POSITIVE SELF REGULATION OF CYTOTOXICITY IN HUMAN NATURAL KILLER CELLS BY PRODUCTION OF INTERFERON UPON EXPOSURE TO INFLUENZA AND HERPES VIRUSES

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Until recently, interferon (IFN)[1] has been viewed primarily as an antiviral agent that activates enzymes to interfere with viral replication in host cells (1). Its importance, however, might lie beyond this direct activation of viral resistance because increasing evidence points to IFN as a regulator of a multitude of cell-mediated immune responses (2). This property of IFN as well as its ability to inhibit certain tumor cells in culture has been the basis for the current interest in its use in the therapy of cancer patients. One of its key immunoregulatory functions is its ability to augment natural killer (NK) cell activity, which may well be an essential part of the early defense mechanism against infections or tumor development (3). The cells responsible for NK activity exist in circulation in man for most of his adult life (3), and a recent study showed similar results in mice, when peripheral blood lymphocytes (PBL) instead of spleen cells were used as the source of NK cells (4).

All classes of IFN (5–7), including recombinant IFN produced in *Escherichia coli* (8), are effective in augmenting NK activity. Analysis with metabolic inhibitors of the mechanism of the NK augmentation process indicated the need for a short period of RNA and protein synthesis after contact with IFN (9, 10) and it seems likely that new production of receptors for target cells and/or lytic enzymes is important in the boosting of reactivity. In addition to IFN, a variety of biological agents can enhance cytotoxicity in NK cells (11–14). These agents presumably perform this function by inducing IFN, but the cell sources of such IFN are unclear. Virus-infected cells are extremely sensitive to NK lysis, and this sensitivity might be due, in part, to IFN induced in the infected cells and/or in the effector cell population (15–17). There is some evidence that human NK cells can produce IFN during contact with K562 tumor cells (18). However, their IFN-producing capabilities to other substances have not yet been explored.

The present study was designed to determine whether human NK cells could produce IFN in response to viruses, such as influenza and herpes simplex, and

[1] *Abbreviations used in this paper:* FBS, fetal bovine serum; IFN, interferon; LGL, large granular lymphocytes; NK, natural killer, PBL, peripheral blood lymphocytes; PBS, phosphate-buffered saline.

subsequently become augmented in their lytic function. We also considered the ability of other cell types to provide helper function, either by IFN production themselves or by proper presentation of the virus to the IFN-producing cell, thus aiding in the augmentation sequence. This aspect was worth investigating because of previous reports that influenza virus can induce IFN in monocytes (19) and that Sendai virus might induce IFN in B cells (20), whereas HSV-1 has been shown to induce IFN in Fc receptor-negative null cells (21). We demonstrate that large granular lymphocytes (LGL), which possess virtually all of the NK activity and comprise ~10% of the total PBL (22), can produce IFN of either α or γ type in response to influenza virus, and the type induced may depend on the donor's previous encounter with the virus. Herpes simplex, on the other hand, induced mainly IFN-α production by LGL. In parallel with IFN production, enhanced NK activity was seen in LGL in all cases after incubation with the viruses, suggesting positive self regulation of function.

Materials and Methods

Viruses. Influenza A/Port Chalmers/1/73 (H3N2) virus, originally obtained from Dr. Geoffrey Schild, National Institute for Biological Standards and Control, Hampstead, England, was prepared by inoculation in fertile hens' eggs (23). Herpes simplex 1 (HSV-1), Rodanus strain, was prepared by inoculation into Vero cell cultures (24). Both viruses were kindly provided by Martha Wells, Food and Drug Administration, Bureau of Biologics, Bethesda, MD.

Peripheral Blood Lymphocytes. Lymphocytes were obtained from three sources, all located at the National Institutes of Health, Bethesda, MD. The lymphocyte concentrates from the Platelet-Pheresis Center were diluted 1:5 in RPMI 1640 medium containing 10% fetal bovine serum (FBS), 100 μg/ml each of penicillin and streptomycin, 2 mM glutamine, 5 mM Hepes, and 2 U/ml of preservative-free heparin. The buffy coats from the blood bank and the leukopacks from the leukopheresis center were diluted 1:2 in phosphate-buffered saline (PBS). PBL were then prepared from the three types of blood preparations by centrifugation on Ficoll-Hypaque at 400 *g* for 30 min at room temperature.

