Lentivirus-Induced Interferon Inhibits Maturation and Proliferation of Monocytes and Restricts the Replication of Caprine Arthritis-Encephalitis Virus

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In this study, we investigated the effect of a lentivirus-induced interferon (LV-IFN) on the interaction of caprine arthritis-encephalitis virus and its host cell, the monocyte-macrophage. LV-IFN was produced in culture supernatant 48 h after adding fresh goat lymphocytes to caprine arthritis-encephalitis virus-infected goat macrophages. The culture supernatant contained IFN activity at a titer of 1:360 as assayed by inhibition of vesicular stomatitis virus-induced lysis of fibroblasts. LV-IFN inhibited in vitro monocyte proliferation and maturation of monocytes to macrophages. Nevertheless, treated monocytes produced prostaglandin E2, a cytokine generally produced by activated macrophages. By inhibiting the maturation of monocytes to the more permissive macrophages, LV-IFN indirectly downregulated virus replication. The cytokine also had a direct inhibitory effect on virus gene expression in mature macrophages. In these cells, LV-IFN blocked the viral life cycle at the level of transcription. Finally, LV-IFN blocked fusion between infected macrophages and highly permissive goat synovial membrane cells. By restricting macrophage maturation, viral replication, and cell fusion, LV-IFN may downregulate the net rate of virus replication in vivo. These functions may contribute to the persistence of the virus in the host by reducing the expression of the viral genome.

Lentiviruses, as their name suggests (L. lenti, slow), cause diseases with prolonged incubation periods and progressively debilitating clinical courses (14). The lentiviruses compose a subfamily of nononcogenic retroviruses which includes the prototype lentivirus, visna-maedi virus of sheep (25) and caprine arthritis-encephalitis virus (CAEV) (9), in addition to equine infectious anemia virus (5), and several immunodeficiency viruses which have been discovered and described in the past decade: the human immunodeficiency virus types 1 and 2 (1, 7), multiple simian immunodeficiency viruses (10, 22, 29), and feline immunodeficiency virus (30).

Lentiviruses of sheep and goats infect cells of monocyte-macrophage lineage (11, 28). The rate of replication of these viruses in the monocyte-macrophage is related to the degree of maturation of the cell. Monocytes contain viral genome in the form of proviral DNA. As these cells mature in vivo to tissue macrophages, the virus begins to replicate, but completion of the virus life cycle is curtailed at some point after transcription of viral RNA so that few infectious virions are produced (11, 26). When infected tissue macrophages are cultivated in vitro, viral replication proceeds unabated (12). This suggests that a tissue factor(s) keeps the replicative cycle of the virus in check.

Previous studies to determine the mechanism of restriction of lentiviral replication in sheep and goats revealed that cocultivation of lentivirus-infected macrophages with lymphocytes resulted in the release of a unique interferon (IFN), termed lentivirus-induced IFN (LV-IFN) (27). Preliminary studies on the function of LV-IFN indicated that supernatant fluids containing this cytokine inhibited lentivirus replication in macrophages. In addition, LV-IFN induced the expression of class II major histocompatibility complex (Ia) antigens on macrophages (17).

In this study, we have demonstrated that LV-IFN inhibited the proliferation and maturation of monocytes. Since completion of the virus life cycle depends on maturation of monocytes to macrophages (26), LV-IFN indirectly inhibited replication of CAEV in goat monocyte-macrophages. In addition, LV-IFN inhibited the replicative cycle of CAEV in already mature goat macrophages at the stage of transcription of viral RNA. Furthermore, LV-IFN caused a dramatic reduction in the fusion function of the virus, thus reducing the efficiency of virus dissemination.

MATERIALS AND METHODS

Virus. CAEV strain CO (CAEV/CO), grown in goat synovial membrane (GSM) cells (24), was used in this study. Virus infectivity, assayed in GSM cells as previously described (9), was 5 × 106 50% tissue culture infective doses (TCID50) per ml.

