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NOTES

Feline Immunodeficiency Virus Decreases Cell-Cell Communication and Mitochondrial Membrane Potential

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The in vitro effects of viral replication on mitochondrial membrane potential (MMP) and gap junctional intercellular communication (GJIC) were evaluated as two parameters of potential cellular injury. Two distinct cell types were infected with the Petaluma strain of feline immunodeficiency virus (FIV). Primary astroglia supported acute FIV infection, resulting in syncytia within 3 days of infection, whereas immortalized Crandell feline kidney (CRFK) cells of epithelial origin supported persistent FIV infection in the absence of an obvious cytopathic effect. An examination of cells under conditions that included an infection rate of more than 90% for either population revealed that the astroglia produced about four times more virus than the CRFK cells. The mitochondrial uptake of the cationic fluorescent dye rhodamine 123 in infected astroglia was less than 45% of that of normal control cells, whereas the MMP of the CRFK cells, which produced about one-fourth as much virus, was 80.8% of that of the normal cells. Cell-cell communication between adjacent cells was determined by the recovery of fluorescence following photobleaching of a single cell. In spite of the lower level of innate cell-cell communication among cultured CRFK cells than among astroglia, viral replication resulted in a 30% decrease in the GJIC of both astroglia and CRFK cells. These studies indicate that cell injury, as defined by an inhibition of MMP and GJIC, can occur as a result of persistent and acute infection with the Petaluma strain of FIV.

Feline immunodeficiency virus (FIV) is a common pathogen of domestic cats throughout the world (24, 25, 37). Clinical signs of FIV infection, such as wasting, diarrhea, lymphoid atrophy, and opportunistic infections, resemble those attributable to human immunodeficiency virus (HIV) infection in humans (24, 37, 38, 42, 46). FIV, however, apparently has a broader range of tropism than HIV, with certain strains readily infecting cells of epithelial origin (such as Crandell feline kidney [CRFK] cells) as well as $CD8^+$ and $CD4^+$ T lymphocytes, macrophages, and astroglia (2, 3, 15, 37). FIV and HIV-1 have also been associated with similar neurologic signs and central nervous system lesions (6, 11, 13–15, 43). FIV has been isolated from both cerebrospinal fluid and brain tissues of naturally and experimentally infected cats (45, 46).

Like HIV, FIV replicates in vitro in astroglia and microglia of the central nervous system but apparently not in oligodendrocytes or neurons (12, 13, 15). Neurologic disease could be the result of FIV- or HIV-induced alterations in astroglial or microglial function. Astroglia, the most abundant cells of the central nervous system, are macroglia that maintain ion concentrations, take up and metabolize neurotransmitters, regulate the differentiation and function of myelinated axons, and aid in the formation of tight junctions between endothelial cells in the blood-brain barrier (21, 27–29, 41). They are also a source for cytokines that are involved in cellular signaling, inflammatory responses, and antigen presentation (19, 20, 22, 34).

The cellular mechanisms for lentivirus-induced lesions are not well studied, although these viruses often induce cell-cell fusion, resulting in multinucleated cells and limited viability in susceptible target cells. In addition, there is evidence suggesting that HIV infection alters membrane integrity and that membrane-HIV interactions play a critical role in the pathogenesis of AIDS (7, 17, 23, 33). Membrane interactions of the host cell with HIV or viral proteins are postulated to cause permeability changes in the membrane, interference with the normal homeostasis of the cell, alteration of ion fluxes, elevation of intracellular Ca^{2+} concentrations, and an increase in membrane unsaturated oleic acid (7, 8, 17, 23, 30, 33, 36).

Virally induced effects of gap junctional intercellular communication (GJIC) and the dissipation of mitochondrial membrane potential (MMP) are two critical end points of cellular injury which are very sensitive to changes in intracellular Ca^{2+} levels. MMP is essential in the generation of energy for cellular homeostasis. Whereas the outer mitochondrial membrane is permeable to most ions, the inner membrane houses the electron transport chain-driven proton pump. The resulting electrochemical gradient facilitates a continuous cycling of H^+, allowing the transport of certain ions, such as Ca^{2+} and K^+, and high-energy phosphates between the cytosol and the mitochondria (31).

