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Immune Interferon Production by Lymphoid Cells: Role in the Inhibition of Herpesviruses

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Bovine peripheral blood lymphocytes (PBL) obtained from infectious bovine rhinotracheitis (IBR) virus- and tuberculin-immunized animals produced large quantities of interferon within 24 h of in vitro stimulation by IBR and purified protein derivative antigens. Separation of PBL into populations enriched in T lymphocytes or B lymphocytes suggested that the T lymphocyte provided the antigen-specific step for immune interferon production. A 2- to 10-fold increase in interferon occurred when lymphocytes were combined with autologous macrophages. Although macrophages, even if treated with antilymphocyte serum to remove any contaminating lymphocytes, could produce some interferon, the augmented interferon produced by macrophage-lymphocyte cultures was not due to an additive effect of interferon from macrophages and lymphocytes. Direct physical contact between macrophages and lymphocytes was required for the production of enhanced levels of interferon. Antigen-antibody complexes of irradiated virus-infected cells in the presence of antibody were as efficient or better at stimulating interferon than was free antigen. Because IBR virus was inhibited by interferon levels stimulated in cultures by IBR antigen, it was suggested that the local production of interferon by immune cells might play a similar role in curtailing virus dissemination in vivo, thus leading to recovery from disease.

If one could define the full spectrum of host defense mechanisms against herpesviruses, their mode of action, and the interplay between them, it might be possible to explain why some individuals are subject to frequent recrudescent herpes whereas others remain asymptomatic. The roles of antibody (1, 3, 24), direct T cell cytotoxicity (34, 42), antibody-dependent cytotoxicity (23, 32, 34, 39), and cell-mediated inhibition of virus cytopathology (19, 30, 31), as well as the role of various lymphokines (25, 26, 50), have been investigated, but their exact function in controlling disease is not known. Much of the data with regard to these parameters is negative; i.e., levels of neutralizing antibody (3, 24), antibody-complement cytotoxicity (25), antibody-dependent cytotoxicity (34), T cell cytotoxicity (34), and lymphocyte transformation (25, 35, 41) do not appear related to the occurrence of recrudescence.

Recently a form of interferon was reported, the induction of which occurred on an immune-specific basis (9, 10, 12, 48). This immune interferon is distinguishable from "classical" interferon not only by its mode of induction and probable cells of origin, but also by biochemical and immunochemical characteristics (12, 48, 51). Merigan and his colleagues have examined the induction requirements for immune interferon from immune human leukocytes exposed to herpes simplex virus antigen (25, 47). The cell providing the antigen-specific step appeared to be a T lymphocyte, but macrophages were required for optimum interferon production. This work could imply that the local release of immune interferon is an important mechanism of controlling the extent of herpesvirus lesions. However, it was not shown whether the amounts of interferon released were protective against herpes simplex virus.

In the present communication, we have examined the question of immune interferon production by bovine herpesvirus-stimulated immune bovine leukocytes. This system has certain advantages in that large cell samples can be repeatedly collected, experimental infections are possible, and, most important, the system uses components almost exclusively of bovine

origin. We demonstrate the kinetics of interferon production and the cell types involved and, in addition, evaluate the activity of interferon produced against the virus that stimulated its production. Our results are discussed in terms of the possible role of immune interferon in controlling herpesvirus infections in vivo.

MATERIALS AND METHODS

Animals and immunization. Young female bovines were immunized once intranasally and then intramuscularly on three separate occasions at monthly intervals with 10^9 plaque-forming units (PFU) of infectious bovine rhinotracheitis (IBR) virus. On two occasions, the intramuscularly injected virus was emulsified in Freund adjuvant containing 10 mg of mycobacterium tuberculosis H37-RA per ml (Difco). These animals were used to obtain both mammary gland macrophages and peripheral blood lymphocytes in all experiments described subsequently. Normal animals were housed separately to prevent possible cross-infection with IBR virus.

Viruses and antigens. Strain P8-2 of IBR virus was prepared as previously described (24). Briefly, confluent Georgia bovine kidney (GBK) cells were grown to confluency in petri dishes (150 mm) and infected with 0.6 ml of IBR virus (approximately 0.1 to 0.5 PFU/cell). Virus was allowed to adsorb at 37 C for 90 min before the addition of fresh culture medium. After 2 days in culture, all the cells showed virus cytopathology and approximately 90% were detached. The cells remaining attached were removed into the culture fluids by vigorous pipetting. All the cells and the culture fluids were subjected to two freeze-thaw cycles. Cellular debris was removed by centrifugation at $1,000 \times g$ for 10 min. The virus was then pelleted by centrifugation at $40,000 \times g$ at 4 C for 1 h. The virus pellet was resuspended in a minimal amount of Puck solution F (PS), and 0.5-ml samples were layered onto a 12-ml gradient of 10 to 30% (wt/wt) sucrose in PS. The gradients were centrifuged at $35,000 \times g$ at 4 C for 40 min. Fractions were collected by puncturing the bottom of the tube and assayed for infectivity. The fractions containing the most infectivity were pooled and repelleted at $40,000 \times g$ for 1 h at 4 C. This constituted purified virus antigen (equivalent to 10^8 PFU/ml). It was inactivated by exposure to ultraviolet irradiation as described previously (29). To obtain infected irradiated cellular antigens, confluent GBK cells were infected with 1 PFU of IBR virus per cell. Twenty-four hours later, the supernatant fluids were removed and the monolayers were irradiated for 5 min at a distance of 12 cm from two General Electric G875 ultraviolet lamps. The cells were then removed from the monolayer with the aid of a rubber policeman and resuspended in PS. Control uninfected cell cultures were treated in a similar manner.

In experiments requiring antigen-antibody complexes, IBR virus or virus-infected cells were treated for 1 h at 37 C with 50 neutralizing units of heat-inactivated bovine anti-IBR antiserum.

Vesicular stomatitis virus (VSV Indiana strain) was cultured and titrated in GBK cells.

Purified protein derivative (PPD) was obtained from Connaught Laboratories, Willowdale, Ontario, as a sterile solution of 4 mg/ml.

