RESTRICTED REPLICATION OF LENTIVIRUSES

Visna Viruses Induce a Unique Interferon During Interaction Between Lymphocytes and Infected Macrophages

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Lentiviruses are a subgroup of retroviruses that are so named because they cause diseases with long incubation periods, insidious onsets, and slowly progressive courses (1). The members of the virus group include visna virus of sheep, caprine arthritis encephalitis virus (CAEV)[1] of goats, equine infectious anemia virus of horses, and, tentatively, the retrovirus associated with acquired immune deficiency syndrome (AIDS) in humans (2, 3). These agents are host specific and cause persistent infections in various cells of the immune system (4–6). In cultured cells they cause productive lytic infections but in vivo they replicate continuously at a restricted, minimally productive level (7–9). The mechanism of this unique type of replication is poorly understood but it sets the pace for the slow onset of chronic disease.

The ruminant lentiviruses, visna and CAEV, are prototypes of the lentivirus group and cause chronic-active inflammation characterized by infiltration and proliferation of mononuclear cells in various organ systems. These include the central nervous system (CNS) (visna), the lungs (maedi), the synovia (arthritis), and mammary glands (mastitis) (4). Examination of virus-cell interactions in these infections has shown (10, 11) that infection is confined exclusively to cells of the macrophage lineage, extending from promonocytes in the bone marrow to specific populations of tissue macrophages. No infection in lymphocytes has been observed either in inflamed tissues or in virus-inoculated cultures of mononuclear cells from blood (11, and Gendelman, H. E., O. Narayan, S. Kennedy-Stoskopf et al., manuscript submitted for publication). Levels of transcription and translation of the viral genome become amplified during maturation of promonocytes to tissue macrophages but, in the latter cells, replication is restricted at some

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[1] *Abbreviations used in this paper:* BLV, bovine leukemia virus; CAEV-CO, caprine arthritis encephalitis virus strain CO; CDV, canine distemper virus; CNS, central nervous system; Con A, concanavalin A; DME, Dulbecco's modified Eagle's medium; GSM, goat synovial membrane; IFN, interferon; LV, lentivirus; LV-IFN, lentivirus-induced interferon; PBM, peripheral blood mononuclear cells; PBM-MO, peripheral blood mononuclear cell–derived macrophage; PBS, phosphate-buffered saline; PFU, plaque-forming unit; PI-3, parainfluenza virus type 3; SCP, sheep choroid plexus fibroblasts; TrMO, SV40-transformed sheep alveolar macrophage; UV, ultraviolet; VSV, vesicular stomatitis virus.

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point after transcription (11, 12). In situ hybridization and infectivity studies have shown that tissue macrophages often contain thousands of copies of viral RNA but produce relatively few infectious particles (9, 11). This type of restricted replication could not be reproduced in macrophage cultures inoculated in vitro (13). We therefore asked whether the restriction of replication in vivo may be mediated by an interferon (IFN) induced during replication of the virus.

We show in this report that sheep and goat lentiviruses induce a unique IFN. Production of the IFN required interaction between lentivirus-infected macrophages and lymphocytes in a cooperative mechanism previously undescribed for virus-induced IFN. We define the specificities in IFN induction of the three participants, lentivirus, macrophage, and lymphocyte, and present a preliminary characterization of the IFN. An accompanying report (next article [14]) describes the biological effects of IFN and its probable role in restricting virus replication and potentiation of the inflammatory disease in vivo.

Materials and Methods

Viruses and Cell Cultures. Two strains of sheep/goat lentiviruses, Icelandic visna virus strain 1514 and CAEV strain CO (CAEV-CO), were mainly used in these studies. Stock preparations of the two viruses were prepared in cultures of sheep choroid plexus fibroblasts (SCP) and goat synovial membrane cultures (GSM), respectively, as previously described (9, 15). Stocks of both cell types were obtained by explantation and subcultivation of choroid plexus (lamb) and synovial membranes (goat) from colostrum-deprived newborn animals. The viruses replicate productively in the respective cultures (visna virus in SCP and CAEV in GSM), achieving titers of $\sim 5 \times 10^6$ TCD_{50}/ml. Replication was accompanied by multinucleated giant cell formation. The viruses were also propagated in cultures of sheep alveolar macrophage cultures transformed (immortalized) by SV40 (TrMO) (13). Alveolar cells were obtained from a Corriedale lamb by broncoalveolar lavage and cultures were inoculated with SV40. Proliferating colonies of macrophages were then propagated in Dulbecco's modified Eagle's medium (DME) plus 20% heated (56°C for 30 min) lamb serum and maintained in DME plus 2% lamb serum. These cells are trypsin dissociable and grow into density-dependent monolayers. In addition to the T antigen of SV40, the cells have nonspecific esterases and surface Fc receptors by which they readily phagocytize antibody-coated material (13). In addition, they express Ia antigens when treated with IFN induced by these lentiviruses (14). Inoculation of these cells with either of the two lentiviruses resulted in productive replication with infectivity titers of $\sim 1 \times 10^5$ plaque-forming units (PFU)/ml from day 3 through day 10, after which the cells degenerated.

