jurkat T cells were infected with ERA (hatched bars) and CVS (gray bars). CCR/CEM (white bars) and Raji (black bars) cells were not infected by either ERA or CVS (data shown only for ERA). (B) Kinetics of ERA (gray bars) and CVS (black bars) production and percentage of NC-positive cells in Jurkat T cells infected at an MOI of 0.1 with either ERA or CVS.

percentage of cells expressing G also started to increase at 18 h postinfection (19%), reaching 70% by day 6, whereas most of the cells (92%) were NC positive at 12 h postinfection. This indicated that expression of G and apoptosis were concomitant events and suggested that G may be the key factor in induction of apoptosis. The results for CVS-infected cultures (Fig. 7, bottom) strongly suggested that NC accumulation and apoptosis induction occur independently, since 90% of the cells contained NC by day 6 (Fig. 7, bottom) and this remained unchanged by day 10 (data not shown), but this did not modify the viability of the culture. Apoptosis did not hinder virus production, as indicated by a comparison between Fig. 3 and 6. An average of 40% of the cells were apoptotic between days 3 and 6 of infection, and the average ERA rabies virus production was 6 log PFU/ml. This suggested that rabies virus-mediated apoptosis did not occur early enough in the infectious cycle to limit virus replication.

These results suggest that initiation of apoptosis is related to expression of the G protein and that apoptosis occurs too late in the infectious cycle to hinder virus replication and production of infectious particles by the infected lymphocytes.

DISCUSSION

In this study, we showed for the first time that the rabies virus infects both mouse spleen lymphocytes and the human T-lymphocyte cell line Jurkat *in vitro*. The attenuated rabies virus strain ERA infects ConA-activated splenocytes and T-cell lines more efficiently than CVS, a highly neurovirulent rabies virus strain. These data suggest that in addition to myocytes and macrophages (5, 32), lymphocytes, activated at the site of inoculation, may be an important target in the periphery, especially for the attenuated rabies virus strain.

The nature of the rabies virus target cell was investigated in this work. The observations that not all spleen lymphocytes or cultured lymphocyte cell lines are susceptible to rabies virus infection and that pretreatment with ConA increases the susceptibility of CD4⁺ CD8⁺ lymphocytes to infection suggest that rabies virus infection of lymphocytes requires either a specific cell phenotype or a certain cellular activation state. The different susceptibilities of lymphocyte cell lines to rabies virus infection may reflect the existence of a rabies virus-specific lymphocyte receptor expressed only by Jurkat T cells. Resistance of the Daudi and Raji cell lines to infection may be due to the absence of the rabies virus receptor on the surfaces of these cells. An alternative hypothesis is that these cells are refractory to secondary infection as a result of Epstein-Barr virus immortalization, as documented for vesicular stomatitis virus (6). The existence of a lymphocyte-specific receptor specific for the rabies virus is strongly supported, however, by the fact that the rabies virus binds to Jurkat T cells but is unable to bind to CEM cells (18a). The identity of the lymphocyte receptor has not yet been determined. Activated lymphocytes are highly permissive to rabies virus infection. This may be due to an improved capacity of the cellular machinery of an activated T cell, compared to a nonactivated T cell, to support rabies virus infection, as reported for other viruses such as vesicular stomatitis virus and measles virus (24, 37). The state of activation may also regulate receptor expression, particularly in lymphocytes, where the expression of clusters of adhesion and accessory molecules, such as ICAM-1, VCAM-1, and CD28, is determined by the cellular activation state.

Rabies virus was found to trigger apoptosis in the Jurkat T cells that it infects. Rabies virus-induced apoptosis in lymphocytes causes well-characterized morphological alterations, such as cellular shrinkage and margination of chromatin, observed by both cytofluorimetry and electron microscopy. Intracellular DNA fragmentation was also detected by DNA electrophoresis at 48 h postinfection, or as early as 18 h postinfection with the more sensitive TUNEL technique. The initiation of apoptosis is controlled by different factors. Most apoptosis initiators can be identified at the primary site of perturbation within the cell (48). For viral infection, two factors have been postulated: apoptosis either results from the direct binding of viral protein to cell membrane receptors (2) or requires *de novo* synthesis of viral RNA or proteins (23, 42, 43). The involvement of a cell surface apoptotic signal is unlikely in rabies virus-induced apoptosis, since binding of UV-inactivated rabies virus particles to the lymphocyte membrane did not induce apoptosis. This result, together with the observation that DNA fragmentation is not detected until 18 h postinfection, suggests that rabies virus-induced apoptosis depends on later events in the virus life cycle. We showed that apoptosis is linked to the capacity of the virus to replicate. ERA rabies virus, which is the most efficient inducer of apoptosis, produced more viral protein inclusions in the cytoplasm than CVS, a poor inducer of apoptosis. Moreover, rabies virus-induced apoptosis occurred only after viral G and N proteins had ac-

