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Circulating human peripheral blood granulocytes synthesize and secrete tumor necrosis factor α

(endotoxin/neutrophil/interleukin 1)

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ABSTRACT **Circulating peripheral blood polymorphonuclear neutrophils (PMNs) have long been considered terminally differentiated cells that do not synthesize or secrete protein. However, work of others and ourselves has shown that PMNs can secrete the cytokine interleukin 1. In the present study we investigated whether circulating PMNs are capable of synthesizing and secreting another cytokine, tumor necrosis factor α (TNF-α). Highly purified (>99% granulocytes) PMNs were isolated from normal human volunteer blood and cultured with or without bacterial lipopolysaccharide (LPS) for up to 24 hr. Cell culture supernatants were collected and tested for TNF-α, and total RNA was isolated from cells at various times after stimulation and assessed for TNF-α mRNA by Northern blot techniques. The results showed that message for TNF-α was produced after 60 min of *in vitro* stimulation with LPS and was maximal at about 4 hr. TNF-α was secreted into the supernatant of unstimulated PMNs from two different donors during 24 hr of culture (35–50 pg/ml), but significantly more (160–190 pg/ml) was secreted by PMNs when stimulated with LPS. PMNs from six other normal volunteers showed significant LPS-stimulated secretion of TNF at 60–180 min of culture. The secreted product also had biological activity against the TNF-sensitive L-M cell line, confirming that PMNs can make and secrete immunologically and biologically active TNF. Since it is also possible for monocytes to synthesize and secrete TNF, the amount of TNF secreted by a monocyte population equal to 20% of the PMNs cultured was measured. The results showed that monocytes at a concentration 20 times that potentially contaminating the PMN populations cultured could not produce as much TNF (unstimulated, 26–65 pg/ml; stimulated, 32–87 pg/ml). The PMN must now be considered a cell capable of altering the acute inflammatory response and modulating the immune response through the synthesis and release of cytokines.**

The polymorphonuclear neutrophil (PMN) is a pivotal cell in the acute inflammatory response. After being released from the bone marrow, PMNs are recruited to the site of inflammation by a myriad of chemotactic factors. Through phagocytosis and the release of various cytotoxic compounds the neutrophil plays a critical role in the removal of invading organisms. Once in the circulation the PMN has been considered a terminally differentiated cell not capable of protein synthesis (1). The various nonspecific factors necessary for defense against invading pathogens have been thought to be manufactured and stored prior to release of PMNs into the peripheral blood. Recently, however, evidence has accumulated that circulating neutrophils may be capable of the selective synthesis of mRNA and proteins (2). Our laboratory and others have shown that the PMN is capable of secreting interleukin 1 (IL-1), a protein with important immunoregulatory functions (3, 4). In this study, we investigated the ability of circulating PMNs to produce another immunoregulatory cytokine, tumor necrosis factor α (TNF-α). The broad range of biological effects attributed to TNF include the induction of fever (5), augmentation of collagenase and prostaglandin E_2 production by fibroblasts (6), stimulation of IL-1 and prostaglandin E_2 synthesis by resting macrophages (7), induction of osteoclastic bone resorption (8), inhibition of lipoprotein lipase (9), and immunostimulation of lymphocytes (10). It has also been shown that TNF acts synergistically with other cytokines such as IL-1 (11, 12).

MATERIALS AND METHODS

Cell Purification and Cell Culture Conditions. The method for cell purification was modified from a method described by Tiku *et al.* (3). Heparinized peripheral blood from normal human volunteers was obtained by venipuncture. The blood was then mixed with an equal volume of dextran 70 (Macrodex, Pharmacia) in 0.9% NaCl and maintained for 1 hr at 37°C in a 5% CO_2/95% air incubator. The supernatant was removed and centrifuged at 275 × *g* for 10 min. The resulting pellet was collected and diluted in 5 ml of RPMI-1640 (GIBCO) supplemented with 2 mM L-glutamine, 10 nM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes), and 1% (vol/vol) antibiotic solution (10,000 units of penicillin, 10,000 μg of streptomycin, and 25 μg of Fungizone per ml) (culture medium). This supernatant was then layered over Ficoll/Hypaque (Pharmacia) and centrifuged at 450 × *g* for 30 min at 22°C. The PMN pellet, free of mononuclear cells, was subjected to erythrocyte lysis in the following manner. Five milliliters of sterile, endotoxin-free H_2O was added and the mixture was gently "vortexed" by hand. Immediately thereafter, 0.5 ml of filter-sterilized 9% NaCl was added to restore isotonicity. Fifteen milliliters of culture medium was then added to the mixture and the solution was centrifuged at 300 × *g* for 10 min. The collected pellet was washed three times in culture medium and finally resuspended at a concentration of 5×10^6 cells per ml.