Peripheral Blood Mononuclear Cells (PBM). Equal volumes of heparinized peripheral blood from sheep or goats and Ca^{++}/Mg^{++}-deficient Hanks' salt solution were mixed and layered onto Ficoll-Hypaque gradients (2.4 parts of 9% Ficoll [Sigma Chemical Co., St. Louis, MO] to 1 part of 33.9% Hypaque [Winthrop Laboratories, Sterling Drugs, Inc., New York]) and centrifuged at 600 *g* for 40 min. Mononuclear cell bands were collected by aspiration, washed by centrifugation at 500 *g* for 5 min in DME, and concentrations adjusted to 1×10^6 cells/ml in medium. When used to produce PBM macrophages (PBM-MO), the cells were suspended in DME plus 20% lamb serum; when they were used as a source of lymphocytes they were suspended in RPMI 1640 plus 10% lamb serum (12).

Nonadherent Cells. Enriched populations of nonadherent cells were obtained by suspending the PBM in RPMI plus 10% lamb serum and incubating them in petri dishes coated with rabbit anti-goat IgG (Miles Laboratories, Inc., Elkhart, IN). Nonadherent cells were removed after two successive 2-h adsorption cycles at 37°C and passaged through nylon wool/glass bead columns as described previously (12). Eluates from these columns were pelleted and resuspended in RPMI plus 10% lamb serum.

PBM Macrophages (PBM-MO). In most cases macrophage cultures were developed in

35-mm^2 tissue culture dishes that were seeded with 3 ml of fresh PBM cell suspension in DME plus 20% lamb serum. Monolayers of macrophages were obtained 7–10 d later. Culture dishes were then inverted, nonadherent cells washed away, and the macrophages inoculated with viruses.

Ovine parainfluenza type 3 (PI-3) virus was obtained from Dr. R. Cutlip, U. S. Department of Agriculture, Ames, IA. The virus was cultivated in GSM cell cultures in which it replicated to a titer of 1×10^5 TCD_{50}/ml (16).

A stock preparation of bovine leukemia virus was kindly provided by Dr. Matthew Gonda, NCI-Frederick Cancer Research Facility, Frederick, MD. The Onderspoort strain of canine distemper virus (CDV) was obtained from Dr. Max Appel, Baker Institute, New York State College of Veterinary Medicine, Ithaca, NY, and the Edmonston strain of measles virus procured from the American Type Culture Collection, Rockville, MD. Both CDV and measles viruses were propagated in vero cell cultures as reported previously (17) with resulting infectivity titers of 5×10^6 and 5×10^5 PFU/ml, respectively. The New Jersey strain of vesicular stomatitis virus (VSV) was kindly provided by Dr. Paula Pitha, Oncology Center, Johns Hopkins Hospital. The virus was propagated in GSM cell cultures. The titer was 5×10^5 PFU/ml.

IFN Assays. GSM cells or TrMO cells were cultivated in 96-well microtiter plates in DME plus 10% lamb serum. At confluence the cultures were rinsed with serum-free DME and inoculated with doubling dilutions of IFN test material in DME. After overnight incubation at 37°C, the inocula were removed, monolayers washed, and each culture was inoculated with 10^4 PFU of VSV and incubated for a further 24 h at 37°C. 24 h after control cultures were inoculated with VSV, they were completely lysed by the virus. The titer of IFN in various supernatant fluids was defined as the highest dilution of the fluid that protected these cell cultures against lysis by VSV.

Column Chromatography. Sephadex G50 (Pharmacia, Inc., Piscataway, NJ) was preswollen in phosphate-buffered saline (PBS), pH 7.4, degassed, and packed into a 60×1 cm column by gravity. The column was rinsed with 10 column volumes of DME and calibrated using known molecular weight marker standards: albumin, ovalbumin, and ribonuclease, which were detected at 280 nm. One ml of stock-induced lentivirus IFN (LV-IFN) supernatant fluid was then applied and eluted with DME plus 1% lamb serum. 2-ml fractions of eluate were collected in a fraction collector and assayed for IFN activity.

Two columns were packed with 1 ml of concanavalin A (Con A)-Sepharose and lens culinaris–Sepharose 4B, respectively, and washed with 10 ml of buffer (0.5 M NaCl, 0.02 M Tris, pH 7.4, 1 mM $MgCl_2$, 1 mM $CaCl_2$), and 1 ml of supernatant fluid containing IFN was applied to each column. This material was applied five times successively to the columns and the final effluent fluids stored for assay. The columns were then washed with 10 ml of buffer described above and any potentially bound IFN were eluted with 2 ml of 1 M α-methyl-D-mannoside. This material was dialyzed against Hanks' salt solution for 2 h and then assayed for IFN.

Animals. Blood from sheep and goats was obtained from animals in a herd that was free from infection with lentiviruses, at the Johns Hopkins Farm. Sheep and goats persistently infected with the lentiviruses were housed in special quarters at the medical school. Serum with virus-neutralizing antibodies was obtained from some of these animals (18).

