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Effect of Bacterial Endotoxin and Interleukin-2 on Human Leu-11$^+$ NK Cells: Ultrastructural and Functional Correlations

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Abstract

Bacterial endotoxin (lipopolysaccharide, LPS) and interleukin 2 (IL-2) are known to stimulate NK cell mediated cytotoxicity against tumor cells. In the present report we sought to correlate the stimulatory effect of IL-2 and LPS on NK cells with ultrastructural changes which occurred as a result of such stimulation. Peripheral blood mononuclear cells (PBMC) were purified from healthy donors by a Ficoll-Hypaque density gradient technique. Leu-11$^+$ NK cells were isolated by flow microfluorometry using a monoclonal FITC conjugated anti-Leu-11a antibody and a FACS II cell sorter. The PBMC were incubated respectively with *E. coli* LPS or recombinant IL-2 (rIL-2) for various time periods. Sorted Leu-11$^+$ NK cells were incubated with LPS for 24 hr. The NK cytotoxicity contained within the PBMC and sorted Leu-11$^+$ cells was assessed by a ^{51}Cr release technique using K562 tumor cells as targets. Leu-11$^+$ NK cells were identified by immunoelectron microscopy using anti-Leu-11a antibody and labelling

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with horseradish peroxidase or colloidal gold. Results showed that both LPS and rIL-2 significantly enhanced the cytotoxic activity of PBMC and sorted Leu-11$^+$ cells. LPS also stimulated *in vitro* production of interferon in the PBMC and caused ultrastructural alterations in Leu-11$^+$ cells. The morphological changes in Leu-11$^+$ cells included increase of dense granules and small vesicles, dilation of the cisternae of rough endoplasmic reticulum and nuclear enevelope, and increased acid phosphatase activity. Recombinant IL-2 induced a significant increase in the number of dense granules, hypertrophy of Golgi apparatus and rough endoplasmic reticulum, and cell proliferation in Leu-11$^+$ cells 7 days after stimulation. These data suggest that: (1) both LPS and rIL-2 activate human NK cell mediated cytotoxicity against K562 tumor cells; (2) the effect of LPS on the enhancement of NK cytotoxicity in PBMC may be a direct and/or indirect process involving production of lymphokines, such as gamma interferon; (3) LPS has a direct effect on Leu-11$^+$ cells; and (4) the LPS or rIL-2 induced ultrastructural changes in Leu-11$^+$ cells correlate directly with the enhanced NK cytotoxicity.

Introduction

Natural killer (NK) cells are defined as a population of lymphoid cells that mediate spontaneous cytotoxicity against neoplasms and against exogenous intruders, such as viruses, bacteria, and parasites. More recent evidence indicates that NK cells also play a significant role in regulation of the growth and functions of hemopoetic and lymphoid cells. Thus, the studies on NK cells have become a major aspect of immunologic research since they were discovered over a decade ago.

NK cells have been found in a wide variety of animal species including some invertebrates (sipunculids, annelids, arthropods) and the majority of vertebrates (Savary and Lotzva, 1986). In man, NK activity is associated with a subset of large granular lymphocytes (LGL) which are characterized by having Fc receptors for IgG and abundant cytoplasm containing numerous azurophilic granules (Timonen et al., 1981; Saksela et al., 1979; Herberman et al., 1979). Functionally only up to about 80% of LGL are active NK cells (Timonen et al., 1982). This finding clearly indicates that LGL represent heterogenous population of cells with varied phenotypes and functional capabilities.

Various surface characteristics and antigens in human LGL have been observed (Allavana and Ortaldo, 1986). Among these surface markers, HNK-1 (Leu-7) and NKP-15 (Leu-11) are the most important markers and have been broadly used to identify human NK cells in the peripheral blood, tissue fluids, and tissues. The recent development of the commercially available monoclonal antibodies against Leu-7 and Leu-11 antigens permits extensive studies on the morphologic and functional properties of human NK cells. Recent studies using two-color flow cytometry show that human NK cells express various combinations of Leu-7 and Leu-11 antigens (Lanier et al., 1983). NK cells with Leu-7$^-$/Leu-11$^+$ phenotype have been found to be the most potent effector cells in human peripheral blood, whereas the Leu-7$^+$/Leu-11$^-$ subset is the least effective (Lanier et al., 1983, 1984; Phillips and Babcock, 1984). Using an immunogold and immunoperoxidase double-labelling method, we have found that human peripheral blood lymphocytes contain approximately 5% Leu-7+/Leu-11$^-$, 15% Leu-7$^+$/Leu-11$^+$, and 9% Leu-7$^-$/Leu-11$^+$ NK cells (Kang et al., 1987a).