Discontinuous Percoll Density Gradient Centrifugation. The separation of LGL, which are large lymphocytes with kidney-shaped nuclei and azurophilic cytoplasmic granules from small mature T cells, was accomplished by the use of a discontinuous Percoll density gradient (18). An essential step before the gradient centrifugation was the rigorous removal of adherent cells that tended to band in the lighter fractions close to those of LGL. More than 99% removal of adherent cells was achieved by incubation of the PBL obtained after Ficoll-Hypaque centrifugation on plastic petri dishes (3003; Falcon Labware, Div. of Becton, Dickinson & Co., Oxnard, CA) or tissue culture flasks (Costar Data Packaging, Cambridge, MA 3275) for 2 h at 37°C and a further incubation on nylon columns for 30 min at 37°C. The cells passing through the nylon columns were then placed on a seven-step discontinuous Percoll density gradient that varied in 2.5% concentrations from 40–55%, as has previously been described in detail elsewhere (25). Constant osmolarity of the Percoll solution and the dilution medium (285 mosmol/kg H_2O) was critical for the successful separation of LGL from the rest of the cells. After centrifugation at 550 *g* for 30 min at room temperature, the bands of lymphocytes were collected and checked for LGL morphology on Giemsa-stained cytocentrifuged slides. In this series of experiments, fraction 3 contained 70–90% LGL, and fractions 6–7 contained ≥95% $OKT3^+$ T cells. Monocytes/macrophages were recovered by gentle scraping of the petri dish-adherent cells with a rubber policeman.

Culture Conditions for the Induction of IFN and Augmentation of NK Activity. Based on preliminary experiments that determined the optimum concentrations of viruses and cells, the following procedure was adopted for routine use. The cultures were carried out in RPMI 1640 medium containing 1% FBS, 100 μg/ml penicillin and streptomycin 2 mM L-glutamine, and 5 mM Hepes. 2 million cells from each of the Percoll fractions as well as an aliquot taken from the nonadherent input cell population before Percoll gradient centrifugation were incubated with

medium alone or with a virus, in a final volume of 1.0 ml in 24-well plates (Costar 3524; Data Packaging) for 18 h at 37°C. The final concentrations of influenza A/PC and HSV-1 were 1:1,000 and 1:100 of the stock solutions, respectively. In addition to these cell cultures, an aliquot of each of the subpopulations was placed in medium and kept in the refrigerator for the same length of time, to provide baseline activities. In most experiments, this 4°C control did not differ from the medium control at 37°C and, therefore, those results are not reported.

At the end of the incubation, the supernatants from all the cultures were collected and frozen at −70°C or immediately tested for the content of IFN. Additional controls were included in preliminary experiments, in which the viruses were incubated in the absence of lymphocytes, and the supernatants were collected for the detection of IFN. These controls were necessary because of the possibility that the viruses might induce IFN production in the human fibroblast test cells during the IFN titrations, thus, giving false positive results. However, the virus supernatants were consistently negative in the IFN assay. After collection of the supernatants, the cells were harvested and transferred to 96-well microtiter plates for mesasurement of NK activity.

Removal of T Cells. To eliminate contaminating T cells from the LGL fractions, 0.2 ml of a 1:4 dilution of OKT3 (Ortho Pharmaceutical, Raritan, NJ) was added to 1×10^7 lymphocytes in 0.1 ml of medium. The mixture was kept on ice for 30 min before addition of 0.1 ml of a 1:4 dilution of rabbit complement (Gibco Laboratories, Grand Island Biological Co., Grand Island, NY). After further incubation for 45 min at 37°C in a shaking water bath, the cells were washed and resuspended to 4×10^6 cells/ml. No correction of the viability was done, so that the original number of cells that could produce IFN or be boosted could be retained. Medium and complement controls from the same fractions were included in most of the experiments. In addition, to check for the efficiency of the procedure for removal of T cells, an aliquot of the nonadherent input cells that contained a large proportion of T cells as well as an aliquot of T cells from fraction 7 were similarly treated, with the appropriate medium and complement controls. Cell viability was then checked on all samples.

Removal of B Cells. The procedure was identical to that mentioned above for OKT3, except that that the antibody used was goat anti-IgM (N. L. Cappel Laboratories, Cochranville, PA), and 0.1 ml of the undiluted reconstituted serum was added to 1×10^7 lymphocytes (26). Because nylon wool-passed nonadherent cells were virtually free of B cells, additional controls consisting of whole unseparated PBL were included to check for the proper elimination of B cells. Cell viability was determined in all samples after the various treatments.