Results

Lack of IFN Production in Lentivirus-infected Cultures. Since IFN induced by viruses is usually produced by virus-infected cells, we inoculated five ovine cell cultures with visna and CAE viruses to determine whether any of the infected cultures would produce IFN. Replicates of three 35-mm^2 tissue culture dishes containing monolayers of primary PBM-MO, TrMO, SCP cells, GSM cells, and fresh PBM suspensions in Teflon bottles (Cole-Parmer Instrument Co., Chicago, IL) were inoculated with each virus at a multiplicity of infection (moi) of 2 and

TABLE I
Lack of IFN Production in Lentivirus-infected Cultures

Stimuli	Cultures				
	PBM-MO	TrMO	SCP	GSM	Fresh PBM
Visna virus	–	–	–	–	–
CAE virus	–	–	–	–	–
PI-3 virus	80	80	ND	ND	ND
Con A	–	–	ND	ND	160

Monolayer cultures of macrophage (PBM-MO and TrMO) and nonmacrophage (SCP and GSM) cell types and suspensions of PBM cells in Teflon bottles were inoculated with viruses (moi = 2) and Con A (10 μg/ml) as indicated. 3 d later supernatant fluids from triplicate samples of each culture were examined for IFN in a VSV-GSM assay system. Numbers are reciprocals of the highest dilution of fluids that protected GSM cells against lysis by VSV and indicate the titer of IFN in the fluid. (–) Indicates no protection of GSM cells with undiluted fluids and therefore no IFN; ND, not done.

incubated at 37°C. Samples of supernatant fluids were collected from each dish daily for 5 d and assayed for IFN within 2 d after storage at 4°C. For controls, replicates of PBM-MO and TrMO were inoculated with PI-3 virus at an moi of 2; a suspension of PBM was inoculated with Con A at a concentration of 10 μg/ml; and samples of supernatant fluids were examined for IFN content as described above.

Results of this experiment (Table I) showed that the lentiviruses did not induce IFN in any of the inoculated cultures. In contrast, both of the macrophage cultures infected with PI-3 virus and the PBM suspension treated with Con A, produced IFN. These results agree with well-established studies showing that retroviruses are poor inducers of IFN in the cell cultures they infect (19). However, as shown above, such cells were capable of producing IFN when appropriately stimulated.

Since neither LV-inoculated macrophages nor LV-inoculated PBM produced IFN, the experiment suggested that, if IFN is produced in vivo, it is unlikely that macrophages, the major cell type infected in the animal, would be the producers. It was possible that cells of another lineage, acting in consort with infected, mature macrophages, could produce IFN. To test this hypothesis, a monolayer of PBM-MO from a normal goat was inoculated with CAEV at an moi of 2 and incubated at 37°C in maintenance medium (DME plus 2% lamb serum). 3 d later the medium was removed and fresh PBM from the same animal were suspended in maintenance medium and added to the cultures of infected macrophages. Examination of the supernatant fluid from these cultures 48 h later showed that IFN with a titer of 1:160 had been produced. Thus, IFN production required interaction between PBM and infected, mature macrophages. This result suggested that the lentiviruses could induce IFN by a mechanism different from the classical pathway requiring that the infected cells be the IFN producer.

Cell Mixtures Required for LV-IFN Production. To dissect the virus-cell interactions required for LV-IFN production, we prepared monolayer cultures of PBM-MO, TrMO, and GSM cells, and infected some of these with CAEV. Freshly dispersed cultures of uninfected TrMO, SCP cells, GSM cells, and freshly prepared PBM were added to replicates of each of the monolayer cultures as

TABLE II
Cell Mixtures Required for LV-IFN Production

Monolayers	Added cell suspensions				
	TrMO	SCP	GSM	Fresh PBM	Nonadherent T cells
Normal PBM-MO	–	–	–	–	–
CAEV/PBM-MO	–	–	–	320	320
CAEV/TrMO	–	–	–	80	ND
CAEV/GSM	–	–	ND	–	–

Cell suspensions of macrophage and nonmacrophage cell types, PBM, and a nonadherent fraction of the PBM were added to monolayer cultures as indicated. The latter cultures included uninfected macrophages and macrophage and nonmacrophage (GSM) cell types that had been inoculated with CAEV (moi = 2) 3 d previously. Supernatant fluids were tested for IFN content 2 d later. IFN was produced only in cultures containing lymphocytes and infected macrophages.

illustrated in Table II. Supernatant fluids were examined for IFN content 48 h later.

The results were clear-cut and showed that IFN was produced only in cultures of infected macrophages to which PBM had been added. Virus-infected macrophages were needed, since addition of PBM to normal macrophages did not stimulate IFN production. Similarly, infection in mature macrophages was essential, since addition of PBM (which contained monocytes) to infected GSM cells did not result in IFN production.

To identify the PBM cell type that was responsible for IFN production, part of a batch of goat PBM containing 5×10^6 cells/ml was fractionated and the nonadherent cells prepared by panning the PBM twice in succession on rabbit anti–goat IgG (Miles Laboratories, Inc.)-coated dishes followed by passage of the nonadherent cells through a nylon wool/glass bead column. As shown in Table II, the addition of nonadherent cells, separated from 5 ml of PBM, to the infected macrophage cultures resulted in production of as much IFN as did 5 ml of unfractionated PBM. The experiment was repeated four times, and examination of the supernatant fluids from the cultures showed IFN with a titer of 1:160 to 1:320. This indicated that the PBM cells which reacted with infected macrophages to produce IFN were most probably T lymphocytes.

Lack of Requirement for Antiviral Immunity or Histocompatibility in Donors of Macrophages and Lymphocytes for LV-IFN Production. Addition of PBM to macrophage cultures infected with any strain of the sheep/goat lentiviruses resulted in IFN production. This included visna virus, four strains of CAEV, and four field strains of sheep viruses obtained in previous studies (10) (data not shown). We therefore used CAEV-CO virus to infect PBM-MO cultures from various animals and added fresh PBM from different sheep and goats to determine whether IFN production was immunologically specific or restricted by histocompatibility. Monolayer cultures of macrophages were derived in 35-mm^2 tissue culture dishes and infected with CAEV at an moi of 2. Three days later 5×10^6 PBM were added and supernatant fluids were examined for IFN content 2 d later.