PBM cells. Peripheral blood was collected by venipuncture from an adult goat into tubes containing sodium EDTA. Peripheral blood mononuclear (PBM) cells were separated on Ficoll-Hypaque gradients (Ficoll, Sigma Chemical Co., St. Louis, Mo.; Hypaque, Winthrop Laboratories, Div. Sterling Drug Inc., New York, N.Y.) as previously described (26) and suspended in Dulbecco modified Eagle medium with 20% lamb serum (D/20/LS). Cells were plated at high density in culture dishes or chamber slides (Lab-Tek; Miles Scientific, Div. Miles Laboratories, Inc., Naperville, Ill.). Monocyte-macrophages used in these experiments were purified by adherence. On the basis of nonspecific esterase staining (16), 98% of the cells which adhered to plastic after 6 h of incubation at 37°C were determined to be of monocyte-macrophage lineage.

Production of LV-IFN. LV-IFN was produced as previously described (27). Briefly, fresh PBM cells were cocultured with CAEV-infected goat macrophages for 48 h. Supernatant fluids containing LV-IFN were then harvested.
clarified by centrifugation, and heated at 56°C for 10 min to inactivate virus. Prior studies had shown that the IFN activity was not affected by heat treatment (27). IFN activity in the fluid was assayed by inhibition of lysis of GSM cells infected with vesicular stomatitis virus as previously described (27). Stock material had IFN titers of 1/160 to 1/320. In all assays, stock LV-IFN was used in the cell culture medium at a concentration of 10% (vol/vol).

**Infectious-center assay.** The infectious-center assay, described previously (26), was used to determine the percentage of infected cells in a culture. Briefly, infected adherent monocytes or macrophages, removed from tissue culture dishes by trypsinization, were added in doubling dilutions to freshly trypsinized GSM indicator cells. Cell mixtures were seeded in duplicate wells of 24-well tissue culture dishes, incubated for 10 days, and observed for the presence of multinucleated giant cells (cytopathic effect). Cytopathic effect at limiting dilution was considered to be caused by a single cell.

**In situ hybridization.** Cloned visna virus DNA was cleaved from the pBR322 plasmid with Ssrl. The 8.6-kilobase fragment was purified by electrophoresis in low-totating-point agarose, and the 32P-labeled DNA was purified by affinity chromatography (NACS Prepac; Bethesda Research Laboratories, Gaithersburg, Md.). The DNA was radiolabeled by nick translation (33) using [32P]dATP and [32P]dCTP (Amersham Corp., Arlington Heights, Ill.), producing DNA fragments of approximately 40 to 70 base pairs, as previously described (20). Specific activities of the radiolabeled DNA probes were greater than 5 × 10^6 cpm/μg.

Cyto centrifuge (Shandon-Southern Instruments, Sewickley, Pa.) preparations were air dried and treated with 0.2 N HCl for 20 min and protease K (1 μg/ml) for 15 min, followed by acetylation for 10 min. The cells were overlaid with radiolabeled DNA (0.2 ng/μl) and incubated for 16 h at room temperature. The preparations were then washed, dried in emulsion (Eastman Kodak Co., Rochester, N.Y.), and exposed for 2 to 5 days. Viral RNA was indicated by silver grains over cells.

**Effect of LV-IFN on monocyte maturation.** During the first few days of in vitro culture, adherent monocytes mature to macrophages, undergoing a variety of morphological, biochemical, and functional changes (8). In this paper, adherent cells cultured for less than 24 h are termed monocytes, cells in culture for 1 to 3 days are called monocyte-macrophages, and those cultured for greater than 3 days are designated macrophages.

(i) **Cell size.** PBM cells were cultured in chamber slides in the presence or absence of LV-IFN (10% [vol/vol]) on days 1, 2, 4, and 6 of culture, nonadherent cells were removed by washing and slides with adherent macrophages were fixed in 100% methanol and stained with Giemsa stain (Hemacolor; Diagnostic Systems, Inc., Gibbstown, N.J.). The longest diameters of 200 cells from each sample were measured using an ocular micrometer. Differences in the size of treated and untreated cells were compared by using t tests.

(ii) **Fc receptors.** Fc receptors on 1-, 2-, 4-, and 7-day-old monocytes and macrophages were quantified by using a rosetting assay with antibody-coated sheep erythrocytes (34). Cells were examined by using phase-contrast illumination, and those with at least three sheep erythrocytes on their surfaces were considered positive. The data from four different experiments were pooled. The percentages of Fc-positive cells in cultures incubated with or without LV-IFN were compared by using paired t tests.