Lymphokines including interferons (IFN) and interleukin-2 (IL-2) have been shown to augment the cytotoxic activity of NK cells (Djeu et al., 1979; Ortaldo et al., 1984; Svedersky et al., 1984; Weigent et al., 1983). In addition, the bacterial endotoxin (lipopolysaccharide, LPS) has also been reported to enhance NK cytotoxicity (Fink et al., 1984; Gangemi et al., 1980; Nowotny, 1985). However, the mechanisms by which LPS and Il-2 exert their effect on the enhancement of NK activity remain unclear. In the present report, we studied the effect of LPS and IL-2 on the ultrastructure of Leu-11+ NK cells by immunoelectron microscopy and sought to correlate these changes with the observed changes in NK cell funtion occurring as a result of this stimulation.

Materials and Methods

Cell preparation

Peripheral blood monuclear cells (PBMC) were obtained by Ficoll-Hypaque centrifugation of heparinized peripheral venous blood from healthy volunteer donors (Boym, 1968). Leu-11+ cells were isolated from PBMC by a FACS II cell sorter using a monclonal anti-Leu-11a antibody conjugated with FITC (Becton-Dickinson Monoclonal Center, Mountain View, CA) according to the method described by Biddison et al. (1981).

Treatment of Cells with LPS and IL-2

PBMC were suspended at a concentration of 1 x 10^6 cells per ml in RPMI 1640 supplemented with 10% heat-inactivated pooled human AB serum (Flow Laboratories McLean, VA), 1% glutamine (Gibco Laboratories, Grand Island, NY), 1% penicillin/streptomycin and 2-mercaptoethanol at a concentration of 5 x 10^{-5} M. Cells were incubated in tissue culture flasks (type 25100, Corning Glass Works, Corning, NY) containing a total of 10 ml medium with 10, 50, and 100 μg/ml LPS in a humidified atmosphere containing 5% CO_2 in air for 24 hr. The controls were cultured under the same conditions in the absence of LPS. Cells were harvested for ultrastructural and cytochemical examinations, and cytotoxicity assays 24 hr after incubation. Culture supernatants were collected for interferon assays.

Purified Leu-11a+ cells were treated with 50 μg/ml LPS in the same manner in microtiter plates at 37°C for 24 hr. Cells were harvested for cytotoxicity assays after incubation.

PBMC were also incubated with recombinant IL-2 (rIL-2)

(Cetus Corporation, Emeryville, CA) at a concentration of 500 International Units (IU) per ml in the same conditions as above for 2 and 7 days. Cells were harvested for immunoelectron microscopic examination and cytotoxicity assays after incubation.

Assay for NK Cytotoxicity

Effector cells were obtained from PBMC which had been washed with RPMI 1640 after incubation with LPS or rIL-2. K562 myeloid cells (American Type Culture Collection, Rockville, MD) were used as target cells for cytolytic assays. Cytotoxicity assays were performed in 96-well v-bottom microtiter plates (PGC Scientific, Gaithersburg, MD), and each effector: target (E/T) ratio was performed in triplicate. Target cells were radiolabeled with 240 µCi of $Na^{51}CrO_4$ for 60 to 90 min at 37°C, washed 3 times and viable target cells in 50 microliters of medium were added to varying numbers of effector cells (in 100 microliters of medium). After incubating in the microtiter plates for 4 to 6 hr at 37°C, 50 microliters of supernatant were removed from each well. In addition, each assay contained target cells incubated with medium alone in the absence of added effector cells (spontaneous release) and target cells incubated in 5% Triton X-100 (maximum release). Percent specific cytotoxicity was calculated as follows:

$$\text{Percent specific lysis} = \frac{\text{Experimental release(CPM)} - \text{Spontaneous release (CPM)}}{\text{Maximum release(CPM)} - \text{Spontaneous release(CPM)}} \times 100$$

The cytotoxicity of the purified Leu-11a+ cells after 24 hr incubation with LPS was assessed by the same procedure. A duplicate assay was performed 5 weeks later.