The experiments are summarized in Table III, each result being an average of three trials. PBM-MO were derived from an immune, persistently infected

TABLE III

Effect of Antiviral Immunity and Histocompatibility in Donors of PBM-MO and Lymphocytes on LV-IFN Production

Exp.	Virus	Source of PBM-MO	Source of lymphocytes	Titer of IFN
1	CAEV	CAEV-infected, immune goat	Autologous	320
2	CAEV	Uninfected goat	Autologous	160
3	CAEV	Uninfected goat	Uninfected sheep	160
4	CAEV	TrMO (sheep)	Uninfected goat	80
5	Bov. leukemia	Uninfected goat	Autologous	–
	Can. distemper	Uninfected goat	Autologous	–
	Measles	Uninfected goat	Autologous	–
6	CAEV	Human	Uninfected goat	–
	CAEV	Bovine	Uninfected goat	–
7	CAEV	Uninfected goat	Human	–
	CAEV	Uninfected goat	Bovine	–

In replicate cultures of experiments 1–7, the specific requirement for participation of lentiviruses, sheep/goat macrophages, and sheep/goat lymphocytes in IFN production are shown. (*1* and *2*) IFN production was not dependent on immunity or prior infection in donors of cells. (*3* and *4*) Histocompatibility between donors of macrophages and lymphocytes was not required. (*5*) There was an absolute requirement for lentiviruses to initiate infection in macrophages. (*6*) Sheep/goat macrophages could not be substituted with bovine and human macrophages. (*7*) Lymphocytes of human and bovine origin did not interact with lentivirus-infected goat macrophages to produce IFN.

goat (Table III, Exp. 1) and an uninfected goat (Exp. 2). Both macrophage cultures were infected with CAEV and then supplemented with autologous PBM 3 d later. Both sets of cultures produced equivalent amounts of IFN. Therefore, production of LV-IFN was not immunologically specific. In Exps. 3 and 4, PBM-MO from the uninfected goat and TrMO from sheep were infected and supplemented with PBM from an uninfected sheep and an uninfected goat, respectively (i.e., infected goat macrophages were complemented with sheep lymphocytes and vice versa). IFN titers in supernatant fluids were equivalent. Therefore, histocompatibility between the donors of macrophages and lymphocytes was not necessary for IFN production. In the next three experiments, we evaluated the effects of substitution of (*a*) lentivirus with other viruses, (*b*) sheep/goat macrophages with macrophages from other species, and (*c*) sheep/goat lymphocytes with PBM from heterologous species. In Table III, Exp. 5, replicate cultures of PBM-MO from the uninfected goat described above were inoculated with bovine leukemia virus (a retrovirus of cattle), canine distemper virus, and measles virus, at an moi of 2, and supplemented with autologous PBM. The latter two viruses were used because they are macrophage-tropic agents. In experiment 6, PBM-MO cultures of human and bovine origin were inoculated with CAEV and supplemented with PBM from the uninfected goat. In experiment 7, PBM-MO cultures from the uninfected goat were inoculated with CAEV and supplemented with PBM of human and bovine origin, respectively. None of the combinations in the latter three experiments resulted in production of IFN. Exp. 5 thus shows that viruses other than the lentiviruses could not induce IFN, probably because they failed to replicate in the macrophage cultures (data not shown). Exp. 6

TABLE IV

Determinants for Production of IFN-inducing Factor in Macrophages

Exp.	Interventions in virus/macrophage system	Titer of IFN
1	None	320
2	Neutralizing antibodies incubated with virus before inoculation of MO culture	–
3	Neutralizing antibodies added to MO culture after infection	160
4	UV irradiation of infected MO culture	–
5	UV-irradiated infected MO culture plus unirradiated supernatant fluid	–
6	Lysate of infected MO culture in infectious supernatant fluid	–
7	Infected MO culture treated with dexamethasone	–

Fresh PBM obtained from the same goat used as the source of PBM-MO cultures were used in all experiments. (*1*) Positive control; addition of PBM to CAEV-infected macrophages resulted in production of IFN with a titer of 1:320. (*2*) Addition of neutralized CAEV to macrophage cultures did not result in infection of the macrophages, and addition of PBM did not result in production of IFN. (*3*) Addition of the specific virus-neutralizing antibodies to macrophage cultures with established infection had no effect on subsequent production of IFN. (*4* and *5*) When infected macrophages were killed by irradiation with UV light, infectious supernatant fluids from the culture did not trigger IFN production by the PBM. (*6*) Lysates of infected macrophages did not induce the PBM to produce IFN. (*7*) Pulse treatment of infected macrophages for 1 h with 100 μg/ml of dexamethazone prevented production of IFN.

probably represents the corollary of Exp. 5 in that CAEV replicated poorly in human and bovine macrophages. Exp. 7 shows that human and bovine lymphocytes did not recognize the IFN-inducing factor (see below) in the infected goat macrophages. Thus, despite the lack of a requirement for histocompatibility between macrophage donors and lymphocyte donors in the ovine-caprine species, restriction appeared at the interspecies level.