(iii) **Nonspecific phagocytosis.** The ability of monocyte-
examined by three methods; supernatant fluids were assayed for infectivity and the cells were examined by both infectious-center assay and in situ hybridization.

(iv) Effect of LV-IFN on fusion of fibroblasts to infected macrophages. Lentivirus-infected macrophages mediate the fusion of other cells, for example GSM cells, that contact them (23). Experiments to determine whether LV-IFN could modulate the fusion process were performed by adding the cytokine to infected macrophages prior to addition of GSM cells or by treating GSM cells with LV-IFN prior to their addition to the infected macrophage cultures. Cultures were fixed and stained 6 h after the addition of GSM to the macrophage cultures, and fusion was assessed by light microscopic examination.

RESULTS

Inhibition of monocyte-macrophage maturation. Untreated adherent cell cultures yielded large numbers of macrophages with abundant, elongate cytoplasmic processes (Fig. 1). In contrast, PBM cells cultured for 6 days in medium containing LV-IFN were few in number and small and rounded, with few cytoplasmic processes. Morphometric studies revealed that the mean longest cell diameter of 6-day-old LV-IFN-treated macrophages was 29.6 μm while that of untreated macrophages was 47.2 μm, a difference which was significant (P < 0.005). Under the same experimental conditions, the development of Fc receptors on LV-IFN-treated macrophages was also inhibited. Only 38% of macrophages cultured in the presence of LV-IFN for 7 days had Fc receptors, in contrast to 76% of macrophages cultured in medium without LV-IFN. LV-IFN had no effect on the phagocytosis of nonopsonized yeast particles.

PGE2 production. Mature, untreated 7- to 9-day-old macrophages did not produce significant levels of PGE2. However, although the maturation of LV-IFN-treated macrophages was stunted, these cells had nevertheless become potent producers of PGE2. PBM cells cultured in the presence of LV-IFN and indomethacin resulted in the usual poor maturation of macrophages, but PGE2 production in these cells was inhibited. From day 7 to day 9 of culture, PGE2 production was 6 and 71 pg for LV-IFN-treated macrophages with and without indomethacin, respectively, and 7 and 5 pg for LV-IFN-untreated macrophages with and without indomethacin, respectively (all values are the pooled results of two experiments). Dulbecco modified Eagle medium, lamb serum, and LV-IFN fluid were tested for PGE2 and did not contain significant levels.

Inhibition of macrophage proliferation. Monocytes seeded in medium containing LV-IFN incorporated significantly less [3H]thymidine than untreated macrophages (P < 0.03) (Fig. 2). Microscopic examination of autoradiographic preparations showed that most of the untreated macrophages had incorporated [3H]thymidine and that these cells had proliferated and formed a monolayer by day 9 of culture. In contrast, LV-IFN-treated macrophages were still sparse, covering less than 25% of the culture dish. Only 10% of treated cells had incorporated [3H]thymidine. These data suggest that LV-IFN inhibited the proliferation of monocytes.

Restriction of virus replication. LV-IFN did not affect the binding and endocytosis of virus to monocyte-macrophages. After 48 h of incubation, approximately 20% of the cells in both treated and untreated groups were infected. However, LV-IFN did restrict virus replication in both monocyte-macrophages and mature macrophages. LV-IFN-treated cells did not produce more than 102 TCID50 of infectious virus at any sampling period. In contrast, untreated infected macrophages produced as much as 104 TCID50 of virus by 5 days after inoculation with CAEV (Table 1). Infectious-center assays indicated that 100% of the cells in both LV-IFN-treated and -untreated groups were infected by day 3 of culture. The disparity in the amount of infectious virus produced by the two groups indicated an inhibitory effect of LV-IFN at some stage of the virus life cycle between endocytosis and virus maturation.