FIG. 4. ERA rabies virus induces apoptosis in Jurkat T cells. (A) Electron micrograph of 48-h ERA virus-infected Jurkat T cells. A, normal live lymphocyte; B, apoptotic lymphocyte with enlarged nucleus showing chromatin margination; C, vacuolized cell releasing apoptotic bodies. Bar, 2 μ m. (B) Agarose gel electrophoresis showing DNA ladder typical of apoptosis. Lanes: S, molecular size references; 1 to 4, DNA preparations from 1- to 4-day ERA virus-infected Jurkat T cells, respectively; 5, DNA extracted from day 4 mock-infected Jurkat T cells; 6, DNA extracted from day 4 ERA virus-infected CCR/CEM T cells.

FIG. 5. Infection and DNA fragmentation in Jurkat T-cell culture. Mock-infected (A), ERA virus-infected (B), and CVS-infected (C) Jurkat T cells were permeabilized after 18 h of culture and stained with FITC-labeled anti-rabies virus NC Ab. Large cytoplasmic inclusions of NC were present in ERA-infected cells, whereas only small inclusions were visible in CVS-infected cells. Apoptosis in 21-h mock-infected (D), ERA rabies virus-infected (E), and CVS-infected (F) Jurkat T cells was analyzed by the TUNEL method. The frequency of apoptotic fragmented nuclei was significantly higher in Jurkat T cells infected with ERA virus than in CVS-infected or mock-infected Jurkat T cells. Magnification, $\times 350$.

cumulated in the cytoplasm. These data strongly suggest that accumulation of viral proteins plays a key role in rabies virus-induced apoptosis, as reported for two other RNA viruses, Sindbis and dengue virus, where apoptosis seems to be regulated by the accumulation of viral proteins in vesicles of the endoplasmic reticulum and Golgi apparatus (7, 23).

The induction of apoptosis may be controlled by a single viral protein. The expression of the chicken anemia virus protein VP3, for example, is sufficient to trigger the apoptotic pathway in T-lymphoblastoid chicken cells (29). Our results on the correlation between expression of ERA virus G protein and apoptosis suggest that the rabies virus G protein is crucial in rabies virus-mediated apoptosis. This is further supported by

the work of Ni et al. on the origin of the rabies virus cytopathic effect in fibroblasts. These authors found that accumulation of G in the endoplasmic compartments regulated cell death (28). The differential capacities of virus strains in inducing apoptosis may be due to major differences in the amino acid sequences and maturation pathways of G in these two viruses (45, 49).

Apoptosis-mediated cellular alterations are regulated by multiple signaling pathways, involving protease and protein kinase activation, and proto-oncogene regulation (21) (see reference 12 for a complete review). In attempts to identify the events located downstream from viral protein accumulation that control apoptosis, NF- κ B activation is often quoted (4, 31). It has been proposed that virally induced apoptosis results from a

FIG. 6. Confocal microscope images of ERA rabies virus-infected Jurkat T cells. Cells were analyzed 3 days after infection with an MOI of 1. (Left panel) Double-labeling assay for rabies virus G protein (green) and TUNEL (red). Apoptotic bodies containing viral G can be observed (white arrows). (Middle panel) Detection of G with a G-specific mouse MAb and FITC-conjugated antimouse Ab. (Right panel) Detection of DNA fragmentation by the TUNEL technique, terminated with Texas red-conjugated streptavidin. Bar, 5 μ m.