Determinants for Production of IFN-inducing Factor in Macrophages. The foregoing experiments suggest that, after infection with lentiviruses, sheep/goat macrophages may have produced a factor which stimulated IFN production in T lymphocytes. In the following series of experiments we attempted to define the macrophage factor in greater detail. CAEV was used to infect macrophages, and PBM-MO and fresh PBM were derived from an uninfected goat. The macrophages were seeded into 35-mm^2 tissue culture dishes and inoculated with virus at an moi of 2. Three d later, 5×10^6 PBM were added and, 2 d later, supernatant fluids were examined for IFN content. Each experiment was performed in triplicate, at least, and the mean value of the IFN titer is reported. Various interventions were introduced at the virus-macrophage interaction before the addition of PBM cells.

The various manipulations are summarized in Table IV: In positive control experiments, addition of the PBM to virus-infected macrophages resulted in production of IFN with a titer of 1:320 (Exp. 1); to prove that virus infection was essential in the macrophages, CAEV was preincubated with neutralizing

antibodies for 1 h before addition to the PBM-MO cultures. The rest of the protocol was unchanged. The PBM-MO did not become infected and no IFN was produced after PBM were added (Exp. 2). In Exp. 3, virus-neutralizing antibodies were added to the PBM-MO culture on day 3 after infection, but 2 h before addition of PBM. The antibodies were maintained in the medium for the duration of the experiment. Before addition of the antibodies, the infectivity titer in the supernatant fluid of the infected PBM-MO was 5×10^4 PFU/ml. No infectivity was detected in the fluid 2 h later when the fresh PBM were added. 2 d later, however, the culture produced IFN with a titer of 1:160 (Exp. 3, Table IV). Thus, virus-neutralizing antibodies had no effect on expression of the factor after infection in the PBM-MO had been initiated. To determine whether the factor was present on the surface of infected macrophages, we removed most of the supernatant fluids from the cultures and irradiated the cells with ultraviolet (UV) light for 3 min immediately before addition of PBM. This treatment completely ablated the factor and resulted in no production of IFN (Exp. 4, Table IV). Replacing the supernatant fluids (which had been removed before irradiation of the cells) to the cultures of irradiated cells did not replenish the factor, and no IFN was produced when PBM were added. Since addition of supernatant fluids from infected macrophages to PBM failed to produce the IFN in the previous experiments (Table I), this was not surprising. However, this experiment ruled out the possibility that the factor was present in supernatant fluids (unlike interleukin 1, which was present; data not shown) and suggested that the factor was strictly cell associated. To determine whether the factor was produced and stored intracellularly, we scraped the infected macrophages from tissue culture dishes into supernatant fluids and the suspensions were briefly sonicated before being added to PBM suspensions. No IFN was produced in these cultures. Thus, the factor was not stored intracellularly and the experiments suggest that living cells may be needed for continuous production of the macrophage factor.

In previous unpublished experiments, we had observed that the steroid, dexamethasone (Sigma Chemical Co.), caused a slight increase in virus yields in infected macrophage cultures. We added the steroid to infected PBM-MO cultures to determine whether the IFN-inducing factor might be increased. In fact, dexamethasone completely abrogated production of the IFN-inducing factor. Infected PBM-MO were pulsed with 100 μg/ml of dexamethasone for 1 h at 37°C, after which the cultures were washed three times with DME before addition of PBM. 2 d later supernatant fluids had infectivity titers of 5×10^5 PFU/ml but no IFN. The mechanism for this inhibition is unclear.

These experiments thus showed that the IFN-inducing factor in macrophages was dependent on lentivirus infection but that synthesis of the product was independent of virus production. The factor was associated only with living macrophages and could be "neutralized" with dexamethasone.

Kinetics of Synthesis of LV-IFN. LV-IFN was produced only after sheep/goat lymphocytes had made contact with lentivirus-infected sheep/goat macrophages. In the following experiments we investigated the kinetics of IFN production by measuring the amount of IFN liberated into the supernatant fluids by cultured lymphocytes that had been separated from infected PBM-MO cultures after

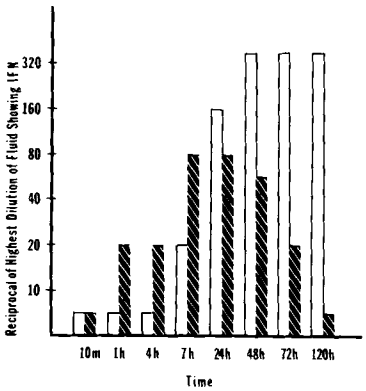

FIGURE 1. PBM were added to infected macrophage cultures in tissue culture dishes and, at times indicated, two dishes were removed for study. The supernatant fluids were tested for IFN and the nonadherent cells transferred to new tissue culture dishes and cultured for a further 24 h at 37°C. Supernatant fluids from these cultures were then tested for IFN. Open bars indicate the amount of IFN produced after the indicated period of cocultivation of the two cell types, and the adjoining dark bars indicate amounts of IFN produced by separated lymphocytes 24 h later. IFN was first detected in cocultures after 7 h and peak levels were found at 48 h. Transferred lymphocytes began to produce IFN after a 1 h exposure to infected macrophages; maximum production required cocultivation for 7 h. After 24 h of cocultivation, the transferred lymphocytes produced decreasing quantities of IFN.