Cells. GBK cells were cultured in Eagle minimal essential medium (MEM). Each liter was supplemented with 2 mmol of glutamine, 10 ml of nonessential amino acids (GIBCO no. 114), 50 mg of gentamicin, and 2.5 g of sodium bicarbonate. For growth, the medium contained 10% fetal calf serum (FCS) and, during interferon assays, 5% FCS. Monolayers for interferon assays were prepared by seeding 50×10^3 cells into each of the 96 wells of a Falcon plastic tissue culture plate (no. 3040). Monolayers were confluent within 24 h and were used at this time for interferon assays. All cultures were incubated at 37 C in a humidified CO_2 (5%) atmosphere.

Preparation of lymphocytes. Blood from normal and immune animals was collected by venipuncture into a syringe containing preservative-free heparin (5 IU per ml of blood collected). The buffy coat was obtained after centrifugation at $800 \times g$ for 20 min at 4 C, and these cells were diluted in PS. These leukocyte-rich cultures were layered onto a 3-ml volume of Ficoll-Hypaque (density at 25 C, 1.077 g/cm^3) (Ficoll-Pharmacia; Hypaque-Winthrope) in a round-bottom centrifuge tube (13 by 120 mm). After centrifugation at $400 \times g$ (at the interface) for 20 min at 25 C, the lymphocyte-enriched cells were collected from the interface. These cells were washed once in PS, and the few erythrocytes were lysed with 0.83% ammonium chloride (5 min at 37 C) and then washed twice more in PS before enumeration. These cells were called peripheral blood leukocytes (PBL) and consisted of 98 to 99% mononuclear cells and the rest polymorphonuclear leukocytes. The PBL were further processed as described previously (30) to remove adherent cells by passage over glass wool and to obtain T and B lymphocyte-enriched cell subpopulations by the nylon wool technique of Julius et al. (17). The nylon wool (Leuko Pak, Fenwal Laboratories, Maston Grove, Ill.) was washed for several days in many changes of double-distilled water and then dried, carded, and packed to the 7- or 8-ml mark of a 12-ml plastic disposable syringe. The entire assembly was sterilized by autoclaving. Glass wool columns were prepared in a similar fashion. Several volumes of warm (37 C) PS containing 5% fresh autologous plasma (AP) were passed through the columns. The air pockets present in the column were removed with the aid of a sterile Pasteur pipette, and the columns were sealed with sterile parafilm and allowed to equilibrate for 45 to 60 min at 37 C. Bovine PBL were suspended in PS + 5% AP at a cell concentration of 50×10^6/ml. Three milliliters of this cell suspension was applied to a glass wool column (equipped with a 23-gauge needle) and slowly allowed to percolate into the column. After all the cells entered, the column was once again sealed and incubated at 37 C for 45 to 60 min. The cells were eluted with 25 to 30 ml of PS + 5% AP, and these constituted glass wool-purified (GWP) cells. The GWP cells were resuspended at a concen-

tration of 50×10^6/ml in PS + 5% AP, and 2 ml was applied to a nylon wool column. The cells were washed into the column with 1 ml of PS + 5% AP. The column was sealed and incubated at 37 C for 45 to 60 min. After incubation, the cells were eluted with 25 ml of PS + 5% AP. Most of the cells that would elute did so in the first 10 ml. These effluent cells were referred to as the T cell-rich subpopulation. Previously we showed that this subpopulation contains between 0.8 and 2.0% immunoglobulin-bearing cells and responds well to T cell mitogens but poorly to lipopolysaccharide (LPS) (30).

The cells that did not elute were called the B cell-rich subpopulation. These cells were recovered by placing the nylon wool in a small beaker, adding 5 ml of PS + 5% AP, and teasing the nylon wool with a sterile 5-ml pipette. The nylon wool was then packed, and the supernatant fluids containing the adherent cells were harvested. This procedure was repeated a second time. These cells consist of between 25 and 42% immunoglobulin-bearing cells and respond well to LPS and poorly to T cell mitogens (30).

The cells obtained by these procedures were invariably >98% viable as determined by trypan blue dye exclusion.

Preparation of macrophage cultures. Bovine mammary macrophages were obtained by infusing 5 ml of PS containing 1% (wt/vol) LPS of *Escherichia coli* O128 B12 (Difco) into each teat canal as described previously (48). Six hours later, the teat canal was flushed to remove polymorphs by infusing an additional 15 ml of PS followed by milking out the fluids. Four days later, 15 ml of PS was once again injected into each teat canal. The udder was massaged and the cells were milked out. Each quarter yielded approximately 50×10^6 cells. The cells were washed three times in ice-cold PS, enumerated, and diluted in MEM + 15% FCS to the correct cell concentration. One milliliter of the cell suspension was seeded into each of the 24 wells of a Linbro plastic tissue culture plate (no. 16-24-TC). After a 6-h adsorption period at 37 C, the nonadherent cells consisting predominantly of lymphocytes and polymorphs were removed by washing the cultures twice with 2 ml of PS, after which fresh MEM + 10% FCS was added. Three days later the cultures were once again washed and used for the production of interferon (see below). To insure the complete removal of all lymphocytes from the macrophage cultures, in some experiments the cells adhering after 3 days in culture were treated for 1 h at 37 C with fresh bovine complement and rabbit anti-bovine lymphocyte serum (ALS) (final concentration, 1/60 ALS and 1/40 complement). The monolayers were then washed (three times) with fresh medium before their use in the production of interferon.

Production of interferon by macrophage-lymphocyte cultures. Established bovine mammary macrophage cultures were washed, and 3×10^6 lymphocytes (PBL, GWP, or T or B cell-enriched subpopulations) in 1 ml of RPMI 1640 + 10% FCS were added to each culture. Simultaneously, ultraviolet-irradiated IBR virus (equivalent to 5×10^6 PFU/culture) or PPD (12.5 μg/culture) was added. Control cultures contained macrophages + lymphocytes, macrophages + antigen, or lymphocytes + antigen (in the absence of macrophages). Cultures were incubated at 37 C in a humidified CO_2 (5%) incubator for 3 days, after which time the culture fluids were gently aspirated, centrifuged to remove any contaminating cells, and stored frozen at −70 C until titrated to determine the levels of interferon.