Direct intercellular communication occurs through gap junctions within the cytoplasmic membrane. These junctions are aggregates of cell-cell channels which join adjacent cells and mediate bidirectional diffusion of inorganic ions and small molecules (<1 kDa) between cells but do not mediate the passage of macromolecules (32). Individual channels between adjacent cells are formed by the alignment of two hexameric

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aggregates of proteins called connexons which form a hydrophilic pore about 1 nm wide. GJIC is involved in the maintenance of cellular homeostasis, growth control, cell repair, and neoplastic transformation (18).

In order to evaluate potential molecular targets of FIV-induced cellular injury, fluorescent probes for specific cellular functions were used to examine the in situ effects of viral replication on MMP and GJIC. The Petaluma strain of FIV acutely infects primary astroglia, producing obvious syncytia and cell death, whereas this strain chronically infects the immortalized CRFK cells in the absence of a cytopathic effect (9, 10, 14, 15, 44).

FIV infection of CRFK cells and astroglia. MMP and GJIC in astroglia acutely infected and CRFK cells persistently infected with the Petaluma strain of FIV were measured. Cultured CRFK cells maintained in Dulbecco's modified Eagle's medium with 10% calf serum, 1× minimal essential medium (Gibco, Gaithersburg, Md.), and 1 mM sodium pyruvate with penicillin and streptomycin (Sigma, St. Louis, Mo.) had been persistently infected for more than 6 months with the Petaluma strain of FIV (NIH AIDS Repository). Astroglia from fetal domestic cats at 50 to 60 days of gestation were prepared as described by Zenger et al. (47) and maintained in Dulbecco's modified Eagle's medium F12 (1:1, vol/vol) with 15% fetal bovine serum. A glial fibrillary acidic protein assay with rabbit anti-bovine glial fibrillary acidic protein antibody (Dako, Carpinteria, Calif.) was used to confirm the astroglial nature of the cultured central nervous system cells (47). Astroglia were inoculated by incubation for 1 h at 37°C with viral stock from supernatant fluid of Petaluma strain-infected CRFK cells having a reverse transcriptase activity of 7.8×10^6 cpm/ml. The astroglia in a 75-cm^3 flask were infected at approximately 50% confluency with 1 ml of the stock virus or with medium. After incubating at 37°C for 1 h, the cells were given fresh medium and observed daily for the presence of syncytia. Uninfected cells were added two or three times per week to maintain viability. Infected astroglia were analyzed at 15 to 20 days postinfection. Both cell types were subcultured at a density of 2×10^5 viable cells in 35-mm-diameter plastic tissue culture dishes 48 h prior to evaluation of MMP or GJIC.

An ACAS 570 interactive laser cytometer (Meridian Instruments, Okemos, Mich.) permitted evaluations of these parameters in individual cells as well as in populations of cells. Therefore, conditions were selected so that a high percentage of cells in the sample population were supporting viral replication. Unlike infected CRFK cells and normal control cells, astroglia infected with the Petaluma strain of FIV began forming syncytia 3 days after subculturing with added viable, uninfected cells. The cultured CRFK cells and astroglia were examined 48 h after subculturing in order to avoid measuring changes that might reflect membrane fusion in the latter cells. At this time, evaluation of the infected cells by indirect immunofluorescence assay indicated that 95% of the CRFK cells and 91% of the astrocytes were infected with FIV (40) and microscopic examination indicated that the cells were well attached and were not showing signs of a cytopathic effect. Although similar percentages of astroglia and CRFK cells were infected, the amount of virus in the supernatants as measured by the reverse transcriptase assay (2) was more than four times greater in the infected astroglia (15.1×10^5 cpm/10^6 cells) than in the CRFK cells (3.49×10^5 cpm/10^6 cells). The intensity of FIV-specific immunofluorescence was also consistently greater in the infected astroglia (data not shown). Therefore, the amount of virus produced by the astroglia was considerably greater than the amount produced by the persistently infected CRFK cells. For each experiment, the cell cultures were divided into one control and at least two treated groups, with three to six culture dishes per group. Means were compared by Duncan's multiple range test for variables and the General Linear Models procedure. The P values of <0.05 were considered significant.