Differential cell smears were prepared on microscope slides. Cells were diluted to a concentration of 10^6 per ml in 12 × 75 mm test tubes using culture medium. A volume of 7% bovine albumin (Sigma) equivalent to the volume of the cells and medium was then added to each tube. This suspension was added to a cytofunnel and then placed into a Cytospin2

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Abbreviations: TNF, tumor necrosis factor; IL-1, interleukin 1; PMN, polymorphonuclear neutrophil; LPS, lipopolysaccharide; NACE, naphthol AS-D chloroacetate esterase; ANAE, α-naphthyl acetate esterase.

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centrifuge (Shandon, Sewickley, PA). Cell preparations were spun for 10 min at 100 rpm. Slides were immediately fixed for 1 min in 100% methanol. Slides were stained with Wright stain for 2 min. They were then stained with Giemsa stain for 4 min, rinsed with H_2O for 3 min, and dried. Cells were enumerated at ×1000 magnification using oil immersion. Cell viability was measured by trypan blue exclusion staining.

Cell purity was also monitored by differential cell counting with Wright staining as described above and by esterase staining for naphthol AS-D chloroacetate esterase (NACE, Sigma) or α-naphthyl acetate esterase (ANAE, Sigma) according to the manufacturer's instructions. Five hundred leukocytes were counted on each slide and percentages of morphologically identified or enzyme-stained cells were determined.

Secretion of TNF. Sixty-milliliter samples of heparinized peripheral blood from two normal human volunteers were obtained by venipuncture and PMNs were purified by the method described above. PMNs were cultured at a final concentration of 5×10^5 cells in 200 μl per well in 96-well flat-bottom tissue culture dishes (Nunc) and stimulated with an optimal dose of *Escherichia coli* lipopolysaccharide (LPS, 5 μg/ml; *E. coli* 055:B5, Difco). Monocyte/macrophage cells were also isolated from these blood samples by adherence of Ficoll/Hypaque-separated mononuclear cells to plastic Petri dishes for 1 hr at 37°C and stimulated in the same fashion except that they were cultured at a final concentration of 10^5 in 200 μl per well. This population represents 20% of the total number of PMNs that were similarly stimulated. The supernatants were collected and TNF-α was measured with an enzyme-linked immunosorbent assay (ELISA) kit (T Cell Sciences, Cambridge, MA).

Isolation of PMN RNA and Northern Blot Analysis. PMNs were isolated as described above from 300 ml of heparinized venous blood obtained from a single donor. Cell purity and viability were measured as above. The isolated PMNs were cultured in Petri dishes (15 × 100 mm, Falcon) at a final concentration of 2×10^6 per ml. With the exception of the control, each dish contained LPS at a final concentration of 5 μg/ml. Cells were harvested and washed at 0, 30, 60, 120, and 180 min.

Total cellular RNA was collected from peripheral PMNs by the guanidinium isothiocyanate/cesium chloride medium (13). In brief, guanidinium isothiocyanate was added to washed cell pellets and vortexed for RNA isolation. The guanidinium solution was layered onto 5.7 M cesium chloride and spun at 116,000 × *g* for 18 hr at 18–24°C. The RNA pellet was collected and resuspended in 440 μl of 0.3 M sodium acetate, 880 μl of ethanol was added, and the mixture was allowed to precipitate at −70°C for 1 hr. The pellet was collected by centrifugation, resuspended in a mixture of 200 μl of H_2O, 20 μl of 3 M sodium acetate, and 440 μl ethanol and allowed to precipitate overnight. The pellet was collected by centrifugation, lyophilized, and dissolved in H_2O. Total RNA concentration and purity were measured by optical density at 260 and 280 nm. Samples were then loaded onto a 2 M formaldehyde/1% agarose gel (20 ng per lane) and subjected to electrophoresis overnight. The formaldehyde-denatured RNA was then transferred to nitrocellulose and hybridized with the prepared radiolabeled probe for human TNF. The nitrocellulose filter was exposed to x-ray film for 5 days and the autoradiograph was developed. The TNF cDNA probe (a gift from Peter Ralph, Cetus) was labeled with ^{32}P by nick-translation (19).