Inactivation of Monocytes. Contaminating monocytes in the LGL preparations were inactivated by incubation with silica or carrageenan at final concentrations of 200 μg/ml, 100 μg/ml or 50 μg/ml for 2 h at 37°C (27).

Titration of IFN. The levels of IFN in the culture supernatants were determined by the inhibition of cytopathic effect by vesicular stomatitis virus in human foreskin fibroblasts when performed at the Bureau of Biologics (28) and by Sindbis virus in human WISH amniotic cells when performed at the University of Texas (26). The titers are expressed in international units, standardized with NIH reference human leukocyte IFN (G-023-901-527).

Neutralization of IFN. Anti-IFN-α, prepared by K. Zoon in sheep against an 18,000 mol wt component of lymphoblastoid IFN, had a titer of 900,000 U/ml (28). Sheep anti-IFN-α, kindly provided by Dr. B. Dalton and Dr. C. A. Ogburn, had a titer of 640,000 U/ml (29). Rabbit anti-IFN-β, kindly provided by Dr. Y. K. Yip and Dr. J. Vilcek (30), had a titer of 1:6,000, whereas sheep anti-IFN-β, obtained from Dr. B. Dalton and Dr. C. A. Ogburn, had a titer of 1:60,000. Rabbit anti-IFN-γ, as previously described (31), had a titer of 1:6,000.

Qualitative identification of IFN in the culture supernatants was performed by incubating each IFN sample with 10–100-fold excess of antiserum of each type for 1 h at room temperature. In some experiments, two or more antisera were added to determine the presence of a mixture of IFN species. The IFN/anti-IFN mixture was then diluted serially at half-log dilutions through six wells of a 96-well microtiter plate. These dilutions were then transferred to wells containing human fibroblasts or WISH cells for determination of residual IFN activity. Laboratory standards of IFN-α, β, γ were included in each assay.

Measurement of NK Activity. NK activity was determined by the release of radioactivity from 5×10^3 ^{51}Cr labeled K562 tumor cells after 4 h incubation at 37°C, with lymphocytes at

effector/target ratios of 50:1 and 10:1 in a 96-well microtiter plate (25). The supernatants were collected by the Titertek harvesting system (Flow Laboratories, McLean, VA). The baseline (autologous) lysis was determined by the addition of unlabeled autologous tumor cells to the labeled targets at the same ratios as lymphocytes to target cells. Percent NK activity was then calculated as follows: ([experimental cpm − autologous cpm]/[total cpm incorporation]) × 100.

The autologous control was usually 6–8%, and the standard error in most groups was <1%. Percent cytotoxicity of >2.0% was usually significantly different from the control at $P < 0.05$. For clarity in presenting data in Results, the standard errors were not included, and percent cytotoxicity <1% was expressed as 0% in all cases.

Results

Induction of IFN and Boosting of NK Activity in Cell Fractions Recovered from Percoll Gradients. Results of initial studies to define the cell population(s) that responded to influenza A/PC and HSV-1 are shown in Fig. 1. The fractions incubated with medium alone showed the typical profile of distribution of spontaneous NK activity, with peak activities in fractions 2–3, in which 70–90% of the cells showed LGL morphology on cytocentrifuged slides. The denser cells, particularly in fractions 5–7, which consisted mainly of small lymphocytes with the characteristics of mature T cells, had no spontaneous NK activity. In the presence of the two viruses, the