Interferon Assays

The supernatant of the culture medium was collected 24 hr following incubation with various doses of LPS. Total interferon was assayed in human KB cells as previously reported (Maheshwari et al., 1980). The titers of interferon were determined against an international standard of human gamma interferon from NIAID, NIH, Bethesda, MD.

Immunoelectron Microscopy

All PBMC samples from different experiments and effector-target conjugates were processed for immunoelectron microscopic identification of Leu-11$^+$ cells according to the procedures described previously (Kang et al., 1985, 1987 a, b). The monoclonal anti - Leu - 11 and anti - Leu - 7 antibodies used for labelling were stained by the reaction product of horseradish peroxidase (HRP) using an ABC method or labeled with 10 or 20 nm colloidal gold via an anti-mouse IgG antibody. Cells labeled with colloidal gold were utilized for acid phosphatase localization (Kang et al., 1985). The samples were embedded in Epon (Poly/Bed, Polysciences, Warrington, PA) following dehydration in a series of graded ethanol solutions. Ultrathin sections prepared with a diamond knife were briefly stained in lead citrate and examined in a JEOL 100 CX transmission electron microscope.

Results

Enhancement of NK Activity and Interferon Production by LPS

Results from the cytotoxicity assays indicate 1.5- to 2-fold increases in the NK cytotoxicity of PBMC treated with LPS for 24 hr as compared to the non-LPS treated controls (Table 1). In some cases, the increase in cytotoxicity correlated directly with increase in LPS concentrations. There was a significant increase in NK activity in sorted Leu-11$^+$ cells incubated with 50 μg/ml LPS for 24 hr as compared to freshly isolated Leu-11$^+$ cells ($p < 0.02$) or Leu-11$^+$ cells incubated *in vitro* for 24 hr in the absence of LPS ($p < 0.03$) (Table 2).

In parallel to NK cytotoxicity, the total interferon levels in the supernatants of the LPS-treated PBMC showed a significant dose-dependent increase with LPS concentrations (Table 1).

Enhancement of NK Activity by rIL-2

A significantly higher percentage of target cells were killed by effectors stimulated with rIL-2 for 2 or 7 days as compared to that of the controls ($p < 0.05$) (Table 3). As seen in Table 3, after rIL-2 stimulation for 48 hr, almost all of

Table 1. Percent of NK specific lysis of K562 cells following 24 hr exposure to varying concentrations of LPS from *E. coli*, 0111:B4

	Donor 1		
	% Killing E/T ratio		Interferon(IU/ml)
	50:1	12:1	
Control	67 ± 4.11	38.73 ± 1.80	13
10 μg/ml	86.09 ± 11.45	70.75 ± 10.57	24
50	84.18 ± 2.40	71.62 ± 6.27	48
100	82.37 ± 8.89	70.23 ± 5.09	48
	Donor 2		
	% Killing E/T ratio		Interferon (IU/ml)
	50:1	12:1	
Control	41.92 ± 3.5	18.09 ± 1.1	0
10 μg/ml	54.11 ± 1.2	24.73 ± 4	13
50	71.36 ± 5.1	43.34 ± 3.4	50
100	100 ± 0.7	51.33 ± 0.9	150

Table 2. Percent of NK specific lysis of K562 cells in purified Leu-11$^+$ cells following 24 hr incubation with 50 μg/ml LPS

	Specific Cytototoxicity (%)*	
	Experiment 1	Experiment 2
Day 0 (prior to incubation)	56.6 ± 4.4	N.D.
24 hr incubation without LPS	56.1 ± 1.6	56.5 ± 3.3
24 hr incubation with LPS	70.1 ± 5.8	65.1 ± 1.7

*E/T ratio, 20:1; N.D., not done

the NK activity was found in the Leu-11+ population which lysed a significantly higher percentage of the targets than did the Leu-11⁻ population ($p < 0.002$). However, there was no significant difference in the percentage of Leu-11+ cells between the rIL-2 stimulated and non-stimulated populations as determined by flow microflurometry.