various intervals of cocultivation. The LV-IFN induction system described above using CAEV, PBM-MO, and PBM from an uninfected goat, was adopted in this experiment. 3 ml of medium containing 5×10^6 PBM was added to tissue culture dishes containing mature PBM-MO that had been infected 3 d previously with CAEV. At various intervals after cocultivation, two dishes with cocultures were removed for study. A sample of supernatant fluid was collected from each dish and assayed for IFN. The nonadherent cells were then transferred from each dish to a centrifuge tube in an excess of DME and sedimented by centrifugation. The cells were then resuspended in 3 ml of fresh RPMI plus 10% lamb serum, added to new tissue culture dishes (without macrophages), and incubated at 37°C for 48 h. Supernatant fluids were then tested for IFN content. Fig. 1 shows the kinetics of synthesis of LV-IFN. No IFN was produced in individual cultures of infected PBM-MO or PBM (data not shown). In cocultures, IFN was detected for the first time 7 h after PBM were added to infected PBM-MO. IFN titers increased to maximum levels by 48 h. After this there was no further increase in the amount IFN in the supernatant fluids.

IFN was produced by the transferred lymphocytes but this required a minimum period of 1 h prior contact with infected PBM-MO. Longer contact of these cells with infected PBM-MO resulted in production of larger amounts of IFN after they were transferred. However, after 24 h of cocultivation, the transferred lymphocytes began to produce decreasing amounts of IFN. Cell counts and viability studies on these cells, using trypan blue exclusion tests, showed that the decine in IFN production was not caused by a reduction in cell numbers or cell death.

These data suggest that the lymphocytes recognize the IFN-inducing factor in infected PBM-MO and begin to produce IFN after a minimum period of

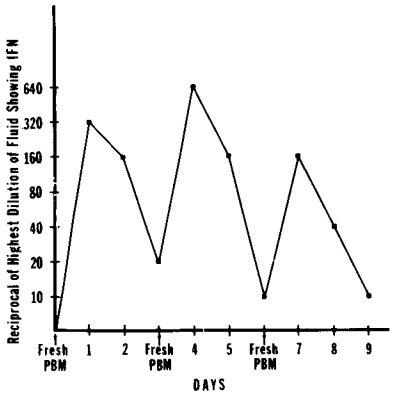

FIGURE 2. Fresh PBM were added to dishes of infected macrophages and supernatant fluids were assayed for IFN daily and discarded. The nonadherent cells were removed, washed by centrifugation, and added back to the original dishes in fresh medium. On days 3 and 6, new PBM were added to the dishes and the daily assay of supernatant fluids continued. New bursts of IFN 24 h after addition of new PBM are indicated.

sensitization. The sensitization is probably asynchronous, starting at 1 h and reaching maximal levels after 24–48 h of cocultivation. The plateau in the IFN level after 48 h of cocultivation suggests that no further IFN production was in progress. On the other hand, the decline in ability of lymphocytes to produce IFN after this period suggests that either the lymphocytes were capable of producing only a specific amount of IFN, and became refractory after this, or that the IFN-inducing factor in infected PBM-MO were exhausted. Two experiments were performed to test this hypothesis: (*a*) "Spent" lymphocytes were transferred, from three dishes in which they had been cocultivated with infected PBM-MO for 3 d and had produced IFN, to three new dishes of infected PBM-MO. No new IFN was produced. Addition of 5×10^6 new PBM to each of these latter dishes of PBM-MO resulted in IFN production to peak levels 48 h later, with titers of 1:160 to 1:320. This showed that the lymphocytes were not capable of producing IFN after having done so once, and that spent lymphocytes did not interfere with the ability of new lymphocytes to produce IFN.

(*b*) To test whether the IFN-inducing factor was exhausted after a single round of IFN production by lymphocytes, an experiment was performed in duplicate in which three suspensions of fresh PBM were added to a single dish of infected macrophages at 3-d intervals. Supernatant fluids were measured for IFN content daily and the lymphocytes were removed, washed by centrifugation, and added back to the original dish in fresh medium. At two successive 3-d intervals, old PBM were replaced with new PBM and the daily determinations of IFN continued. Fig. 2 shows that new bursts of IFN were produced 24 h after addition of new PBM. Furthermore, infected macrophages maintained their IFN-inducing capacity through more than one cycle of IFN production. We concluded that the decline in IFN production was due to exhaustion in the lymphocyte population.

Preliminary Characterization of LV-IFN. Because of its highly stable physical nature (see below) LV-IFN was produced in a 200 ml batch that was aliquoted and stored frozen at −70°C. Samples were then removed for different experi-

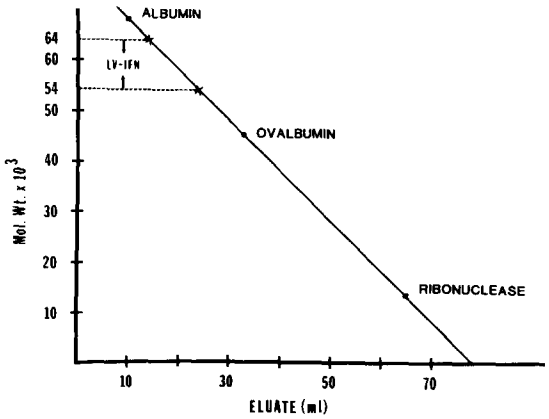

FIGURE 3. 1 ml of stock serum-free supernatant fluid containing LV-IFN that had been heat treated and stored frozen was applied to a 60-cm-long column containing G-50 Sephadex, equilibrated with DME and 1% lamb serum. Eluates were collected in 2 ml fractions and assayed for IFN. All IFN activity was found in three consecutive fractions ranging in molecular weight from 54,000 to 64,000. Albumin, ovalbumin, and ribonuclease were used as molecular weight markers.