TNF Bioassay. TNF was measured for biological activity on the cell line L-M (ATCC CCL 1.2) by the method of Kramer and Carver (14). Cells were cultured in RPMI-1640 with 1% antibiotic solution and 1% Bacto-Peptone (Difco). Twenty-four hours before assay, L-M cells were washed, diluted in test medium, and added to all wells of assay plates at 5×10^4 cells per well. On the day of assay, plates were washed, and samples to be tested were diluted from 1:2 to 1:128 in medium as above, but with the addition of actinomycin D (1 μg/ml), and added to the assay wells. Plates were incubated for another 20 hr and the degree of cytotoxicity determined by the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) dye reduction method (15). For this, 25 μl of MTT solution (5 mg/ml) was added to each well, and the plates were incubated for a further 4 hr. Formazan crystals were dissolved in acidified 10% SDS and color was read on a multiscanning ELISA reader (Molecular Devices, Mountain View, CA) using $A_{570} - A_{650}$. Recombinant human TNF (T Cell Sciences) was used as a standard for determination of the quantity of TNF contained in the supernatants. This standard contained 1000 pg/ml and was diluted to 1 pg/ml in $\log_2$ dilutions. The best-fit curve was a semilogarithmic curve [logarithm of concentration vs. $(A_{570} - A_{650})$] and had a correlation coefficient of 0.985. Sample TNF quantity was determined from this standard curve by using the results obtained at a dilution of 1:4, with the computer software package SOFTMAX (Molecular Devices).

RESULTS

PMN Purity. Cells isolated by the described method contained more than 99% granulocytes as detected by enzyme staining (Table 1). NACE is reported (17) to be specific for cells of granulocytic lineage. ANAE staining revealed less than 1% monocyte contamination in the PMN preparation. ANAE is an enzyme detected primarily in monocytes and is virtually absent from granulocytes (18). Wright staining revealed a 99.4% granulocyte population (Table 1) that consisted of 98.9% PMNs and 1% eosinophils. Five hundred cells from the isolated cell population were counted for each enzyme stain and for Wright-stained cytospin preparations.

TNF Secretion. As measured by ELISA, culture supernatants from PMNs stimulated for 24 hr with *E. coli* LPS contained more than 196 pg of TNF per ml as compared to TNF standards (Table 2). Secretion of TNF was detected in PMN and monocyte/macrophage populations (Table 2). PMNs were isolated as above from 60 ml of heparinized venous blood from two different normal human volunteers. Monocyte/macrophages were also isolated from the same blood samples and stimulated in the same fashion except that they were cultured at a final concentration of 10^5 in 200 μl per well. This population represents 20% of the total number of PMNs that were similarly stimulated. The data showed that a quantity of monocytes equal to 20% of the PMNs cultured produced significantly less TNF.

The kinetics of TNF secretion were measured using PMNs isolated from blood of six other normal volunteers. Significant secretion of TNF measured by ELISA was found at 60 min after stimulation in culture with LPS and increased thereafter (Fig. 1). There was expected variation among volunteers. The same supernatants were tested in the L-M cell bioassay and similar results were found (Table 3). PMNs secreted a mean of 13.2 pg/ml of TNF at 0 hr and at 60–180 mins secreted significantly more TNF (21.8–36 pg/ml). These

Table 1. Purity of PMN preparation

Stain	No. positive	No. negative	% granulocyte	% monocyte/ macrophage
NACE	497	3	99.4	0.6
ANAE	4	496	99.2	0.8
Wright	—	—	99.4	0.6

Isolated purified granulocyte populations were stained for NACE and ANAE activity or with Wright stain; 500 cells were counted per slide. NACE is detected primarily in granulocytes, and ANAE in monocytes.

Table 2. Secretion of TNF by PMNs and monocytes/macrophages (M/M)

Cells	TNF-α, pg/ml	
	Donor A	Donor B
PMNs		
Unstimulated	50.9	35.4
Stimulated	158.9	196.4
M/M		
Unstimulated	66.4	26.2
Stimulated	87.2	31.8

PMNs (5×10^5 per ml) or monocytes/macrophages (10^5 per ml) were cultured in medium alone (unstimulated) or in medium containing *E. coli* LPS (stimulated). After 24 hr, supernatants were collected and assayed for TNF-α by ELISA.

results showed that LPS-stimulated PMNs secreted TNF that was both immunologically and biologically active. It should be noted that the bioassay for TNF measures not only TNF-α, but also TNF-β (14).