Interferon assays. Interferon was assayed by determining its ability to inhibit the plaque formation by VSV in GBK cell cultures. Confluent GBK cells were exposed to twofold dilutions of interferon samples diluted in MEM + 5% FCS. After 24 h, the interferon samples were aspirated and the monolayers were infected with VSV. After a 2-h absorption period, virus was removed and the monolayers were overlaid with MEM containing 1% methylcellulose (23) for a further 24 h. The methylcellulose overlay was removed by vigorously inverting the plate. The monolayers were washed with saline, fixed, and stained with 1% gentian violet in 70% ethanol. Excess dye was removed by washing in distilled water, the plates were dried, and interferon was quantitated by recording the dilution of interferon that was capable of inhibiting plaque by 50% over that of controls. Thus, 1 unit of interferon inhibited VSV plaque formation by 50%. All samples from any one series of experiments, done in quadruplicate or six replicates, were titrated at one time to reduce variability within the assay. An interferon standard was included to compare reproducibility from day to day. The results are expressed as geometric mean units.

Parabiotic chambers. In experiments designed to physically separate macrophages from lymphocytes, the macrophages were cultured in Linbro plates (no. 16-24-TC) as described previously. Plastic cylinders (12-mm diameter) containing a 0.4-μm membrane (Nucleopore no. 40 CPR 047 00, General Electric, Pleasanton, Calif.) on one side were then inserted into the wells containing macrophages. The distance between the Nucleopore membrane and the macrophages was 2 mm. Lymphocytes were then placed into the upper part of the chamber. Stimulating antigen was included on either the side of the macrophage, the side of the lymphocytes, or both.

ALS. Preparation of ALS has been described previously (33). Briefly, rabbits were immunized subcutaneously at 2-week intervals with 10^8 macrophage-depleted PBL. One week after a third injection of PBL enriched in T cells, by nylon wool separation (24), animals were bled. Sera were heat inactivated at 56 C for 30 min, and each 1 ml of serum was adsorbed twice with 5×10^7 cultured adherent bovine mammary cells (consisting of >98% macrophages). Sera were frozen until used. The cytotoxicity titer of the ALS was 1:160 against lymphocytes and 1:10 against macrophages as determined by the microcytotoxicity test (43), using fresh bovine serum as a source of complement. The ALS was used at a final concentration of 1/60 and complement at 1/40.

Blastogenesis of macrophage-lymphocyte cultures. Combination lymphocyte-macrophage cul-

tures (described above) were stimulated with 50 μl of antigen (approximately 5 × 10^6 PFU of IBR and 12.5 μg of PPD). Twenty-four hours before termination of cultures, they were pulsed with 1 μCi of [*methyl*-^{3}H]thymidine (specific activity, 5 Ci/mmol; Amersham/Searle, Oakville, Ontario). At the end of the experiment, the cells were precipitated with 10% ice-cold trichloroacetic acid and further processed as described previously (29).

Preparation of Fc rosetting cells. Sheep erythrocytes (RBC), free of leukocytes, were washed three times with PS and a 5% suspension mixed with a subagglutinating amount of rabbit anti-sheep RBC serum (1:900 final dilution). After incubation at 37 C for 60 min with frequent gentle agitation, cells were washed three times and made into a 1% suspension. Equal volumes of treated sheep cells and lymphocytes (3 × 10^6/ml) were mixed, centrifuged at 200 × *g* for 5 min, and left for 10 min at room temperature. The cells containing rosettes were gently resuspended and applied to a Ficoll-Hypaque gradient (conditions as described above). After centrifugation, the pellet containing rosetting cells was treated with 0.83% NH_4Cl to lyse RBC. The lymphocytes were washed three times and then used in the interferon assays. These short incubation periods detect only very few erythrocyte rosettes, and these latter rosettes are extremely fragile (14).

RESULTS

Interferon production by lymphocyte-macrophage cultures stimulated by antigen. Whereas in the absence of specific antigen neither PBL, macrophages, nor combination cultures produced interferon (<4 units/ml), in the presence of antigen (IBR or PPD) detectable interferon production occurred with all three types of cultures (Fig. 1). Levels from PBL-macrophage combination cultures were always higher, varying from 2- to 10-fold between experiments, than from cultures of PBL alone. Antigen-stimulated immune PBL invariably gave detectable interferon (>16 units), but levels from stimulated macrophages from the same animals were minimal or undetectable (<8 units). Interferon production from antigen-stimulated PBL depleted of adherent cells, by glass wool filtration, was less (two- to fourfold) than from stimulated unfractionated PBL (Fig. 2B). Combination or individual cultures of PBL and macrophages from nonimmunized animals failed to produce interferon upon antigen stimulation, indicating that the interferon detected above was of immune origin. Not shown in Fig. 1 are the results of experiments in which allogeneic combinations of immune lymphocytes and normal macrophages were investigated for antigen-induced interferon production. These results were irregular, with some interferon (mean, 7 units) always being produced in the absence of antigen. However, interferon levels after antigen stimulation were higher than in

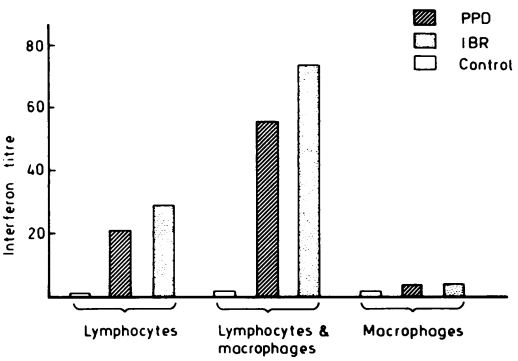

FIG. 1. *Interferon production by immune lymphocytes and macrophages. Cultures were prepared, as described in the text, containing 1 × 10^5 macrophages and 3 × 10^6 PBL. These were maintained in the presence of IBR virus (equivalent to 5 × 10^6 PFU/culture) or PPD (12.5 μg/culture) antigens, or in the absence of antigen, for 3 days. One unit of interferon was capable of inhibiting VSV plaque formation by 50%. The results are expressed as the mean of quadruplicate determinations.*