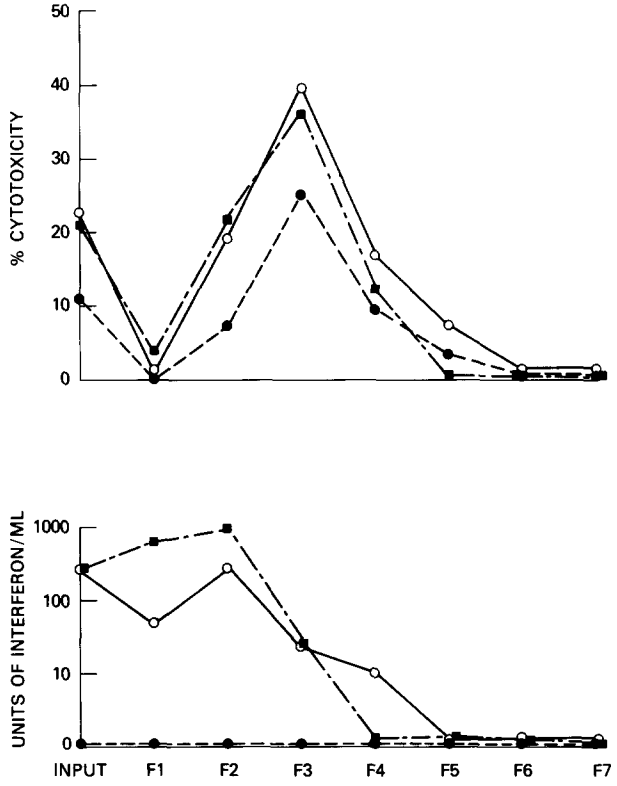

FIG. 1. Augmentation of NK activity and induction of IFN in lymphocytes fractionated by discontinuous Percoll gradients. Each lymphocyte fraction was incubated with medium (●), influenza A/PC virus (■), or HSV-1 (○) for 18 h at 37°C before they were tested for production of IFN and for augmentation of NK activity at a 10/1 effector/target ratio.

nonadherent input cells showed IFN production and boosted NK activity. After these cells were fractionated according to density, only the LGL-enriched fractions 2–3, which initially contained spontaneous NK activity, responded by IFN production. The level of IFN detected was remarkably high, particularly in LGL cultures containing influenza A/PC. As expected, NK activity was also augmented in these same fractions. There was no quantitative correlation between the level of IFN production and the degree of augmentation of NK activity, suggesting (as has been previously reported) (5) that small amounts of IFN may suffice to produce maximum cytotoxicity. IFN was also detected in fraction 1, which was probably produced by monocytes, as will be seen in more detail in experiments to be discussed below. A consistent observation was the lack of IFN production or boosted cytotoxicity in T cells of fractions 5–7.

Kinetics of IFN Production and Boosting of NK Activity. The lack of a T cell response might have been due to insufficient time of incubation with the viruses. Moreover, proper presentation of the virus by adherent accessory cells might have been necessary for stimulation of T cells. To rule out these possibilities, LGL from fraction 3 and T cells from fractions 6–7 were cultured in the absence or presence of 10% monocytes for 1–4 d, with optimum doses of influenza A/PC or HSV-1. In parallel, adherent monocytes, either at the 10% level or at a 10-fold higher concentration, were incubated with viruses (Table I). Again, the LGL responded to both viruses by IFN production and augmented cytotoxicity, and peak levels were observed by day 1. Addition of monocytes did not enhance either function, although monocytes were also capable of producing IFN. It is interesting to note that monocytes, at the concentration added to provide helper function or at the 10-fold higher concentration, still did not produce the levels of IFN production seen with LGL. T cells showed no response in the presence of monocytes, even after 4 d of culture. The low levels of IFN seen in the mixture of T cells and monocytes can be accounted for by production by the monocytes alone.

Elimination of Contaminating T Cells from LGL. Although small mature T cells were not involved in IFN production under the conditions of our assay, other T cells of

TABLE I

Kinetics of IFN Induction and Enhancement of NK Activity by Viruses

Virus	Days of culture	Percent cytotoxicity (units of IFN/ml)*					
		LGL		T cells		Monocytes‡	
		Without monocytes	With 10% monocytes	Without monocytes	With 10% monocytes	10%	100%
None	1	15.3 (0)	12.4 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Influenza A/PC		29.6 (3,125)	25.1 (3,125)	0 (0)	0 (200)	0 (125)	0 (625)
Herpes simplex 1		30.9 (1,250)	25.5 (1,220)	0 (0)	0 (125)	0 (125)	0 (250)
None	4	18.7 (0)	11.3 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Influenza A/PC		28.8 (2,000)	24.6 (3,125)	0 (0)	0 (200)	0 (200)	0 (625)
Herpes simplex 1		28.3 (1,250)	23.2 (1,250)	0 (0)	0 (200)	0 (250)	0 (400)

* Effector/target ratio of 10/1.

‡ Monocytes were incubated with each virus at the concentration that represented 10% (2×10^5 cells/ml) or 100% (2×10^6 cells/ml) of the lymphocyte concentrations.

medium size that could contaminate the LGL fractions might serve as the source. To eliminate this possibility, LGL and the nonadherent input cells were both treated with OKT3 plus complement before incubation with influenza A/PC or HSV-1. As seen in Table II, this treatment eliminated 75% of the input cells, 100% of the cells in fraction 7, and 25% in the LGL-containing fraction. (In other experiments, 10–40% of the LGL fractions were killed, depending on the purity of the samples. This level of OKT3-positive cells is often seen in LGL fractions that have not been further fractionated with 29° E rosetting). The treated LGL were still as potent as the medium or complement control cells in producing IFN and exhibiting enhanced cytotoxicity. The input fraction, when treated with OKT3 plus C, often produced low levels of IFN, (~25 U) in the absence of either virus. This low level might be due to the IFN-inducing capability of OKT3 itself on T cells. An accompanying enhancement of NK activity was also seen.