RNA Production. Northern blot analysis of PMN TNF mRNA was carried out at 0, 30, 60, 120, and 180 min after stimulation with *E. coli* LPS (Fig. 2). LPS-induced TNF mRNA production was undetectable during the first 30 min of culture. At 1 hr, significant levels were produced, which peaked and then diminished over the next two hr.

DISCUSSION

The major cell producing TNF in human peripheral blood has been thought to be the monocyte. The granulocytes have the same stem cell as monocytes and share other functions such as phagocytosis and killing of bacteria and have been shown to produce IL-1 (3, 4). The results presented here show unequivocally that the mature circulating human granulocyte is capable of synthesizing and secreting the cytokine TNF-α. The cell population studied was more than 99% PMNs. Furthermore, even a monocyte population 20% the size of the PMN population did not secrete TNF equivalent to that secreted by PMNs from the same donors.

In order to evaluate whether the secreted TNF had been manufactured and stored by the PMNs prior to release into the circulation or manufactured after they became circulating cells, Northern blot analysis of stimulated cell populations was performed. The results show that circulating PMNs are

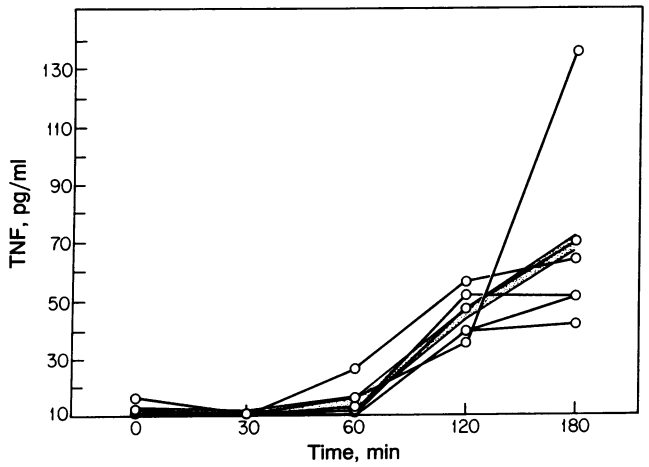

FIG. 1. Kinetics of secretion of TNF-α measured by ELISA. PMNs were cultured at 5×10^5 per well with LPS, and culture supernatants were harvested at times from 0 to 180 min. PMNs from six different volunteers are represented by solid lines. Mean TNF values are indicated by stippled area. Values at 120 and 180 min differ significantly from 0 time value (paired *t* test).

Table 3. Bioassay of TNF secreted by PMNs

Time	TNF, pg/ml	*P**
0	13.2 ± 1.9	—
30	9.6 ± 1.7	0.5
60	21.8 ± 2.3	0.03
120	36.6 ± 8.1	0.02
180	33.0 ± 4.9	0.005

PMNs were cultured with LPS for various times, and supernatants were assayed for cytotoxicity toward L-M cells.
*By paired *t* test, compared with 0 time.

indeed capable of synthesizing TNF mRNA after exposure to endotoxin for 60 min (Fig. 1). We were unable to detect significant levels of TNF mRNA at either time 0 or at 30 min. This demonstrates that the circulating PMN has the capability of synthesizing TNF mRNA and that it is not preformed.

The production of TNF by PMNs may play an important role at the site of infection. TNF is a natural stimulant of neutrophils that promotes adherence to endothelial cells and to particles, leading to increased phagocytosis, respiratory burst activity, and degranulation (16). In prior studies, investigators examined the effects of exogenous TNF on PMN function on the assumption that the source of the protein *in vivo* was the monocyte/macrophage. We propose that the production of TNF (as well as IL-1) by PMNs plays an autocrine role in PMN function. The release of TNF by neutrophils that arrive first at the site of inflammation would help to potentiate the recruitment of other PMNs to the area. It would also facilitate the adherence of the PMNs to the vascular endothelium, thus allowing a population of cells to collect in a short period of time. TNF stimulation of these cells would also prime them for phatocytosis once they reach the inflammatory site.