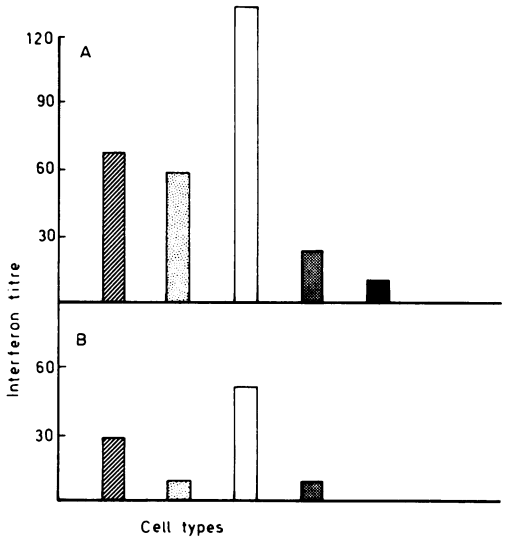

FIG. 2. *Interferon production by PBL and cell subpopulations. Various cell separation techniques (see text) were used to obtain PBL (▨), GWP (▩), T cell-enriched (□) or B cell-enriched (■) subpopulations of cells. (A) 3 × 10^6 lymphocytes were added to established cultures of macrophages (1 × 10^5 cells/culture). (B) 3 × 10^6 lymphocytes in the absence of macrophages. All cultures were stimulated with 5 × 10^6 PFU equivalents of ultraviolet-inactivated IBR virus and harvested 72 h later. Interferon production by 1 × 10^5 macrophages in the presence of IBR antigen is shown in the upper panel (■).*

cultures without antigen (four- to eightfold) but less than antigen-stimulated autologous combinations by two- to eightfold. Combinations of normal lymphocytes and "immune" macro-

phages gave variable and low levels (average, 4 units) of interferon both in the presence or absence of antigen. The influence of antigen dose on immune interferon production is shown in Table 1. Both PPD- and IBR-stimulated lymphocytes produced more interferon with increasing concentration of antigen. Above a certain concentration of antigen (PPD, 25 μg/ml; IBR, 5 × 10^6 PFU/ml), the rate of increase per unit of antigen declined.

In Fig. 2 are the results of experiments designed to investigate the nature of the cell type producing the interferon. Antigen-stimulated enriched T cell subpopulations always gave higher levels of interferon than either PBL- or B cell-enriched populations. This was true both in the presence (Fig. 2A) and absence (Fig. 2B) of autologous macrophages. Because the B cell-enriched subpopulation still contained many T cells (about 40% of the T cells found in PBL as measured by the E rosette technique), it is possible that the interferon produced by these cells was also of T cell origin. A further enrichment of B cells was achieved by isolating Fc receptor-bearing cells by forming Fc rosettes with antibody-coated RBC followed by isolation of rosetting cells by Ficoll-Hypaque flotation. Under the conditions used, only very few cells formed direct E rosettes, and they were very unstable (14). Upon antigen stimulation, these B cell-enriched subpopulations produced threefold less interferon than did nylon-adherent cells (Table 2), further supporting the notion that the origin of antigen-induced interferon was the T cell.

The results shown in Fig. 1 and 2 indicate that whereas T cells are needed for antigen-induced interferon production, macrophages are required to amplify the response. Non-macrophage cells would not suffice for this amplification – neither combinations of T cells with autologous fibroblasts nor with heterologous GBK cells gave enhanced interferon levels as compared with stimulated T cells alone (data not shown).

TABLE 1. *Effect of antigen concentration on interferon titers produced by macrophage-lymphocyte culture*[a]

Antigen	Concn	Interferon titer	Units of interferon/unit of antigen
PPD	0.5 μg	4	
	2.5 μg	9	3.6[b]
	5.0 μg	24	4.8
	25 μg	52	2.1
	50 μg	66	1.32
	100 μg	62	0.62
IBR	2 × 10^5 PFU equivalents	<4	
	5 × 10^5 PFU equivalents	11	22[c]
	10^6 PFU equivalents	37	37
	5 × 10^6 PFU equivalents	78	15.6
	10^7 PFU equivalents	94	9.4
	2 × 10^7 PFU equivalents	116	5.8

[a] Cultures contained 10^5 mammary macrophages and 3 × 10^6 PBL. Cultures were harvested 3 days post-stimulation.
[b] Units of interferon per microgram of PPD.
[c] Units of interferon per 10^6 PFU equivalents of ultraviolet-irradiated virus.

TABLE 2. *Interferon production by subpopulations of peripheral blood lymphocytes*

Antigen	Cell type		
	Effluent[a]	Adherent[b]	Fc bearing[c]
IBR (5 × 10^6 PFU)	64	14	<4
PPD (12.5 μg)	16	7	ND[d]

[a] Cells present in the effluent after nylon wool separation.
[b] Cells adhering to the nylon wool column.
[c] Adherent cells further purified to remove non-Fc-bearing cells (see text).
[d] ND, Not done.

Influence of number of macrophages and lymphocytes on immune interferon production. The observation that antigen-stimulated glass wool-depleted PBL gave less interferon than unfractionated cells prompted experiments to quantitate the mononuclear phagocyte requirement for optimum interferon production. As few as 10^4 autologous mammary macrophages (0.3%) augmented the response of 3 × 10^6 T cells (Fig. 3). Maximum augmentation occurred with 10^6 macrophages (highest number tested). Macrophages alone at concentrations of 5 × 10^5 or greater also produced interferon upon antigen stimulation. This effect was not entirely due to residual contaminating T cells since removal of these cells by treatment with ALS and complement reduced, but did not abolish, interferon production (Table 3). That induction was not caused by contaminating endotoxin in the antigen preparations was indicated by experiments showing that antigen-stimulated macrophages from nonimmunized animals did not produce interferon (Table 3). Our results indicate, therefore, that some macrophages may be specifically "armed" (26) and that these cells respond to antigen and produce interferon.