Elimination of Contaminating Monocytes from LGL. Because LGL-enriched fractions contain small percentages of monocytes, it was necessary to further eliminate the possibility that some monocytes contaminating the LGL fractions might have provided helper function or served as the IFN source. Therefore, the LGL-enriched fractions were incubated with silica or carrageenan for 2 h at 37°C and then cultured with influenza A/PC or HSV-1. Monocytes alone were also treated in a similar fashion, to monitor the efficacy of the monocyte-toxic agents. As seen in Table III, monocytes produced 125 U and 50 U/ml of IFN with influenza A/PC and HSV-1, but this function was lost after treatment with 100–200 μg/ml of silica or 200 μg/ml of carrageenan. Of interest was the ability of silica to inhibit NK activity in LGL but not IFN production, when used at 200 μg/ml. Silica at lower doses and carrageenan at all doses used did not interfere with either of the functions of LGL.

Further proof of the ability of LGL alone to respond to viruses was provided by the simultaneous treatment of LGL with OKT3 plus complement and silica (Table IV). Removal of both T cells and monocytes from LGL had no effect on the IFN-producing capability or the enhancement of cytotoxicity.

Elimination of Contaminating B Cells from LGL. The input cells used in the fractionation procedure had first been passed through nylon wool, which is highly efficient in removing B cells. Because there have been some reports that B cells are capable of producing IFN, particularly to Sendai virus (20) or to heterologous tumor cells (26), we examined the possibility of a small amount of contamination of LGL with B cells

TABLE II

Effect of OKT3 plus Complement on IFN Production and Enhancement of Cytotoxicity in LGL

Cell population*	Treatment	Viability	Percent cytotoxicity (units of IFN/ml)‡		
			None	Influenza A/PC	Herpes simplex 1
Nonadherent	Medium	98%	17.5 (0)	27.1 (625)	25.4 (125)
	OKT3 + C	25%	32.1 (25)	33.9 (1,000)	29.5 (125)
	C	99%	11.4 (0)	29.3 (625)	28.4 (125)
LGL	Medium	98%	28.7 (0)	40.3 (2,000)	43.8 (625)
	OKT3 + C	74%	25.6 (0)	33.1 (2,000)	36.2 (625)

* Fraction 7, containing virtually 100% T cells, showed 0% viability after treatment with OKT3 + C.
‡ Effector/target ratio of 10/1.

TABLE III

Effect of Monocyte-Toxic Agents on IFN Production and Enhancement of Cytotoxicity in LGL

Cell population	Treatment	Concentration	Percent cytotoxicity (units of IFN/ml)*		
			None	Influenza A/PC	Herpes simplex 1
		μg/ml			
Monocytes	Medium	—	0 (0)	0 (125)	0 (50)
	Silica	200	0 (0)	0 (0)	0 (0)
	Silica	100	0 (0)	0 (0)	0 (0)
	Silica	50	0 (0)	0 (50)	0 (40)
	Carrageenan	200	0 (0)	0 (0)	0 (0)
	Carrageenan	100	0 (0)	0 (0)	0 (0)
	Carrageenan	50	0 (0)	0 (125)	0 (50)
LGL	Medium	—	17.3 (0)	27.6 (1,250)	28.1 (250)
	Silica	200	2.7 (0)	2.0 (1,250)	1.8 (250)
	Silica	100	13.8 (0)	24.5 (1,000)	28.5 (250)
	Silica	50	14.0 (0)	23.9 (1,250)	26.7 (200)
	Carrageenan	200	15.4 (0)	29.6 (1,000)	26.8 (250)
	Carrageenan	100	14.2 (0)	30.9 (1,250)	29.5 (250)
	Carrageenan	50	14.8 (0)	28.4 (1,250)	26.4 (250)

*Effector/target ratio of 10/1.