To determine whether LV-IFN would inhibit virus replication in mature macrophages, the above experiments were...
repeated in monocytes which had matured to macrophages prior to LV-IFN treatment and virus inoculation. Infectious-center assays showed that by 3 days after inoculation, 100% of the cells in both treated and untreated groups had become infected. Results of in situ hybridization showed that fewer of the LV-IFN-treated macrophages contained demonstrable viral RNA (Fig. 3) and that these cells also had significantly fewer grains than untreated cells ($P < 0.001$). On day 6, the mean numbers of autoradiographic grains per cell were $53 \pm 28$ and $142 \pm 33$ for LV-IFN-treated and -untreated cells, respectively. (Grains over 50 positive cells were counted; numbers are the means plus or minus standard deviations for three experiments.) There were correspondingly lower viral titers in the supernatants of cultures incubated with LV-IFN. These experiments demonstrated that whereas LV-IFN did not prevent infection in macrophages, it had a direct inhibitory effect on viral replication in mature macrophages, blocking replication at the level of transcription. The methods used did not allow us to determine whether the reduced number of transcripts was caused by inhibition of transcription or selective enzymatic degradation of viral RNA.

Inhibition of fusion of GSM cells to infected macrophages.

When infected macrophages were treated with LV-IFN 24 h prior to the addition of GSM cells, fusion of uninfected cells to infected macrophages proceeded unabated (Fig. 4). However, when GSM cells were treated with LV-IFN prior to their addition to infected macrophages, fusion was abrogated. This suggested that LV-IFN protected the GSM cells from infection via contact with virus-infected macrophages.

**DISCUSSION**

In this paper, we describe the diverse functions of a unique IFN which is produced in vitro by lymphocytes in concert with lentivirus-infected macrophages. LV-IFN has also been shown to be present locally in infected tissues such as synovium (O. Narayan, unpublished data). Preliminary characterization of culture supernatants containing the IFN-like cytokine(s), LV-IFN, indicated that IFN activity was associated with a 54- to 64-kilodalton nonglycosylated protein and that LV-IFN was heat stable (56°C for 10 min) and acid stable (pH 2) (27). The cellular requirements for production of LV-IFN are akin to those of gamma IFN (IFN-γ) in that both monocytes and lymphocytes are required for its production. Monoclonal antibodies directed against Ia antigen block the production of LV-IFN (Narayan, unpublished), indicating that, like IFN-γ, its manufacture is class II restricted. However, LV-IFN differs from IFN-γ in that it is a nonglycosylated protein and is acid stable. In this respect, it resembles IFN-α. Further characterization of LV-IFN has been hampered by the lack of reagents to identify cell markers in goats. The recent development of monoclonal antibodies to identify sheep macrophages and T-cell subsets (19) should help us identify the cell which elaborates a similar LV-IFN in sheep.

In vitro studies to determine the functions of LV-IFN revealed that it mediates many of the effects currently ascribed to IFNs. It has antiproliferative effects, it is a potent immunomodulator, and it has restrictive effects on the replicative cycle of the virus in the macrophage, both by limiting expression of the viral genome and by preventing virus-induced cell-to-cell fusion.

Antiproliferative effects are common to all classes of IFNs and have been described for a variety of cell types, including

**TABLE 1. Restriction of CAEV replication in monocyte-macrophages cultured in the presence or absence of LV-IFN**

<table>
<thead>
<tr>
<th>Time (days)</th>
<th>$\log_{10}$ TCID$_{50}$ of CAEV$^a$</th>
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<tbody>
<tr>
<td></td>
<td>LV-IFN+</td>
</tr>
<tr>
<td>1</td>
<td>0.6</td>
</tr>
<tr>
<td>2</td>
<td>0.9</td>
</tr>
<tr>
<td>3</td>
<td>1.9</td>
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<tr>
<td>4</td>
<td>0.4</td>
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$^a$ Figures represent CAEV produced by LV-IFN-treated (LV-IFN+) or -untreated (LV-IFN-) monocytes or macrophages during 5 days of culture.
as measured by cell size and expression of several macrophage markers (2).

The pathogenesis of lentiviral infection is closely tied to monocyte proliferation and maturation (26). Monocytes are not permissive for lentiviral replication but contain the viral genome as proviral DNA (28). As they mature, they become more permissive for viral replication (11). LV-IFN, by altering the process of monocyte proliferation and maturation, may have profound effects on the expression of viral genome in a variety of tissues, thus affecting disease expression during infection.