ments. The batch was prepared by a slight modification from previously described methods. PBM-MO and PBM from a normal goat were used for the process; 100 ml of blood was used to produce PBM-MO cultures and 200 ml for preparation of PBM. The CAEV-CO-infected cultures of PBM-MO were rinsed with serum-free DME before the addition of PBM in serum-free DME. Supernatant fluids collected 2 d later were clarified by centrifugation at 10,000 *g* for 10 min, heated at 56°C for 30 min to inactivate the virus, distributed in vials, and frozen at −70°C. A vial of this material had a titer of 1:320 after thawing and was considered stock IFN.

An aliquot of the stock IFN material described above, treated with trypsin (Gibco Laboratories, Grand Island, NY) at a concentration 10 μg/ml for 15 min at 37°C, lost all biological activity. This proved the protein nature of LV-IFN. The size of the protein was determined by gel filtration through Sephadex G50. One ml of stock LV-IFN was applied to the column and eluted with DMEM plus 1% lamb serum. The eluate was collected sequentially in 2-ml fractions and each was examined for IFN activity. Biological activity was found in three consecutive fractions, with an estimated molecular weight of 54,000–64,000 (Fig. 3).

Experiments to determine whether the LV-IFN is glycosylated were performed by testing for binding of the IFN to Con A–Sepharose and lens culanaris–Sepharose in two successive tests. 1 ml of stock LV-IFN was passed five times successively over each column and the final effluent retained for an assay for residual IFN. Potentially bound LV-IFN was eluted off the columns with 2 ml of 1.0 M α-methyl-D-mannoside. Assays showed that all of the IFN was present in effluent fluid and none in the eluate. Thus, the LV-IFN did not bind to either lentil column, suggesting it is not glycosylated.

Because of its unusual origin, the LV-IFN was compared with IFN induced in ovine macrophages by PI-3 virus and in PBM cultures by Con A, respectively. Table V shows the differences among the three IFN. LV-IFN was the most

TABLE V
Comparative Properties of Different Ovine IFN

Supernatant fluids	Assay systems		pH 2 24 h*	56°C 30 min*	Freeze/ thaw*
	VSV/GSM	VSV/TrMO			
LV-IFN	320	160	160	160	160
PI-3–IFN	80	20	80	40	<10
Con A–IFN	80	10	<10	<10	<10

Supernatant fluids containing LV-IFN (from frozen stock), IFN resulting from inoculation of TrMO with PI-3 virus, and IFN resulting from treatment of goat PBM with Con A at 10 μg/ml for 48 h, were compared in various tests as indicated. IFN in the three fluids protected GSM against VSV but LV-IFN was more effective than the other two in protecting macrophages against lysis by VSV.

* IFN assays were performed using a VSV-GSM assay system. IFN titers after various treatments are self explanatory.

stable of the three in resistance to low pH, heat, and freeze-thawing. Furthermore, it was the only IFN that protected macrophages against lysis by VSV. It is clear that the LV-IFN has properties that include those of both of the other two in addition to unique properties of its own.

Discussion

We have shown in this report that the lentiviruses of sheep and goats induce an IFN by a hitherto unrecognized mechanism involving a sequential interaction of three participants: the lentivirus, macrophages, and lymphocytes. This indirect induction process extends the parameters by which viruses induce IFN. Classically, IFN induced by virus is a product of the infected cell, with the types of IFN (alpha or beta) being dependent on the infected cell type, and the amount being dependent on the virus used for infection (20–22). In this direct system, paramyxoviruses, which include PI-3, are good inducers (23), while retroviruses are poor inducers (19, 20). We confirmed these findings in our experiments. The indirect system represents a major departure from the classical concept; it is a much more efficient IFN induction system and the amount of IFN produced is not dependent on the number of virus particles used. Rather, as seen in this study, minimal virus replication need occur in the macrophages to induce the IFN-inducing factor. This factor is then capable of activating IFN production in many lymphocytes. This amplification system resulted in relatively high titers of IFN induced by the lentiviruses. In the animal this would provide a mechanism for continuous production of IFN during restricted virus replication.

The first step in the LV-IFN induction process involved the specificity of the viruses. This may be partly attributable to the nonlytic type of infection these agents cause in sheep/goat macrophages. Although measles and canine distemper viruses replicate in macrophage cultures in their respective hosts (canine [24] and human [25]), their life cycles did not go to completion in ruminant macrophages. PI-3 also replicated poorly in these cells and, although it induced IFN directly in the macrophages, addition of PBM to the infected macrophages did not result in production of an IFN similar to LV-IFN, (i.e., the IFN did not protect TrMO against lysis by VSV; data not shown). The IFN induction process was not peculiar to retroviruses in general, since bovine leukemia virus, a

retrovirus known to be extremely oncogenic in sheep (26), was incapable of inducing the IFN-inducing factor in sheep macrophages. The lentiviruses of ruminants therefore seemed uniquely endowed to induce this factor in species-specific macrophages.