TABLE IV

Effect of Removal of both OKT3+ Cells and Monocytes on IFN Production and Enhancement of Cytotoxicity in LGL

Cell population	Treatment	Concentration	Percent cytotoxicity (units of IFN/ml)*		
			None	Influenza A/PC	Herpes simplex 1
		μg/ml			
Monocytes	Medium	—	0 (0)	0 (200)	0 (50)
	Silica	200	0 (0)	0 (0)	0 (0)
	Silica	100	0 (0)	0 (0)	0 (0)
LGL	Medium	—	32.7 (0)	44.7 (3,125)	43.8 (1,000)
	Silica	200	1.2 (0)	0 (3,125)	0 (0)
	Silica	100	28.4 (0)	38.5 (3,125)	37.6 (1,250)
	OKT3 + C	—	32.9 (0)	38.1 (3,125)	42.2 (1,250)
	OKT3 + C/silica	200	0 (0)	0 (3,125)	0 (1,000)
	OKT3 + C/silica	100	29.5 (0)	40.7 (2,000)	39.8 (1,000)

* Effector/target ratio of 10/1.

that might contribute to IFN production. LGL were thus treated with anti-IgM plus complement, before incubation with the viruses. Table V shows that ~20% of PBL but <1% of nylon wool-passed cells and <1% of LGL were killed by this treatment. The treated cells showed unimpaired IFN production and enhanced NK activity.

Characterization of IFN. Antigenic typing of the IFN produced by LGL and monocytes in response in influenza A/PC and HSV-1 was performed in two separate laboratories, at the University of Texas (Table VI) and at the Bureau of Biologics (Table VII), using different sources of antisera prepared against IFN α, β, and γ. The data from both laboratories were comparable and showed that the IFN produced in LGL or monocytes in most cases was readily neutralized by anti-α and not by anti-β

TABLE V

Effect of Anti-IgM plus Complement on IFN Production and Enhancement of Cytotoxicity in LGL

Cell population	Treatment	Viability	Percent cytotoxicity (units of IFN/ml)*		
			None	Influenza A/PC	Herpes simplex 1
Unseparated PBL	Medium	99%	5.5 (0)	12.3 (200)	14.3 (125)
	Anti-IgM + C	78%	8.6 (0)	15.4 (250)	14.8 (125)
	C	98%	6.2 (0)	14.9 (250)	13.3 (100)
Nonadherent	Medium	98%	12.6 (0)	19.1 (250)	20.1 (50)
	Anti-IgM + C	99%	11.5 (0)	19.3 (125)	19.5 (50)
	C	99%	11.8 (0)	20.2 (125)	18.8 (40)
LGL	Medium	99%	28.1 (0)	35.9 (400)	37.7 (250)
	Anti-IgM + C	98%	26.3 (0)	46.4 (625)	36.1 (125)

* Effector/target ratio of 10/1.

TABLE VI

Neutralization of IFN Induced in LGL and Monocytes by Influenza and HSV-1 Viruses

Source of IFN	Day of culture	Stimulus	Residual IFN activity after treatment with*							
			Medium	anti-α‡	anti-β‡	anti-γ§	anti-α and anti-β	anti-α and anti-γ	anti-β and anti-γ	anti-α, β and anti-γ
LGL	1	A/PC	3,000	<10	3,000	3,000	<10	<10	3,000	<10
		HSV	3,000	<10	3,000	3,000	<10	<10	3,000	<10
	4	A/PC	3,000	<10	3,000	3,000	<10	<10	3,000	<10
		HSV	3,000	<10	3,000	3,000	<10	<10	3,000	<10
Monocytes	1	A/PC	3,000	<10	3,000	3,000	<10	<10	3,000	<10
		HSV	100	<10	100		<10	<10	100	<10
Standard IFN-α			6,000	<100	6,000	6,000	ND‖	ND	ND	ND
Standard IFN-β			3,000	1,000	100	6,000	ND	ND	ND	ND
Standard IFN-γ			6,000	6,000	6,000	<100	ND	ND	ND	ND

* Undiluted cell culture supernates were preincubated with each antiserum for 1 h at room temperature before they were titered for IFN on WISH cells challenged with Sindbis virus.

‡ Anti-α and β were obtained from Dr. B. Dalton and Dr. C. A. Ogburn.

§ Anti-γ was prepared by G. J. Stanton and colleagues.

‖ Not done.

and anti-γ. In some individuals, however, their LGL responded to influenza A/PC by producing IFN that was not neutralized by anti-α or anti-β, but completely by anti-γ, as shown in Table VII. Further analysis of one of these individuals showed that he was seropositive to influenza A/PC.