Effects of viral replication on MMP. The mitochondrial incorporation of the cationic fluorescent dye rhodamine 123 is dependent on the presence of an intact electrochemical gradient across the mitochondrial inner membrane. Therefore, a decrease in fluorescence values indicates a dissipation of MMP (4, 16). The cationic fluorescent dye rhodamine 123, prepared as a 2-mg/ml stock in ethanol, was used as a probe for examining the dissipation of MMP (4, 16). After overnight incubation at 37°C, the infected and uninfected control cultured cells were washed four times with Dulbecco's phosphate-buffered saline (PBS) and incubated for 30 min at 37°C with rhodamine 123 diluted from the stock solution to 5 μg/ml in serum-free and phenol red-free medium. The cells were subsequently washed four times in serum-free medium without phenol red, and the fluorescence intensity was immediately evaluated with the ACAS 570 laser cytometer at an excitation wavelength of 488 nm. Eight areas per dish in three dishes per experimental group were examined in experiments from two different days. The mean fluorescence intensity was determined for 50 to 60 cells per treatment group to provide a relative measure of MMP based on the Nernstian distribution of the dye (16).

A significant difference ($P < 0.05$) in the incorporation of rhodamine 123 in the infected CRFK cells and astroglia compared with the uninfected cells was observed (Fig. 1). The mean fluorescence intensity in the infected astroglia was 44.9% of that of the uninfected controls, and the intensity in the infected CRFK cells was 80.8% of that of the uninfected controls (Fig. 2). While the MMPs of normal astroglia and CRFK cells were comparable, the incorporation of rhodamine 123 in infected astroglia was consistently less than that in the infected CRFK cells. In addition, although equivalent numbers of cells were infected, the amount of virus produced as measured by reverse transcriptase activity was about four times greater in the acutely infected astroglia. MMP may be quantitatively affected by the amount of replication because infection of astroglia which produced more virus than the CRFK cells resulted in nearly twice the decrease in MMP compared with that in corresponding uninfected control cells. Alternatively, the greater impact of infection on the astroglia could be the result of fundamental differences in the two cell types. The inhibition of MMP may not, however, be a requirement for a productive FIV infection because under conditions in which the MMP in infected cells could be maintained at a normal level (with camptothecin treatment), the amount of virus produced was equivalent to that in infected CRFK cells with the dissipation of MMP (unpublished data).

Effects of viral replication on GJIC. Fluorescence recovery in photobleached cells is dependent on the ability of non-membrane-permeating carboxyfluorescein to flow to bleached cells from the neighboring cells through gap junctions. GJIC was evaluated with the ACAS 570 by an assay of fluorescence recovery after photobleaching (1, 44). The cells were washed four times with PBS and stained for 15 min at 37°C with 5-carboxyfluorescein diacetate diluted to 10 μg/ml (from a 2-mg/ml stock solution in dimethyl sulfoxide in serumless Dulbecco's modified Eagle's medium without phenol red. The 5-carboxyfluorescein diacetate is taken up by cells and converted to carboxyfluorescein, which is able to diffuse through gap junctions but not through nonjunctional membrane. The cultures were rinsed four times and maintained in serumless,

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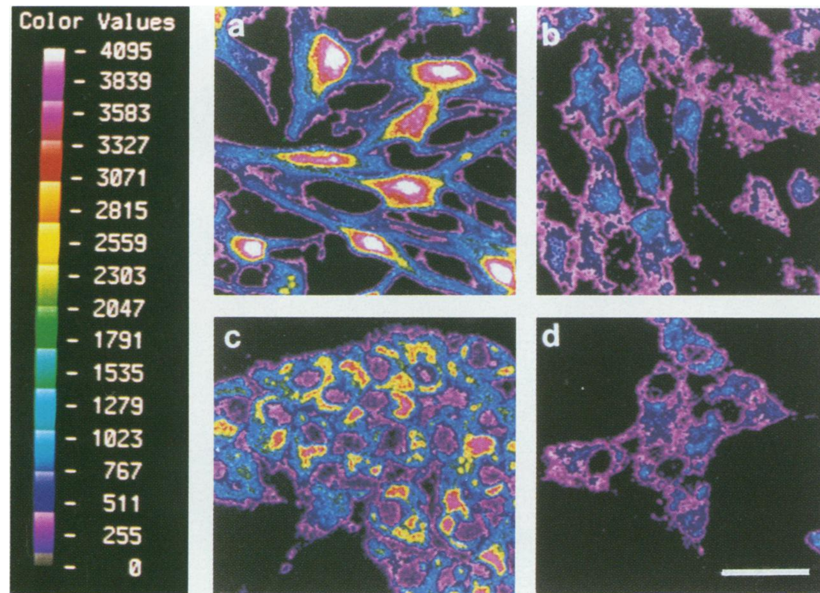