Other cells within the circulation and those resident in the inflamed tissues would inevitably be affected by TNF secreted by PMNs. Both IL-1 and TNF have been shown to be immunostimulatory to lymphocytes. Thus, immunomodulation through the stimulation of these cells, which mount a conventional immune response, could be directly attributed to the PMNs. These effects could be seen systemically as well as locally. Because of the large number of PMNs in the circulation, those in the marginating pool, and those infil-

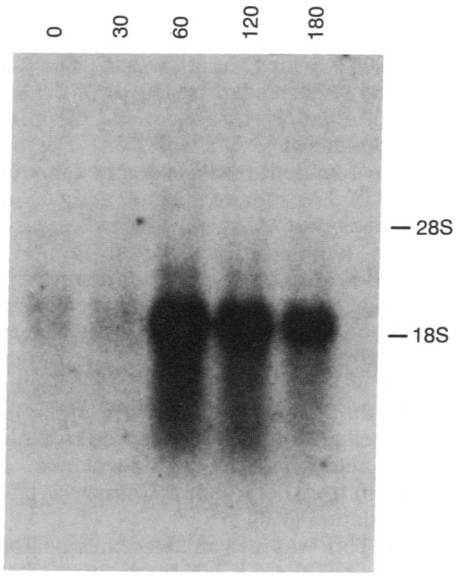

FIG. 2. Northern blot analysis of TNF mRNA at various times after stimulation with *E. coli* LPS. PMNs from a single donor were cultured (2×10^6 cells per ml) with LPS (5 μg/ml) for 0, 30, 60, 120, or 180 min. Positions of 28S and 18S rRNA are indicated.

trating into the site of inflammation, the *in vivo* production of TNF by this cell population could be substantial, contributing to the numerous metabolic effects TNF elicits in the host in response to acute infection.

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1. Bainton, D. F., Ullyot, J. L. & Farquhar, M. G. (1971) *J. Exp. Med.* **134,** 907–934.
2. Jack, R. M. & Fearon, D. T. (1988) *J. Immunol.* **140,** 4286–4293.
3. Tiku, K., Tiku, M. L. & Skosey, J. L. (1986) *J. Immunol.* **136,** 3677–3685.
4. Dubravec, D., Rodrick, M. L., Grbic, J. & Mannick, J. A. (1988) *J. Dental Res.* **67,** 151.
5. Dinarello, C. A., Cannon, J. G., Wolff, S. M., Bernheim, H. A., Beutler, B., Cerami, A., Figari, I. S., Palladino, M. A., Jr., & O'Connor, J. V. (1986) *J. Exp. Med.* **163,** 1433–1450.
6. Dayer, J. M., Beutler, B. & Cerami, A. (1985) *J. Exp. Med.* **162,** 2163–2168.
7. Bachwich, P. R., Chensue, S. W., Larrick, J. W. & Kunkel, S. L. (1986) *Biochem. Biophys. Res. Commun.* **136,** 94–101.
8. Bertolini, D. R., Nedwin, G. E., Bringman, T. S., Smith, D. D. & Mundy, G. R. (1986) *Nature (London)* **319,** 516–518.
9. Torti, F. M., Dieckman, B., Beutler, B., Cerami, A. & Ringold, G. M. (1985) *Science* **229,** 867–869.
10. Chouaib, S., Bertoglio, J., Blay, J. Y., Marchiol-Fourigault, C. & Fradelizi, D. (1988) *Proc. Natl. Acad. Sci. USA* **85,** 6875–6879.
11. Elias, J. A., Gustilo, K., Baeder, W. & Freundlich, B. (1987) *J. Immunol.* **138,** 3812–3816.
12. Stashenko, P., Dewhirst, F. E., Peros, N. J., Kent, R. L. & Ago, J. M. (1987) *J. Immunol.* **138,** 1464–1468.
13. Chirgwin, J. W., Przybyla, A. E., MacDonald, R. J. & Rutter, W. J. (1979) *Biochemistry* **18,** 5294–5299.
14. Kramer, S. M. & Carver, M. E. (1986) *J. Immunol. Methods* **93,** 201–206.
15. Meager, A., Leung, H. & Woolley, J. (1989) *J. Immunol. Methods* **116,** 1–17.
16. Klebanoff, S. J., Vadas, M. A., Harlan, J. M., Sparks, L. H., Gamble, J. R., Agosti, J. M. & Waltersdorph, A. M. (1986) *J. Immunol.* **136,** 4220–4225.
17. Yam, L. T., Li, C. Y. & Crosby, W. H. (1971) *Am. J. Clin. Pathol.* **55,** 283–287.
18. Sariban, E., Mitchell, T. & Kufe, D. (1985) *Nature (London)* **316,** 64–66.
19. Rigby, P., Dieckmann, M., Rhodes, C. & Berg, P. (1977) *J. Mol. Biol.* **113,** 237–241.