To determine the number of immune lymphocytes required to produce 1 unit of interferon, various numbers of lymphocytes were added to cultures containing a fixed number of autologous macrophages (10^5/culture). As few as 10^5 T lymphocytes produced detectable amounts of interferon, and optimum production occurred with 3 × 10^6 cells per culture (Fig. 4). An approximate calculation revealed that over the T cell concentration of 10^5 to 3 × 10^6 cells

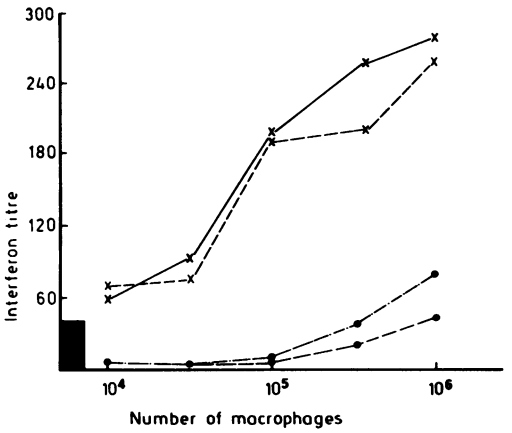

FIG. 3. *Effect of varying the number of macrophages on interferon production by lymphocyte-macrophage cultures. Combination cultures contained 3 × 10⁶ T cell-enriched lymphocytes. The results of two experiments are shown. The response of T cells with macrophages (×——× and ×——×) is compared with the response of macrophage alone (● and ⊙). Interferon levels were determined 72 h after the addition of antigen. The solid block shows interferon levels produced by T cells in the absence of macrophages.*

TABLE 3. *Effect of antilymphocyte serum (ALS) and complement (C) on interferon production by bovine mammary marcophages*[a]

Stimulant (no. of macrophages)	Immune macrophages			Normal macrophages (control)
	ALS[b]	ALS + C[c]	Control	
IBR[d]				
(5 × 10⁵)	3.5[e]	3.5	4	3
(1 × 10⁶)	28.5	18	32	5
(5 × 10⁶)	50	36	57	9
PPD[f]				
(5 × 10⁵)	3	3.5	3	3.5
(1 × 10⁶)	18	9	14	4
(5 × 10⁶)	36	18	36	8.5

[a] Bovine mammary macrophages were cultured for 3 days, at the cell numbers indicated, before being treated with the respective reagents.

[b] Treatment was for 1 h at 37 C. ALS was used at a final dilution of 1/60.

[c] Treatment for 1 h at 37 C. Fresh bovine serum was used as a source of complement. Complement treatment alone had no effect on interferon production.

[d] Stimulated with approximately 5 × 10⁶ PFU of IBR virus.

[e] Units of interferon; geometric mean of six replicates.

[f] Stimulated with 12.5 μg of PPD.

per culture, it took 0.5 × 10⁴ to 2 × 10⁴ cells to produce 1 unit of interferon (Fig. 4). At 10⁷ T cells per culture, a substantial increase in the number of cells was required to produce 1 unit of interferon. Presumably, at high cell densities lymphocyte death was occurring because of depletion of nutrients and possibly release of toxic products. Those cultures were invariably more acid than cultures containing 3 × 10⁶ or fewer lymphocytes.

Kinetics of interferon production. In antigen-stimulated immune combination cultures containing optimum numbers of lymphocytes and autologous macrophages, interferon production was first detectable as early as 8 h and peak levels were attained between 24 and 48 h of culture (Fig. 5). The pattern of response was similar for PBL except that levels of interferon

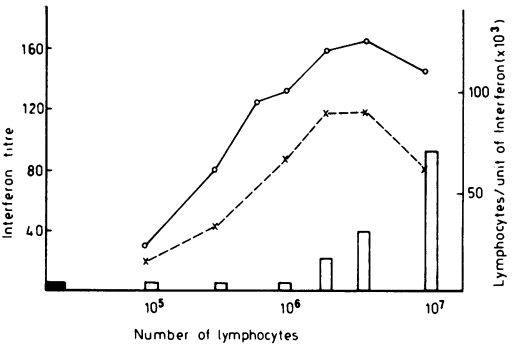

FIG. 4. *Effect of varying the number of lymphocytes on interferon production by macrophage-lymphocyte cultures. Various numbers of PBL (×) or T cell-enriched subpopulation (○) were added to a constant number (1 × 10⁵) of autologous macrophages. The cultures were harvested 72 h after the addition of 5 × 10⁶ PFU equivalents of ultraviolet-irradiated IBR virus. Control cultures (■) contained 1 × 10⁵ macrophages. The number of lymphocytes required to produce 1 unit of interferon at various lymphocyte concentrations is indicated by the histogram (▣).*

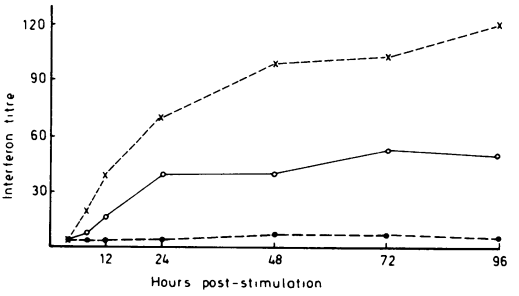

FIG. 5. *Kinetics of interferon production in macrophage-lymphocyte cultures. Cultures containing 1 × 10⁵ macrophages plus 3 × 10⁶ T cell-enriched lymphocytes (×) or 3 × 10⁶ PBL (○) were stimulated with 5 × 10⁶ PFU equivalents of ultraviolet-irradiated IBR virus. At various times, post-stimulated duplicate cultures were harvested and the amount of interferon was quantitated. The amount of interferon produced by macrophage cultures in the presence of antigen (●) is shown.*

were always lower and were not detectable until 12 h after stimulation. That the interferon produced by 24 to 48 h was remarkably stable was evidenced by the observation that peak interferon levels were maintained in cultures kept for 7 days (data not shown). The kinetics of immune interferon production was examined in several animals. Although the levels varied, the kinetics of production did not.

Relationship between blastogenesis and interferon production. To determine whether there was a direct relationship between antigen-induced proliferation and interferon production, PBL and syngeneic macrophages were stimulated with both a viral antigen (IBR) and a nonviral antigen (PPD). Although the lymphocytes were capable of producing interferon as well as undergoing blastogenesis in response to both antigens, the quantity of interferon produced was not related to the degree of proliferation (Table 4). Thus, high quantities of interferon were produced in response to IBR antigen even though only moderate proliferative responses were occurring. Whereas peak interferon production occurred at 24 to 48 h of culture, peak proliferation occurred at 4 to 5 days. The proliferative response to PPD was greater than to IBR, but PPD was a poorer stimulant of interferon production.