FIG. 1. Decreased rhodamine 123 uptake in FIV-infected astroglia and CRFK cells indicates a decrease in MMP. The relative fluorescence intensity scale is shown on the left. Fluorescence from rhodamine 123 is shown in a field of uninfected (a) and infected (b) astroglia and uninfected (c) and infected (d) CRFK cells. Bar, 50 μm.

phenol red-free medium before microscopic fields containing aggregates of cells were selected. Single cells, when present, served as photobleached negative controls. Single cells or small isolated groups of nonphotobleached cells were used to monitor potential background photobleaching resulting from image scans and/or the presence of uncleaved dye. Two to three abutting cells were selected from each field to monitor fluorescence transfer at an excitation wavelength of 488 nm. One or two cells from each field were photobleached while a single cell or small group of cells were left unbleached and demarcated as positive controls. Photobleaching was done by delivering a high-intensity laser beam, which reduces the carboxyfluorescein due photochemically in the selected cells and results in a reduction of fluorescence (20 to 40% of the original value, depending on the cell type). Sufficient bleaching of fluorescence to measure recovery without causing visible cell damage at the light-microscopic level was the criterion used to determine laser strength, number of bleaches per cell, and scan and bleach intensities. A series of five postbleach image scans were generated. One scan was taken immediately postbleach, and the subsequent four scans were taken at 1-min intervals to measure the redistribution of intracellular fluorescence through gap junctions. At least three analyses of cells from each of six dishes (15 to 20 cells) per treatment group were conducted in two separate experiments. Fluorescence levels were quantified by computer-assisted evaluation of data.

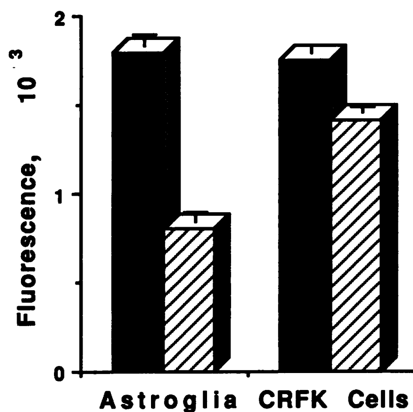

FIG. 2. The effects of FIV infection on the mean uptake of rhodamine 123. The fluorescence intensity means and standard errors for 68 uninfected astroglia and 232 uninfected CRFK cells are represented by the solid bars, and those for 57 infected astroglia and 222 infected CRFK cells are represented by the hatched bars.

Estimation of a rate constant (k) for fluorescence recovery was performed by fitting the percent fluorescence intensity at a given time, $F(t)$, to the following equation: $F(t) = F_{eq}(1 - e^{-kt}) + F(0)$, where F_{eq} represents the percent fluorescence recovery of the bleached cell at equilibrium, and e^{-kt} is the estimate of the rate constant at a given time, and $F(0)$ is the percent fluorescence intensity immediately following photobleaching (1). The value of F_{eq} depends on the number of contacting cells and the initial level of bleaching. Data from at least 30 cells from each treatment group were pooled to obtain the mean k values by curve fitting regression analysis, which permits extrapolation of fluorescence recovery versus time.

The GJIC was monitored in both uninfected and infected CRFK cells and astroglia. The cells were examined 24 h before any syncytia could be detected in the cultured astroglia. Figure 3a and b represents a field of uninfected and of infected astroglia, respectively, before and immediately after photobleaching and at 4 min after photobleaching, showing the recovery of fluorescence. The cell labelled 3 in each field was left unbleached as a positive control, and those labelled 1 and 2 were bleached. The GJIC was lower in the infected astroglia (cells 1 and 2 in Fig. 3b) than in the uninfected astroglia (cells 1 and 2 in Fig. 3a). Cell 2 of the infected population nearly ceased to communicate with adjacent cells.

At any time up to 4 min after photobleaching of a population of either cell type, the mean fluorescent recovery was less in the infected cells than in the corresponding uninfected cells