Table 3. Effect of rIL-2 on NK activity of PBMC

	%Specific Lysis Effector: Target Ratio		
Donor 1	50:1	25:1	12:1
Day 0	73.2 ± 2.4	54.2 ± 8.0	50.1 ± 3.5
Day 2	92.8 ± 1.5	89.1 ± 2.9	77.7 ± 3.1
Day 7	92.5 ± 6.9	88.3 ± 0.2	75.3 ± 3.8
Donor 2	100:1	50:1	25:1
Day 0	47.7 ± 9.3	57.2 ± 7.6	48.5 ± 4.0
Day 2	100.0 ± 4.0	83.0 ± 4.3	63.2 ± 0.9
Day 7	83.3 ± 3.4	83.1 ± 1.8	85.3 ± 1.4

Ultrastructure and Ultracytochemistry of Leu-11+ Cells following Exposure to LPS or rIL-2

Two NK subsets bearing Leu-11 antigen in human peripheral blood have been reported (Lanier et al., 1983), including Leu-7⁻/Leu-11+ and Leu-7+/ Leu-11+. There are no differences in the ultrastructure betwen these two subsets (Kang et al., 1987a). Both subsets have well defined Golgi complex, rough endoplasmic reticulum, numerous mitochondria, many membrane-bound dense granules, vacuoles, centrioles, parallel tubular arrays (PTA), and paracrystalline arrays (Figs. 1, 2). Some ultrastructural alterations were observed in Leu-11+ cells 24 hr after incubation with LPS. The cisternae of rough endoplasmic reticulum, nuclear envelope and Golgi saccules showed distinct dilation. Numerous small vesicles and many large dense granules were often found in the cytoplasm of Leu-11+ cells treated with 100 μg/ml LPS (Fig.3). Tubuloreticular inclusions (TRI) were observed in the cisternae of rough endoplasmic reticulum of Leu-11+ cells exposed to 50 and 100 μg/ml LPS for 24 hr (Fig. 4). No ultrastructural differences were found in the effector-target conjugates between the LPS-treated and control samples. Frequently more than one Leu-11+ effector cell was seen conjugated to a single target cell (Fig. 5).

Increase of acid phosphatase activity was observed only in Leu-11+ cells which were treated with 50 or 100 μg/ml LPS for 24 hr. The reaction product of the enzyme was observed in the Golgi saccules and vesicles, cisternae of rough endoplasmic reticulum and nuclear envelope, dense granules, vacuoles, and vacuoles containing paracrystalline arrays (Fig. 6).

With respect to the effect of rIL-2 on the ultrastructure of Leu-11+ cells, no discernible changes were observed 48 hr after treatment with rIL-2. However, the number of dense granules was significantly increased in Leu-11+ cells exposed to rIL-2 for 48 hr as compared to that of non-stimulated cells ($p < 0.004$). In contrast, there were marked changes observed in the size and ultrastructure of Leu-11+ cells


(1) A
B
C
D
PTA
E
e

Fig. 1. PBMC incubated with anti-Leu-7 and anti-Leu-11 antibodies. Leu-7$^+$/Leu-11$^+$ cells are stained by both colloidal gold and HRP on the cell surface. Gold grains indicate Leu-7 antigen, whereas HRP represents Leu-11 antigen.

A. A normally exposed micrograph of a Leu-7$^+$/Leu-11$^+$ cell depicting intense HRP staining on the cell surface and detail ultrastructure. Note numerous mitochondira, dense granules (arrows), and reniform nucleus with a distinct nucleolus. X 14,300.

B. An underexposed micrograph of the same cell showing dense distribution of gold grains on the cell surface. X 14,300.

C. An underexposed micrograph of a Leu-7$^+$/Leu-11$^+$ cell showing gold grains on the cell surface. X 44,280.

D. A normally exposed micrograph of the same cell shown in Figure 1C depicting well defined Golgi apparatus, centriole (C), and coated vesicles (arrows). X 44,280.