The second specific step in the IFN induction process was the response of the macrophage. Although the lentiviruses replicated productively in several cell types of the natural host, including SCP fibroblasts and synovial membrane cells, only infected macrophages produced the IFN-inducing factor. The fact that both alveolar and blood macrophages produced the factor suggests that infection in any macrophage from this species may suffice for this function. However, the nature of the factor remains to be characterized. The use of virus-neutralizing antibodies to study production of the factor clearly established that infection with lentiviruses was necessary but that infectious virus was not essential. The failure of UV-inactivated infected macrophages and lysates of infected macrophages to supply the factor is compatible with a labile substance produced in small quantities by the infected macrophages. A similar observation was made in a previous study (10) in which fusion of SCP fibroblasts by certain field isolates of lentiviruses required continuous presence of macrophages. The fusion factor in macrophages was also cell associated and was produced in limited quantities. Whether the fusion factor and the IFN-inducing factor in the infected macrophages are the same is unknown. It is possible that the IFN-inducing factor is a lysosomal enzyme or an enzyme-modified protein on the macrophage cell surface. Dexamethasone, which inhibits the IFN-inducing factor, may have a role in this process, given its ability to stabilize lysosomal membranes. Such hypothetical stabilization of lysosomes could prevent loss of enzymes and prevent production of the factor.

The third sequential step in the LV-IFN induction process involved the interaction of T lymphocytes with the infected macrophages. We have already discussed the suggestion of a sudden burst of IFN production by the lymphocytes after contact with the infected macrophages, together with the observation that the macrophage-to-lymphocyte signal was neither immunologically specific nor restricted by histocompatibility. However, restriction at the species level was at play, since neither bovine nor human lymphocytes recognized the signal to produce IFN. This requirement of participation between lymphocytes and macrophages for the production of LV-IFN has some resemblance to the interaction of similar cells in the production of mitogen-induced gamma IFN (27). However, this may be coincidental, since immunological parameters were not involved in the production of LV-IFN and the LV-IFN had physical properties distinct from gamma IFN.

One of the questions arising early in the study was the type of IFN induced by the lentiviruses. However, the lack of molecularly defined sheep/goat IFN and lack of typing sera made characterization of the LV-IFN difficult. The presumptive classification is based on a comparison with the IFN induced by PI-3 virus and Con A, which are similar to classical IFN (21). Thus, the IFN induced by PI-3 virus infection of goat macrophages fits the general descriptions of human and murine alpha IFN, as determined by the macrophage cell source, the resistance of the IFN to low pH, and the relative heat stability. Similarly, the

IFN resulting from Con A stimulation of PBM resembles gamma IFN, as determined by the mitogen inducer, the producer cells, and its lability to pH 2 and heat. In contrast, the LV-IFN had properties overlapping those of both the preceding two IFN, in addition to some unique properties. Its resistance to low pH and heat, molecular size, and nonglycosylated nature resemble alpha IFN, while synthesis by macrophage-dependent T lymphocytes resembles gamma IFN. This analogy is made stronger by the ability of LV-IFN to induce expression of Ia antigen in cultured macrophages (14), a phenomenon associated with gamma IFN (26). It is doubtful that LV-IFN represents a mixture of alpha and gamma IFN because it possesses the unique properties of resistance to freeze-thawing and the ability to protect macrophages from lysis with VSV.

The LV-IFN induction process is highly relevant to events in the infected animal. Since monocyte-macrophages are the main target cells in vivo for virus replication, and since the lesions in disease consist of infiltrations of lymphocytes and macrophages, conditions would be ideal for local synthesis of IFN. However, LV-IFN was not found in extracts of inflamed tissues. Nevertheless, as described in a companion study (14), the ability of LF-IFN to inhibit lentivirus replication in cultured macrophages, and to induce expression of Ia antigens of the major histocompatibility complex in macrophages, is consistent with findings in infected tissues where virus replication is restricted and Ia antigen expression in macrophages is at a high level. LV-IFN may therefore be produced in inflamed tissues in quantities too small to be detected in the IFN biological assay but effective enough at the cellular level to restrict virus replication in macrophages and influence the cellular immune responses to the virus.

Summary

Lentivirus infections are characterized by a persistent, restricted type of virus replication in tissues. Using sheep and goat lentiviruses, whose target cells in vivo are macrophages, we explored virus-host cell interactions to determine whether an interferon (IFN) is produced during virus replication in vivo which causes restricted replication. We show that the lentiviruses were incapable of inducing IFN directly in any infected cell, including macrophages and lymphocytes. However, after infection with these viruses, sheep and goat macrophages acquired a factor that triggered IFN production by T lymphocytes. Only sheep/goat lentiviruses were capable of inducing the factor and, although these viruses replicated productively in various cell cultures of the natural host animal, only infected macrophages developed the IFN-inducing factor. The factor was produced continuously and was strictly cell associated, requiring direct contact with lymphocytes. The lymphocytes responded with a single, sudden release of IFN beginning 7 h after cocultivation and reaching peak values at 48 h, after which they ceased production and became refractory. IFN production was not immunologically specific and did not require histocompatibility between donors of the two cell types. The IFN is a nonglycosylated protein of molecular weight 54,000–64,000, and is stable to heat and acid treatments. These findings identify a unique IFN and a new method for virus induction of IFN. The novel two-stage process of induction provides a mechanism for local amplification and continuity of production of IFN in vivo. This is compatible with infection in the animal

whose lentivirus-induced pathologic lesions consist of accumulations of lymphocytes and infected macrophages in target tissues.

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