Discussion

Recently available techniques using discontinuous Percoll density centrifugation for isolation of human LGL, which are primarily responsible for NK function in PBL, have enabled us to examine the NK-IFN system in detail. In particular, we have been able to direct our research to answer the questions: (*a*) whether IFN may be produced by LGL in response to inducers such as viruses; (*b*) whether helper cells may be required for optimum IFN production in LGL; (*c*) whether other classes of lymphoid cells may produce virally-induced IFN; (*d*) whether IFN can activate non-LGL to

TABLE VII

Comparison of IFN Induced in Cells from Donors Seropositive and Seronegative to Influenza

Donor	Source of IFN	Stimulus	Day of culture	Residual IFN activity after treatment with*			
				Medium	Anti-α‡	Anti-β§	Anti-γ‖
Seronegative to A/PC	LGL	A/PC	1	250	<5	250	250
		HSV-1		250	<5	250	250
		A/PC	4	250	<5	250	250
		HSV-1		250	<5	250	200
	Monocytes	A/PC	1	200	<5	200	200
		HSV-1		125	<5	125	125
Seropositive to A/PC	LGL	A/PC	1	250	250	250	<5
		HSV-1		200	<5	200	200
		A/PC	4	250	250	250	<5
		HSV-1		250	<5	250	250
	Monocytes	A/PC	1	250	<5	250	250
		HSV-1		125	<5	125	125
	Standard IFN-α			500	<5	500	500
	Standard IFN-β			500	500	<5	500
	Standard IFN-γ			250	250	250	<5

* Cell culture supernatants diluted to an initial concentration of 125–250 IFN U/ml were preincubated with each antiserum for 1 h at room temperature before they were titered for IFN on human foreskin fibroblasts challenged with vesicular stomatitis virus.

‡ Anti-α serum was prepared by K. Zoon and colleagues.

§ Anti-β serum was obtained from Dr. Y. K. Yip and Dr. J. Vilcek.

‖ Anti-γ serum was prepared by G. J. Stanton and colleagues.

acquire NK function; and (*e*) whether more than one species of IFN can be induced in a single subset of lymphoid cells. The results presented here showed that LGL were remarkably well-equipped to respond to influenza and HSV-1. The response was twofold: a rapid induction of IFN and a parallel enhancement of NK activity. Peak levels of IFN, $\sim 1 \times 10^3$ U/10^6 cells, were induced within 24 h of exposure to either virus, and addition of monocytes to the cultures did not enhance IFN production or raise NK levels further. Other lymphocyte fractions isolated from the Percoll gradient, which made up 90% of the input nonadherent cells, exhibited neither IFN nor NK function in the absence or presence of viruses. We considered the possibility that the failure to observe some response in these non-LGL fractions might have been due to either of two factors (*a*) a slower kinetics in the production of IFN; or (*b*) the lack of a helper population. However, incubation for up to 4 d, with or without the addition of monocytes, did not alter the pattern, and no IFN or lytic activity was observed in the small T cell fractions.

On the other hand, monocytes responded to the viruses by IFN production, but they did not acquire the ability to lyse K562 target cells. The levels of IFN were usually below those produced in LGL. The ability of monocytes to produce IFN during overnight culture with influenza virus has been described earlier (19), and our data are consistent with that previous report. It is interesting to note the IFN produced

in leukocytes on a large scale for clinical trials are virally induced and might in large part be a product of LGL, with only a smaller contribution from monocytes.

The purity of the LGL population was critical for definitive identification of these cells as the source of viral-induced IFN. To leave little doubt about the capability of LGL to produce IFN, the LGL fractions were subjected to further purification by removal of contaminating T cells, monocytes, and B cells. Removal of T cells from the LGL by monoclonal OKT-3 plus complement had no effect on IFN production or enhancement of NK activity. Similarly, incubation of LGL with 100 μg/ml of silica or various concentrations of carrageenan, which inactivated monocytes, did not interfere with the two functions. Of interest was that higher levels of silica could inhibit spontaneous NK activity in LGL, but their IFN-producing capability was not blocked. This result might explain previous in vivo observations of potent inhibition by silica of NK activity in mice (32). It might represent a direct sublethal toxic effect of silica on NK cells rather than the elimination of monocytes that are required for the maintenance of in vivo NK activity. Simultaneous treatment of LGL with OKT3 plus complement and silica provided further proof of the ability of LGL to function alone in response to viruses.