E. A portion of a Leu-7$^+$/Leu-11$^+$ cell showing a parallel tubular array (PTA), a multivesicular body, and fine tubular structures (arrows). Inset is an underexposed micrograph showing gold grain. X 37,200.

following stimulation with rIL-2 for 7 days (Fig. 7). The size of these cells greatly increased (10.0 ± 0.6 μm). Rough endoplasmic reticulum (Fig. 8) and Golgi complex (Fig. 9) became highly hypertrophied (Fig. 8). The number of dense granules increased significantly in the stimulated cells by having 5.7 ± 2.5 granules per cell section as compared to 1.2 ± 0.8 granules per cell section in the unstimulated cells. Mitotic figures were frequently observed in the stimulated Leu-11^{+} cells at this stage (Fig. 10).

Table 4. Presence of NK activity in Leu-11 positive and Leu-11 negative populations following stimulation of PBMC for 48 hours with IL-2

Donor 1	E/T Ratio	Leu-11-Positive	Leu-11-Negative
	50:1	59.2 ± 4.4	3.8 ± 0.3
	25:1	44.6 ± 3.8	1.0 ± 0.5
	12:1	28.1 ± 0.9	0 ± 0
	6:1	14.0 ± 3.2	0 ± 0
Donor 2	50:1	53.5 ± 1.4	4.2 ± 0.7
	25:1	35.8 ± 2.4	2.3 ± 1.0
	12:1	22.4 ± 1.1	1.1 ± 0.4
	6:1	8.4 ± 2.4	0 ± 0

Fig. 2. PBMC incubated with anti-Leu-7 and anti-Leu-11 antibodies.

A. A Leu-7⁻/Leu-11⁺ cell is stained only by HRP on the cell surface. Numerous PTA, large vacuoles (arrows), and mitochondria are seen in the cell. X 17,500. Inset is a higher magnification of a vacuole (a) containing a degenerated PTA. X 32,900.