Requirement for physical contact between lymphocytes and macrophages for enhanced interferon production. The results thus far indicate that T lymphocytes produce interferon but that the response is enhanced by the presence of macrophages. Enhancement occurred with numbers of macrophages that alone failed to produce interferon on antigen stimulation. Macrophages could enhance the response in several ways, which include: (i) optimizing antigen presentation (27); (ii) releasing processed antigen or soluble factors to trigger cells (28); or (iii) improving the culture conditions such as also occurs by adding 5×10^{-5} M 2-mercaptoethanol (2ME) to cultures (31, 32). To differentiate possibility (i) from (ii) and (iii), three types of experiments were performed. In the first approach, the effect of adding culture fluids from autologous antigen-stimulated or unstimulated macrophages to PBL was investigated. This approach did not lead to enhanced interferon production (Table 5). The addition of 5×10^{-5} M 2ME also failed to consistently enhance interferon production by antigen-stimulated PBL. Finally, if macrophages were physically separated from lymphocytes by Nucleopore membranes (0.4-μm pore size), augmentation of interferon production did not occur (Fig. 6). Taken together, these approaches would tend to discount the role of macrophages as supplying a soluble augmenting factor or improving culture conditions but support the

TABLE 5. *Interferon production by lymphocytes cultured in the presence or absence of macrophage supernatants*

Culture fluid additives	Interferon titer	
	Unstimulated lymphocytes	Stimulated lymphocytes[a]
Control MEM[b]	<4	32
Control supernatant[c]	<4	28.5
Stimulated supernatant[d]	±4	36
2ME (5×10^{-5} M)	±4	32

[a] 12.5 μg of PPD per ml was added to the cultures.
[b] 20% MEM added to lymphocyte cultures.
[c] 20% culture fluids obtained from 5×10^5 macrophages cultured for 3 days in MEM.
[d] 20% culture fluids obtained from 5×10^5 macrophages stimulated by PPD (12.5 μg/ml) for 3 days.

TABLE 4. *Stimulation of immune lymphocyte-macrophage cultures by IBR virus and PPD antigens*[a]

Antigen	Stimulation on day:									
	1		2		3		4		5	
	Counts/min ($\times 10^{-3}$)	IF[b]	Counts/min ($\times 10^{-3}$)	IF	Counts/min ($\times 10^{-3}$)	IF	Counts/min ($\times 10^{-3}$)	IF	Counts/min ($\times 10^{-3}$)	IF
IBR[c]	7.4	98	7.5	132	18.5	128	45.4	ND[d]	88.4	102
PPD[e]	9.4	32	11.8	42	45.4	36	160	ND	158.1	28
–	9.8	<4	5.2	<4	6.5	<4	8.6	ND	4.7	<4

[a] Cultures contained 5×10^5 mammary macrophages and 3×10^6 PBL. Antigens were added at the initiation of culture. Interferon titers were measured on one set of quadruplicate cultures at the times shown, and the proliferative response was measured on a second set of cultures. To measure proliferation, cultures were pulsed with [^{3}H]thymidine for 18 h and terminated at the times shown.
[b] Interferon titer of quadruplicate determinations.
[c] 10^6 PFU/culture.
[d] ND, Not done.
[e] 12.5 μg/culture.

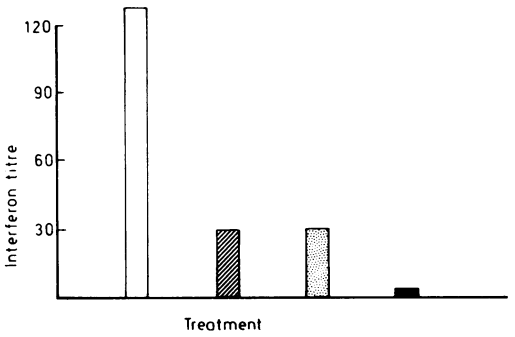

FIG. 6. *Requirement for physical contact between macrophages and lymphocytes for the maximum interferon response. 3 × 10⁶ T cell-enriched lymphocytes were cultured with IBR virus while in contact with 1 × 10⁵ macrophages (□) or separated by Nucleopore membranes containing antigen on both sides of the Nucleopore membrane (▨) or only on the side of the lymphocytes (▩). The quantity of interferon produced by 1 × 10⁵ macrophages after stimulation with antigen was minimal (■).*

possibility that they are involved in a process requiring cell contact such as antigen presentation.

Interferon production by virus-infected cells and antigen-antibody complexes. In vivo, persons with recrudescent herpes have antiviral antibody (3, 24), so one would expect to find virus antigen-antibody complexes (7). In addition, virus membrane antigens are found on the surface of cells before the appearance of infectious virus or intercellular viral dissemination (1, 19). If such cells with membrane antigen could stimulate an early interferon response, then this could result in the protection of uninfected cells.

Experiments were, therefore, designed to determine whether antigen-antibody complexes and virus-infected cells could stimulate interferon production. The results indicate that antigen-antibody complexes are as efficient as free antigen in stimulating interferon (Table 6). Furthermore, infected ultraviolet-irradiated cells were better interferon stimulators than was free antigen or antigen-antibody complexes. This was the case both in the presence and absence of antibody (Table 6). It remains to be determined whether the enhanced interferon production by infected irradiated cells was because of higher antigen concentration or whether the effect was attributable to optimal presentation of antigen to responder lymphocytes.

Interferon production did not occur in the following control combinations: nonimmune lymphocyte-macrophage cultures stimulated by antigen-antibody complexes or infected irradiated cells; and immune lymphocyte-macrophage cultures stimulated by antibody alone or uninfected irradiated cells.

Inhibition of virus replication by immune interferon. If the interferon produced by immune lymphocytes upon antigen stimulation is to be an effective defense mechanism, the virus inducing it must be susceptible to inhibition by interferon. The effect of interferon on a single cycle of replication of the inducing IBR virus and of VSV is shown in Table 7. Although IBR virus was slightly less sensitive to immune interferon than was VSV, there was a slight reduction (27%) in virus yield even from cells pretreated with interferon for only 6 h. The percentage reduction of IBR virus yield increased to 86% when cells were pretreated for 24 h. The most marked reductions of IBR virus yields (99.9%) were evident in cultures pretreated for 24 h followed by the addition of a fresh source of immune interferon for the duration of the replication cycle.