TABLE 1. Apoptosis mediated by UV-inactivated virus

T cells	Mean no. of TUNEL-positive cells/500 screened cells $\pm$ SD (% of cells undergoing apoptosis)			
	Control	Serum-deprived cells	Cells treated with UV-irradiated ERA virus at:	
			1 μ g	5 μ g
Jurkat	80 $\pm$ 31 (16)	145 $\pm$ 42 (29)	77 $\pm$ 5 (15)	67 $\pm$ 17 (13.4)
CCR/CEM	60 $\pm$ 20 (12)	172 $\pm$ 84 (34)	47 $\pm$ 12 (9)	55 $\pm$ 9 (11)

cascade of events, starting with the accumulation of viral proteins in the endoplasmic reticulum or Golgi complex, which induces an oxidative stress that activates NF- κ B and in turn triggers apoptosis (11, 23). The possibility that accumulation of rabies virus G protein in intracytoplasmic structures causes oxidative stress, inducing apoptosis by acting on the NF- κ B signaling pathway, deserves further study.

The ERA virus and other attenuated rabies strains (SAD and SAD mutants) trigger an efficient immune response that contributes to the efficiency of live vaccines (1, 18, 38, 41). The facts that the ERA virus infects a peripheral target and that apoptosis occurs too late to limit viral production are consistent with the notion that the advantage of live vaccines is their ability to amplify the initial inoculum. However, it is paradoxical that ERA induces apoptosis in the lymphocytes it infects but also triggers a strong immune response. This may be explained by the fact that ERA infects only a fraction of splenocytes in vitro, and this may not be sufficient to impair the capacity of lymphocytes to mount an efficient immune response. Nevertheless, lymphocyte apoptosis may modulate the immune response in some way. The early stages of apoptosis are associated with alterations in cell membrane symmetry exposing phosphatidyl serine molecules. These molecules have been reported to have chemoattractant properties for macrophages (47). Thus, it is possible that apoptotic cells may initiate an immune cascade mechanism, resulting from local activation of macrophages. In addition, cell fragmentation may release viral antigens, including NC, that accumulate in the cytoplasm. Recognition of NC, which has been shown to be a strong adjuvant, may also boost the immune response (3, 13). Moreover, we observed that apoptotic bodies contain viral antigens, as already described for Sindbis virus infection (34). The fact that apoptotic bodies contain huge amounts of antigen suggests that they have a role in the immune response in viral antigen presentation. We speculate that induction of apoptosis by the ERA rabies virus strain may augment the presentation of viral antigen, efficiently activating the immune system. Thus, in this hypothesis, lymphocyte apoptosis may be an important mechanism in antigenic presentation and recognition. By contrast with the attenuated rabies virus strain, the highly neurovirulent CVS multiplies less efficiently in lymphocytes and is a poor inducer of apoptosis. CVS triggers almost no host reaction, due to the absence of apoptosis induction. As a result, CVS may easily invade the nervous system, with only a minor host reaction.

Infection of lymphocytes and induction of apoptosis that does not limit viral production are key factors in the efficacy of live rabies vaccines. The construction of a live attenuated vaccine could take advantage of these properties: (i) apoptosis does not limit virus production, and therefore amplification of the antigenic mass is not restricted, and (ii) apoptosis enhances the immune response, including macrophage activation, the cytokine cascade, and improvement of antigenic presentation

FIG. 7. Correlation between apoptosis and rabies virus G protein expression in ERA virus-infected (top) and CVS-infected (bottom) Jurkat T cells. The kinetics of TUNEL-positive cells in virus-infected (dark bars) and mock-infected (gray bars) Jurkat T cells from 12 h to 6 days postinfection are shown. The percentage of cells undergoing apoptosis is given. The percentages of cells expressing rabies virus NC (□) and G (◆) measured in permeabilized cells with FITC-labeled anti-NC Ab and with anti-rabies virus G MAb are also shown. MAb PVE was used to detect ERA virus G protein, and MAb 8-2 was used to detect CVS G. By contrast to the results presented in Fig. 6, where unfixed cells were stained with anti-G MAb, cells in this experiment were permeabilized. Therefore, anti-G MAb stained both surface G and G molecules in the cytoplasm. From 18 h to 6 days postinfection, the number of TUNEL-positive cells among ERA virus-infected Jurkat T cells differed significantly ($P < 0.005$) from that in noninfected cultures.

by fragmentation of infected T lymphocytes. Targeting of a specific subset of lymphocytes subjected to further apoptosis could be a promising approach to improve live-vaccine immunogenicity.

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