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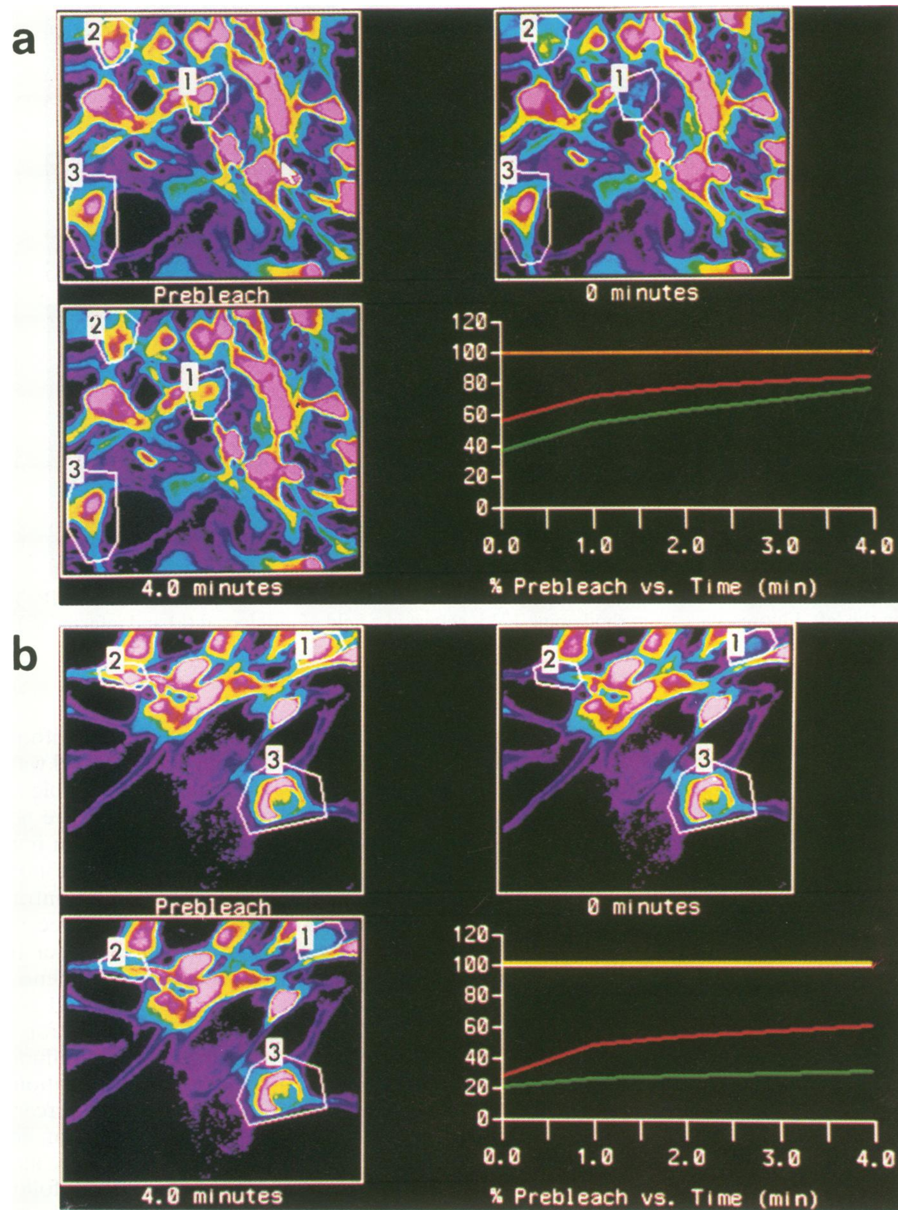

FIG. 3. Effects of FIV infection on GJIC in astroglia as measured by the recovery of fluorescence after photobleaching of individual uninfected (a) and infected (b) astroglia. A single field is shown before (prebleach), immediately after (0 min), and at 4 min following bleaching of cells labelled 1 and 2. Cell 3, which was not bleached, was used as a reference control. The graphs represent the recovery of fluorescence from each cell. Green line, cell 1; red line, cell 2; yellow line, cell 3.

(Fig. 4a). The mean communication rates values (Fig. 4b) indicated that the GJIC between infected cells was significantly less than that between the corresponding uninfected cells (P, <0.03 for CRFK cells and <0.02 for astroglia). The rates of recovery for the infected CRFK cells and infected astroglia were 71.5 and 70.1%, respectively, of the rates for the corresponding uninfected cells.

Although the magnitude of virally induced reduction in both cell types was similar, GJIC in infected and uninfected astroglia was greater than that in the CRFK cells examined. The mean percent recovery of astroglia ranged from about 37% at zero time to between 67 and 77% at 4 min after bleaching compared with less than 20% to between 35 and 45% in the CRFK cells. The difference in the communication rates of the two types of cells was statistically significant ($P < 0.0001$).

It has been suggested that the closing of gap junctions is a mechanism for isolating injured cells (32). Although the GJIC of infected cells within tissues is not known, a possible explanation for the GJIC decrease in infected cell culture could be that cells tend to seal themselves off as a response to infection, in general, rather than as a function of the presence of a virus or viral replication in the cell.