DISCUSSION

We have demonstrated that lymphocytes from herpesvirus-immune animals, upon stim-

TABLE 6. *Interferon production by immune PBL in response to purified IBR antigen or irradiated IBR-infected cells in the presence or absence of anti-IBR antibody*

Stimulating antigen	Interferon titer[a]		
	Expt 1[b]	Expt 2	Expt 3
IBR	50	64	64
IBR-antibody complex[c]	64	64	72
Infected cells[d]	256	256	ND[e]
Infected cells + antibody	228	203	ND
Uninfected cells[f]	3.0	2.5	ND
Antibody	3.0	3.5	ND

[a] Mean titer per milliliter of quadruplicate cultures. Not shown are controls where nonimmunized lymphocyte-macrophage cultures were stimulated with infected irradiated cells or antigen-antibody complexes. These cultures did not produce detectable interferon.

[b] Different preparations of IBR antigen were used in each of the three separate experiments. The same animals were used in each of the three experiments.

[c] IBR virus was reacted for 1 h at 37 C with 50 neutralizing units of anti-IBR antisera. This concentration of antiserum neutralized an equivalent amount of live virus.

[d] GBK cells were infected with 1 PFU/cell. Twenty hours later the cells were irradiated for 5 min by two General Electric G875 ultraviolet bulbs at a distance of 12 cm. 10^4 irradiated infected cells were added per culture.

[e] ND, Not done.

[f] 10^4 uninfected irradiated cells were added per culture.

TABLE 7. *Effect of bovine lymphocyte interferon on single-cycle extracellular virus yields of IBR and VSV*

Treatment	IBR		VSV	
	PFU/ml	% Reduction	PFU/ml	% Reduction
No interferon	2.2×10^5		5×10^6	
Interferon, 6 h[a]	1.6×10^5	27	2×10^6	60
Interferon, 24 h[b]	3×10^4	86.4	3×10^4	99.4
Interferon, 6 h + 20 h[c]	8.5×10^4	61.4	3.5×10^5	93
Interferon, 24 h + 20 h[d]	2.1×10^2	99.9	7.5×10^3	99.9

[a] GBK monolayers treated for 6 h with 64 units of bovine lymphocyte interferon as determined by the VSV plaque reduction assay. Interferon was removed and cells were infected with 0.5 PFU of virus per cell. Cultures were harvested 20 h later.

[b] Same as *a* except that cells were treated with interferon for 24 h before infection.

[c] Same as *a* except that after virus adsorption, interferon-containing medium was added for a further 20 h.

[d] Same as *b* except that after virus adsorption, interferon-containing medium was added for a further 20 h.

ulation with specific viral antigen, produce interferon of sufficient magnitude to be inhibitory to virus replication in vitro. These results are consistent with the hypothesis that immune interferon production may speed recovery from viral disease by limiting viral spread in vivo. Whereas immune PBL, isolated by Ficoll-Hypaque flotation, produced interferon upon either IBR or PPD antigen stimulation, enhanced production occurred if additional autologous macrophages were included in cultures. Macrophages alone failed to produce interferon at numbers sufficient to enhance the PBL response, although at high cell concentrations some interferon was produced by antigen-stimulated macrophages. The macrophage requirement for maximum antigen-induced interferon production was also evidenced by experiments showing that PBL depleted of adherent cells by glass wool filtration produced scant interferon. Our results are in accordance with those of Rasmussen et al. (25), who similarly showed that immune interferon production by human lymphocytes was optimized by the presence of macrophages.

The cell type providing the antigen-specific step was presumably a T cell. Thus cell subpopulations enriched in T cells by the nylon column technique of Julius et al. (17) produced more interferon than did the T cell-depleted nylon-adherent subpopulation. On further depletion of T cells from the latter population, even lower quantities of interferon were produced. Taken together, these findings support those of Valle et al. (47), with herpes simplex-induced interferon production from human lymphocytes, that T cells provide the antigen-specific step for immune interferon production. However, because purified T cells still contain some cells of the mononuclear phagocytic series (about 1%) and their response was enhanced by adding extra macrophages, our results cannot be taken to affirm that interferon was actually produced by T cells. If T cells were producing interferon, then presumably a subset distinct from those undergoing antigen-induced blastogenesis was concerned. Thus, the interferon response occurred early (by 8 h) and peaked between 24 and 48 h, whereas blastogenic responses were barely detectable at 24 h and peaked 4 to 5 days after antigen stimulation. Moreover, the observation that the same population of T cells proliferated more in response to PPD than to IBR antigen, yet the reverse was true with respect to interferon stimulation, also suggests subset differences between T cells measured by the two assays. However, it is possible that the early higher interferon response of IBR-stimulated cells was sufficient to suppress the later proliferative responses of the same cells, since immune interferon is known to be immunosuppressive (2). Solution to the question of whether different T cell subsets are involved in antigen-induced blastogenesis and interferon production must await refinements in cell separation technology.

Macrophages are required for both interferon production and antigen-induced blastogenesis (6, 25, 45), but how such cells function in these responses has yet to be clarified. The results of our present investigation suggest that the macrophage provides a function requiring cell contact rather than a "viability-promoting function" such as can be mimicked by the addition of 2ME to cultures (4, 22, 40). We showed that the addition of neither macrophage-conditioned media nor 5×10^{-5} M 2ME to lymphocyte cultures led to an elevation of induced interferon levels. Even the physical separation of macrophages from lymphocytes by Nucleopore membranes prevented macrophage enhancement. Either macrophages could be functioning as accessory cells presenting antigen to responsive T cells in an optimum configuration, or they could be the actual cells producing interferon on instruction by T cells. If the latter in-