In contrast to its inhibitory effects on monocyte maturation and viral replication, LV-IFN induced functions in macrophages which are usually associated with cell activation. Earlier studies showed that LV-IFN caused increased Ia antigen expression on macrophages, thus potentially increasing the antigen-presenting capacity of these cells (17). This report demonstrated the induction of PGE2 secretion by LV-IFN-treated macrophages, another function associated with activated macrophages (3). PGE2 has a variety of immunosuppressive functions, including the induction of cells of the suppressor-cytotoxic phenotype and suppression of T-cell proliferation (6, 13, 15). Local secretion of PGE2 by macrophages in target organs may explain the finding that inflammatory exudate cells from target organs of infected animals contain large numbers of T8+ cells and reduced T4+ cells (S. Kennedy-Stoskopf, M. C. Zink, and O. Narayan, J. Clin. Immunol. Immunopathol., in press). Curiously, PGE2 also inhibits mononuclear phagocyte proliferation (21), suggesting the possibility of a feedback loop, enhancing the inhibitory effect of LV-IFN on these cells.

In addition to its antiproliferative and immunomodulatory effects, LV-IFN inhibited CAEV replication in both monocytes and macrophages at the level of transcription. Whether this block involved a direct inhibition of transcription or enzymatic degradation of transcripts was not determined. These studies showing suppression of virus replication in mature macrophages suggest that LV-IFN produced locally in infected target organs may be responsible for the suppression of viral replication which is observed in vivo.

A final effect of LV-IFN described here involved its potent effect in inhibiting cell-to-cell fusion. The formation of multinucleated giant cells which occurs by cell fusion, is a characteristic cytopathic effect of lentiviruses and is mediated by the glycoprotein in the viral envelope (18). Studies in our laboratory (M. C. Zink, unpublished data) have shown that viral RNA is able to pass directly from an infected to an uninfected cell prior to the production of mature virus particles. Treatment of GSM cells with LV-IFN abrogated fusion, whereas incubation of macrophages with LV-IFN had no effect. Resistance to fusion may be imparted by a mechanism involving IFN-induced stabilization of GSM plasma membranes (31). In vivo, locally produced LV-IFN may inhibit cell-to-cell transmission of virus. This may explain the finding that multinucleated cells are rarely seen in inflamed target tissues of lentivirus-infected ruminants.

LV-IFN appeared to have both beneficial and harmful effects on the host animal (Fig. 5). By inhibiting macrophage maturation, viral replication, and cell fusion, LV-IFN may downregulate virus replication in vivo. This may contribute to the long incubation period of the disease by postponing the onset of disease. On the other hand, by inducing Ia antigen expression and PGE2 secretion by macrophages in tissues, LV-IFN may not only enhance the antigen-presenting capacity of macrophages (with the attendant possibility for autoimmune disease) but contribute to the influx of

![FIG. 4. (A) Untreated GSM cells fuse to CAEV-infected macrophages, forming large syncytia. (B) GSM cells treated with LV-IFN do not fuse to the infected cells. LV-IFN was added to CAEV-infected macrophages 24 h prior to the addition of uninfected GSM cells. After 6 h of incubation, the cells were fixed, stained, and examined for cytopathic effect. In a separate experiment, GSM cells were treated with LV-IFN for 24 h prior to their addition to infected macrophages, and again the cultured cells were observed for fusion.](http://jvi.asm.org/Downloaded from http://jvi.asm.org/ on November 6, 2017 by guest)
FIG. 5. Lentivirus-infected monocytes become more permissive for viral replication as they mature to macrophages. Mature cells produce abundant viral RNA (●) and express viral antigen (○). Infected macrophages express a portion of the viral antigens within the context of their class II major histocompatibility complex antigens (◆). This is a prerequisite for the subsequent production of LV-IFN by lymphocytes. The various effects of LV-IFN are interdependent. LV-IFN enhances the expression of class II major histocompatibility complex antigens on the macrophage (a), which stimulates the further production of LV-IFN. At the same time, it inhibits the viral replicative cycle, both by inhibiting monocyte proliferation and maturation (b) and by blocking transcription (c). The resulting reduction of viral protein expression downregulates LV-IFN production. Finally, LV-IFN stimulates monocyte-macrophages to produce PGE₂ (d), which itself has an inhibitory effect on monocyte proliferation (e).

T cells that compose a large portion of the mononuclear cell infiltrates seen in affected tissues.

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LITERATURE CITED