B. A higher magnification of the same cell showing numerous PTA. X 25,000.

C. A vacuole from a different Leu-7⁻/Leu-11⁺ cell containing paracrystalline inclusions and a degenerated PTA. X 60,200.

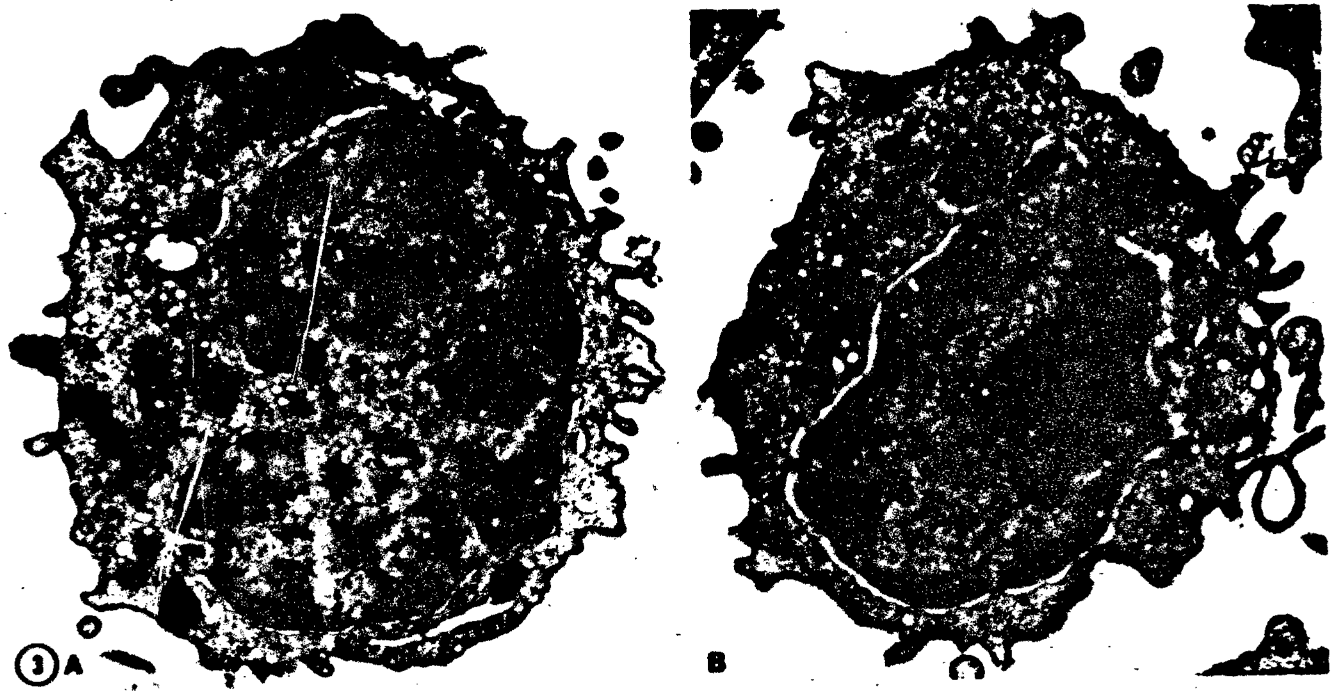

Fig. 3. Leu-11+ cells 24 hr after exposure to 100 µg/ml LPS.

A. Numerous small cytoplasmic vesicles and membrane-bound dense granules (average diameter, 480 nm) (arrows) are found in the majority of the cells. X 17,000.

B. Some Leu-11+ cells contain a few large membrane-bound dense granules (average diameter, 1030 nm) (arrows). X 20,000.

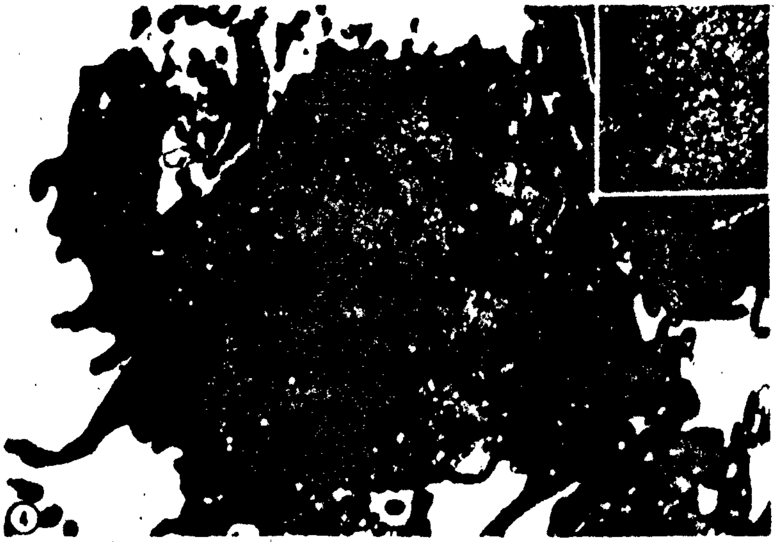

Fig. 4. Leu-11$^+$ cells exposed to 50 or 100 μg/ml LPS for 24 hr. Tubuloreticular inclusions (TRI) are found in the cisternae of rough endoplasmic reticulum (arrowhead). Inset is a higher magnification of the TRI. X 18,000; inset X 58,800.

Fig. 5. PBMC incubated with K562 target cells following 24 hr exposure to LPS (same samples used for cytotoxicity assay). More than one Leu-11$^+$ cell (NK) is often observed [illegible]

Fig. 6. Localization of acid phosphatase in Leu-11$^+$ cells exposed to 100 ug/ml LPS. Reaction product of the enzyme is localized to dense granules (g), vacuoles, and vacuoles containing paracrystalline inclusions (arrowheads). X 56,000.