terpretation is correct, the factor must only react over short distances, since we should have noticed enhanced interferon production in the membrane-separated cultures. It is also possible that both mechanisms occur, with some macrophages being required for the initial antigen processing and presentation and the same cells later producing interferon on stimulation by T cells. That macrophages can function in antigen processing and presentation is a well-known phenomenon in immunology (5, 13, 27, 45, 46). In many cases this function of macrophages occurs to the maximal degree only if macrophages and responder cells share the same genotype (27, 28, 38). This was shown to be the case in the present investigation for antigen-induced interferon production. Allogeneic macrophages were severalfold less effective than were autologous cells at enhancing the antigen-induced interferon response of T lymphocytes. At present our results do not permit us to distinguish between the several hypotheses that have been put forth to explain the restricting effect that histoincompatibility has on cell interactions (18, 27, 37, 52). However, for two reasons we do not favor the hypothesis of Zinkernagel and Doherty (52) to explain the restricting effect of histocompatibility on T cell antiviral cytotoxicity. They suggest that T cells recognize not just the viral antigen but also a virus-induced change in histocompatibility antigens of the target cell. In the system we describe, not only does the time seem short for such a change to occur (8 h), but also one would perhaps not expect an inactivated virus to induce such changes. We are currently further examining the Zinkernagel-Doherty hypothesis by comparing the interferon responses to infected macrophage and non-macrophage-stimulating cells of varying histocompatibility and antigen density.

Optimum antigen presentation could be the explanation for why allogeneic virus-infected bovine kidney cells stimulated higher levels of interferon from T cells than did free antigen. Virus-free allogeneic cells did not have a similar effect. An alternative explanation, that the effect occurred because infected cells provided more antigen, was not supported by dose response data that indicated a plateau effect as free antigen was increased beyond a certain level. Experiments are underway using carrier cells of varying surface antigen concentration to try and distinguish between the effects of antigen dose and antigen presentation.

Our finding that immune PBL can produce interferon upon antigen stimulation has implications for the interpretation of previous in vitro observations on anti-herpesvirus immunity. Thus recently we demonstrated that virus cytopathology in vitro could be inhibited by immune T cells (30, 31). The mechanism of inhibition was not established, but we had disregarded a role for interferon mainly on the basis of the reports that herpesviruses were both poor inducers of interferon and insensitive to its effects (15, 36, 44). However, these notions were generated from observations with classical interferon, not immune interferon. These two interferons can be distinguished biochemically and immunochemically, as well as by differences in the means of induction (9, 10, 12, 47, 48, 51). It is possible that IBR virus may differ in susceptibility to the two species of interferon; we are currently investigating this possibility. Certainly IBR viral replication was inhibitable by immune interferon. Moreover, inhibitory concentrations were generated and maintained in antigen-stimulated lymphocyte cultures for up to 7 days. An additional reason for disregarding interferon as being responsible for lymphocyte-mediated inhibition of virus cytopathology was the observation that in order to demonstrate inhibition, it was mandatory to leave the immune lymphocytes in continuous contact with virus-infected cells. Removal of immune lymphocytes from virus-infected cells results in a rapid development of virus cytopathology (31). Similar effects were noted by Lodmell et al. (19). Furthermore, inhibition did not occur in cultures pretreated with immune cells together with inactivated viral antigen if the cells were removed before virus infection. Since one would normally expect interferon-treated cells to remain refractory to virus infection for a considerable length of time, we felt that suppression could not be due to interferon. However, in the present study we observed that inhibition of IBR replication was far greater if interferon was maintained during the entire replication cycle than if the culture were pretreated with interferon and then removed before virus infection (the usual regimen to demonstrate interferon). Thus the demonstration of reliable interferon-mediated inhibition of IBR virus replication required the continuous presence of interferon. These observations would imply that interferon may have been responsible for the immune lymphocyte-mediated suppression of virus replication observed previously (31) and may also explain the claim that IBR virus is resistant to the effects of interferon (44). The leukocyte-mediated inhibition of herpes simplex replication described by Lodmell et al. (19) was also shown to be at least in part mediated by interferon (20).

The major problem with all anti-herpesvirus-immune parameters is that they fail to totally eliminate the virus from infected individuals. Persistent virus, on reactivation from the latent state, can give rise to recrudescent disease (8, 21). Presumably, although immune interferon also does not lead to complete "cure," it might enhance the recovery process in vitro. Because individuals subject to recrudescence have high levels of circulating antibody (3, 24, 25), maybe recovery is delayed in these individuals because such antibody could block viral antigen from stimulating sufficient interferon. However, this was shown not to be the case since interferon stimulation by either antigen-antibody complexes or virus-infected cells in the presence of antibody was equal to or even better than stimulation by free antigen. If immune interferon plays an important role in controlling the extent of recrudescent disease after virus reactivation, one reason why some persons suffer frequent recrudescence could be that there is a subtle defect in immune interferon production. Rasmussen et al. (25) have related inducible interferon levels to the appearance of a further recrudescence, but their work did not clarify whether a defect in immune interferon production was present during clinical disease. It would be instructive to compare individuals of varying herpes history not only for the amount of immune interferon generated during various stages of disease, but also to relate the time and extent of interferon stimulation to the virus-host cell events. This type of in vitro study has been used to assess the potential importance of other immune parameters in IBR virus immunity (50), as well as in herpes simplex (19). If deficiencies in interferon production are found in those subject to frequent herpes, it may be possible to select vaccination protocols that would qualitatively change a person's immune status such that the T cell subset producing interferon is sufficiently expanded to control herpes diseases.

In conclusion, we propose that immune interferon may play an important role in limiting spread of herpesvirus, thus speeding recovery from recrudescent disease. The possible sequence of events after virus reactivation could be a localization of recirculating T lymphocytes in the region of virus replication. Subsets of these T cells, upon reaction with antigen, antigen-antibody complexes, or virus-infected cells, would release some interferon, whereas others would generate chemotactic factors capable of attracting macrophages. The arrival of these latter cells in the lesion would considerably enhance the interferon response. In individuals not subject to disease recrudescence, these early responses may be sufficient to limit the extent of the disease such that clinical signs do not become apparent. Maybe in those subject to recrudescence, the early responses are insufficient to control herpes dissemination and clinical signs develop. Further work is needed both to evaluate the above working model and to describe the relationship and interplay of the immune interferon defense mechanism with other parameters of host anti-herpesvirus immunity.

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