Digital imaging procedures that have proven valuable in examining mechanisms of mycotoxin and heavy metal cytotoxicity have been exploited to examine the effects of viral replication on critical cellular functions (5, 39). These sensitive

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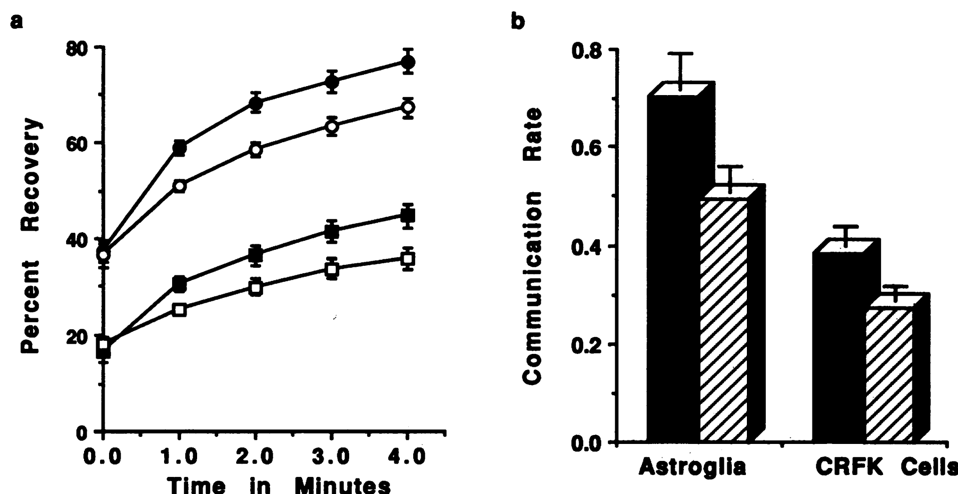

FIG. 4. Effects of FIV infection on the GJIC of a population of cells. (a) Mean percent fluorescence recoveries following photobleaching of 32 uninfected (closed circles) and 29 infected (open circles) astroglia and 26 uninfected (closed squares) and 27 infected (open squares) CRFK cells. (b) Mean communication rates (per minute) calculated from panel a, with standard errors. Uninfected cells are represented by the solid bars; infected cells are represented by the hatched bars.

molecular procedures utilize vital fluorescent probes to determine virally induced alterations in cellular homeostasis. Replication of the Petaluma strain of FIV was consistently associated with quantitative decreases in the MMP and GJIC in cultured cells. It appears that these alterations are general consequences of infection with this virus because decreases were observed in two cell types which differ in origin, function, and susceptibility to FIV replication. The astroglia were primary cell cultures that supported an acute infection, producing large multinucleated cells, and the CRFK were immortalized cultured cells supporting persistent FIV infection in the absence of syncytia or other signs of a cytopathic effect. Since an ultrastructural analysis of gap junctions was not done, it is not possible to determine whether reduced GJIC was due to disruption of gap junctions or to reduced permeability of channels due to other causes, such as cytoplasmic acidification or altered phosphorylation of channel proteins.

The contributions of these alterations to a cytopathic effect are not known. Membrane-associated changes could be related to cell-cell fusion and syncytium formation. Viral replication may directly alter GJIC and MMP or, alternatively, alter the homeostasis of interacting end points. Zenger et al. (47) have found other indicators of cell injury in astroglia; e.g., FIV infection of astroglia decreased cytoplasmic glutathione levels and altered Ca^{2+} homeostasis (47). Because glutathione plays a crucial role in protecting cells against free radicals and electrophiles and in maintaining membrane integrity (26, 35), its reduced activity could contribute to the observed injury in mitochondrial membranes. Differences in intracellular Ca^{2+} stores were detected following ionophore-induced Ca^{2+} fluxes. Decreases in MMP could, therefore be due to mitochondrial uptake of Ca^{2+} and/or changes in plasma membrane function. These differences in the sequestering of calcium could be associated with a calcium-induced loss of MMP (47).

These studies examined intracellular molecular alterations induced by the replication of the Petaluma strain of FIV. Similar studies with other strains of FIV will determine whether these cellular changes are a common property of FIV or a peculiarity of this strain. Such indicators of cell injury could provide the tools to characterize the mechanisms for acute and persistent infections and discriminate the vital functions that determine virus-induced injury and cell death.

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