Analysis of the antigenic characteristics of the IFN induced by the viruses with a battery of antisera against IFN α, β, and γ showed that LGL and monocytes, in most individuals, produced IFN-α to both viruses. However, the LGL of a individual with detectable serum antibodies against influenza A/PC, indicating previous exposure to the virus, produced IFN-γ. This is consistent with the recent report of Ennis et al. (33), who used unfractionated PBL from volunteers who were vaccinated with influenza. Our results are also in line with those of Greene et al. (34), who observed only IFN-α in HSV-seronegative individuals but IFNα and IFNγ in seropositive individuals when their PBL were stimulated with HSV-1. A surprising finding was the high levels of IFN-α, and particularly of IFN-γ, from LGL cultured with influenza A/PC. We routinely detected 1,000 U/10^6 LGL and at times obtained 10,000–15,000 U/10^6 LGL, similar to the findings of Ennis et al. (33), suggesting that this might be an efficient means of producing IFN-γ in quantities large enough for research or clinical purposes. The other quite intriguing aspect of this suggestion of IFN-γ production by LGL is that it provides an indication for a specific-immune function by these cells, which have been considered by us and some other investigators to be within the T cell lineage.

The observation that LGL responded to influenza and HSV-1 by IFN production is an important finding because of the implication that NK cells are under positive self-regulation, being able to produce IFN that can in turn activate cytolytic function of these cells. The efficiency of the NK cell system is reflected in their apparent lack of a requirement for helper cells of another cell type, either for the IFN production stage or for the activation of the lytic mechanism. A second finding of equal importance was the lack of augmentation of cytotoxicity in T cells, even in the presence of monocytes that can provide IFN as the boosting agent. IFN appears to enhance cytolytic function only in LGL, indicating that IFN-activatable precursor NK cells do not exist in non-LGL fractions. There has been some suggestive evidence that the human cell type that produced IFN on exposure to virus-induced tumor cells was the same as the cytotoxic effector cell (12). Our results now firmly establish human LGL as the source of both virus-induced IFN production as well as NK

activity. These findings mirror observations made in the mouse that IFN and NK activity are confined to the same set of $Ly5^+$, $Qa5^+$, asialo GM_1^+ spleen cells (17). However, it remains to be determined whether the same subpopulations of LGL with NK activity also are responsible for the IFN production in response to viruses.

Our data do not indicate whether there is a recruitment of new NK cells from an inactive population in the LGL fraction or whether cytolytic activity is enhanced in already active NK cells. Minato et al., in the mouse, have shown a conversion of precursor NK to the active state (17). In the human, Targan and Dorey (35) as well as Timonen et al. (36) have shown that both recruitment of new effector cells and activation of lytic function take place. Timonen et al. (36), in particular, have shown in great detail that the two phenomena occur in the LGL population. Future experiments with cloned LGL cultures should provide a better insight into the exact mechanism of NK augmentation.

Another aspect of considerable interest in the present findings was the ability of LGL to produce more than one type of IFN. It would be of interest to determine whether the different types of IFN are made by separate subpopulations or whether, depending on the nature of and previous exposure to the stimulus, the same cells can produce different IFN species. In any event, these data strengthen the suggestion that NK cells might be important in natural resistance to various microorganisms, particularly in the early stage of infection.

Summary

Augmentation of natural killer (NK) activity by influenza A/PC and HSV-1 viruses appears to be caused by the induction of interferon (IFN) within the NK cell population itself. These viruses induced high levels of IFN production by human large granular lymphocytes (LGL) that could be readily isolated from peripheral blood by Percoll density gradients. These LGL, which have been previously shown to account for and to be highly associated with endogenous NK activity, became augmented in their lytic function during the 18-h period that IFN was induced. Non-LGL helper cells did not appear to be required in the NK-IFN system (either T cells, B cells, or monocytes). Removal of these latter cell types by treatment with OKT3 plus complement, anti-IgM plus complement, or preincubation with silica or carrageenan had no effect on the ability of LGL to respond to the viruses. Production of IFN was also detected, albeit at lower levels, from monocytes cultured for 18 h with viruses, but no cytotoxic activity was induced. On the other hand, T cells, even in the presence of monocytes, showed neither property, and longer cultures, with virus up to 4 d, still did not alter the pattern. The IFN produced by both LGL and monocytes were predominantly IFN-α, as assessed by neutralization assays with antisera to IFN-α, -β, and -γ. In an individual with detectable serum antibodies to influenza A/PC, however, the IFN induced in LGL appeared to be γ, presumably because of specific recognition of the virus. These data suggest an efficient positive self-regulatory mechanism in NK cells that may be readily switched on by viruses.

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