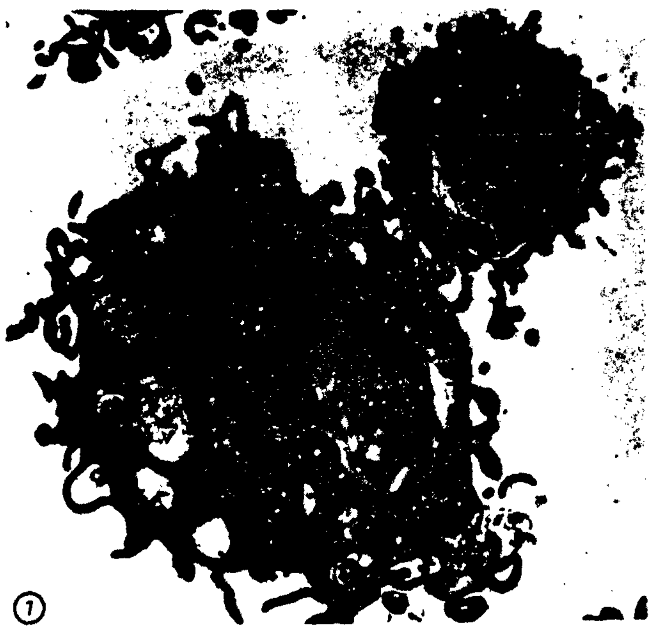

Fig. 7. Blastoid Leu-11+ cells were observed 7 days after incubation with rIL-2. A large blastoid cell with large vacuoles (v) and a non-stimulated Leu-11+ cell are shown in the micrograph. X 18,350.

Fig. 8. Leu-11+ cells after 7 day incubation with rIL-2. Elaborated Golgi apparatus (G) and numerous dense granules (arrows) are seen in the cell. X 11,000.

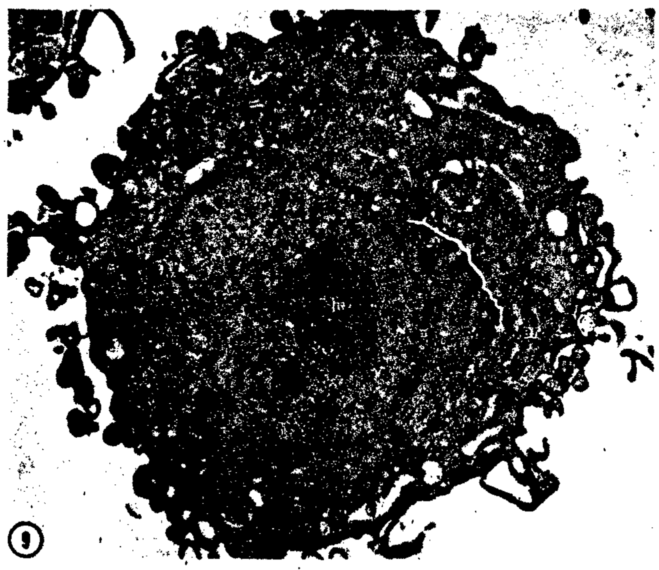

Fig. 9. Leu-11^{+} cells treated with rIL-2. Rough endoplasmic reticulum (er) is highly elaborated 7 days after treatment. X 10,000.

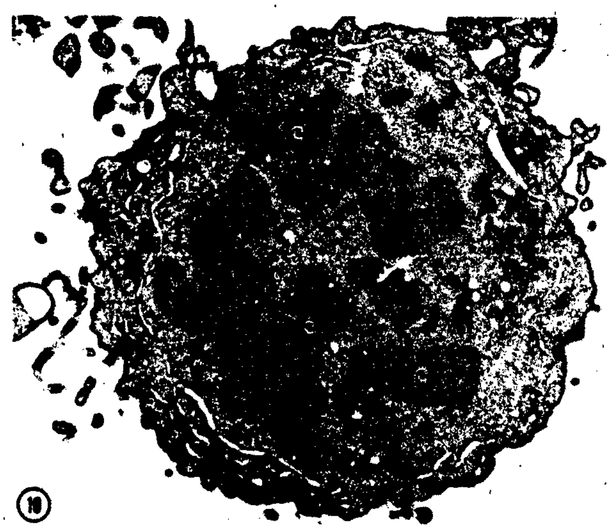

Fig. 10. Leu-11^{+} cells exposed to rIL-2. Cell proliferation was observed 7 days after incubation. Chromosomes (C), elaborated rough endoplasmic reticulum (er), and dense granules (arrows) are observed in a dividing cell. X 10,000.

Discussion

It is well established that interferons (IFN) including gamma interferon (IFN-r) activate NK cell mediated cytotoxicity against tumor cells (Herberman et al., 1979; Lucero et al., 1981; Brunda and Davatelis, 1985). In the present study, the positive correlation of enhanced NK cytotoxicity with production of IFN suggests that IFN is involved in the enhancement of NK cytotoxicity in PBMC treated with LPS. The effect of IFN on NK cells is also indicated by the formation of TRI in Leu-11$^+$ cells. TRI are proven markers of IFN stimulation in human peripheral blood lymphocytes (Grimley et al., 1985). Production of IFN-r as a result of stimulation with LPS has been shown in T cells following exposure to IL-2 or stimulation by macrophages (Blanchard et al., 1986). Reports have also shown that LGL produce IFN-r following IL-2 stimulation (Trinchieri et al., 1984; Ortaldo et al., 1984; Young and Ortaldo, 1987). In this regard, IL-2 is essential to the production of IFN-r by T cells or LGL (Handa et al., 1983). In fact, LPS may indirectly induce production of IL-2 by T cells (Simon and Lee, 1985) and LGL (Pistoia et al., 1983) via stimulation with IL-1 which is produced by macrophages/monocytes (Arend et al., 1985; Dinarello et al., 1985; Haeffner-Cavaillon et al., 1984) and LGL (Herman and Rabson, 1984).

In addition to the indirect effect of LPS on the enhancement of NK cytotoxicity, LPS may also exert a direct effect on human NK cells as indicated by the increased NK cytotoxicity of sorted Leu-11$^+$ cells and our previous observations of the incorporation of LPS by these cells (Kang et al., 1987c).

Elaboration of Golgi apparatus and rough endoplasmic reticulum in Leu-11$^+$ cells following exposure to LPS suggests active synthesis of new materials possibly for fabrication of dense granules (Farquar et al., 1986) or for production of IFN (Djeu et al., 1982). In fact, increased acid phophatase activity was observed in Leu-11$^+$ cells which were exposed to higher doses of LPS. Dense granules contain lysosomal enzymes such as acid phosphatase and arylsulfatase (Kang et al., 1987a; Zucker-Franklin et al., 1983; Babcock and Phillips, 1983) which are believed to be involved in NK cell mediated cytolyis of target cells (Neighbour et al., 1982; Nocera et al., 1983; Frey et al., 1982; Carpen et al., 1981, 1982; Zucker-Franklin et al., 1983). Increase in the number of dense granules may facilitate the lytic ability of NK cells.

Results from the present study showed that rIL-2 significantly enhanced NK cytotoxicity against K562 target and caused increase of dense granules, hypertrophy of Golgi apparatus and rough endoplasmic reticulum, and mitosis in Leu-11⁺ cells. Similar observations of the feeect of rIL-2 on the ultrastructure of NK cells were also recently reported in LGL (Zarcone et al., 1987). All these ultrastructural changes are believed to be implicated in the enhancement of NK cytotoxicity as described earlier. In addition, we have recently observed that rIL-2 also enhances the binding of Leu-11⁺ effectors to K562 target cells (Carl et al., 1987).

Although both IFN-r and rIL-2 have been reported to enhance NK cytotoxicity, the ability of IL-2 to directly affect the cytolytic activity of NK cells has been controversial. Ortaldo et al. (1984) and others (Shiiba et al., 1984; Weigent et al., 1983) reported that the enhancement of cytolytic activity in NK cells by IL-2 is a consequence of triggering IFN-r production. On the other hand, Trinchieri et al. (1984) and other investigators (Svedersky et al., 1984; Van de Griend et al., 1986; Kabelitz et al., 1985) suggested that IL-2 induced enhancement of NK cytolytic activity is IFN independent since antibodies against IFN-r do not prevent enhancement of cytolytic activity by IL-2.

In summary, results from the present studies indicated that: (1) both LPS and rIL-2 effectively enhance NK cytotoxicity in PBMC against K562 tumor cells; (2) both LPS and rIL-2 cause similar ultrastructural changes in Leu-11⁺ NK cells, these changes correlate with NK activity; (3) the effect of LPS on the enhancement of NK cytotoxicity may be a direct and/or indirect process; (4) interferon is implicated in the augmentation of cytotoxicity by LPS.

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