Suppression of Natural Killing In Vitro by Monocytes and Polymorphonuclear Leukocytes

REQUIREMENT FOR REACTIVE METABOLITES OF OXYGEN

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ABSTRACT Natural killer cells spontaneously lyse certain tumor cells and may defend against malignancy. We have previously shown that natural killing (NK) by human peripheral blood mononuclear cells (PBMC) is suppressed in vitro by phorbol diester tumor promoters, including 12-O-tetradecanoylphorbol-13-acetate (TPA). We here demonstrate that suppression of NK is mediated by monocytes or polymorphonuclear leukocytes (PMN) and that suppression is dependent on the generation of reactive forms of molecular oxygen (RO), particularly hydrogen peroxide (H_2O_2). NK was suppressed not only by TPA but also by opsonized zymosan (yeast cell walls), which, like TPA, was not toxic to PBMC. Both TPA and zymosan stimulated the production of superoxide anion (O_2^-) and H_2O_2 by PBMC. Production of RO correlated with suppression of NK. When PBMC were depleted of monocytes, the production of RO and the suppression of NK were both markedly reduced. Suppression could be restored by monocytes or PMN, both of which produced RO in response to TPA or zymosan. Suppression of NK was dependent on RO. Monocytes or PMN from a patient with chronic granulomatous disease, whose cells cannot generate RO, did not mediate suppression of NK. Suppression was also reduced in glucose-free medium, which did not support the generation of RO. Suppression of NK by TPA was inhibited by catalase. Bovine superoxide dismutase had a limited effect on suppression, even in high concentration, and tyrosine-copper (II) complex, which also enhances dismutation of O_2^- to H_2O_2, had almost no effect on suppression. When H_2O_2 was directly generated enzymatically from glucose oxidase and glucose, NK was suppressed and suppression was reversed by catalase. NK was also suppressed by the enzymatic generation of O_2^- from xanthine oxidase and xanthine, but suppression under these conditions was again inhibited by catalase and not by superoxide dismutase, indicating that suppression was due to the secondary formation of H_2O_2 from O_2^-. These results indicate that H_2O_2 is important in suppression of NK. Myeloperoxidase did not appear to play a role in suppression because inhibition of this enzyme by sodium azide, cyanide, or aminotriazole did not prevent suppression of NK. Suppression of NK was reversible; after exposure to zymosan, NK could be partially restored by the addition of catalase and superoxide dismutase or by the removal of zymosan. These studies demonstrate cellular regulation of NK by monocytes or polymorphonuclear leukocytes and indicate a role for RO in immunoregulation.

INTRODUCTION

Natural killer cells are mononuclear cells that spontaneously lyse selected tumor cells, virus-infected cells, and even normal lymphocytes (1–4). Natural killer cells can be distinguished from mature T cells, B cells, or monocytes, although features of both T cells and monocytes have been identified in some or all natural killer cells from mice or humans (5–10). Cytotoxicity

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A preliminary report of part of this work was presented at the Annual Meeting of the American Federation for Clinical Research and published in abstract form in 1981 (*Clin. Res.* **29**: 376A).

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Received for publication 20 April 1981 and in revised form 23 November 1981.

by natural killer cells can be stimulated by interferon (11) or interleukin-2 (12), and in the mouse, can be suppressed by a variety of agents including strontium-89 (13), carrageenans (14), silica particles (14), *Corynebacterium parvum* (15), estrogens (16), hydrocortisone (17), and prostaglandins (18). Certain of these agents appear to induce cellular suppression of natural killing (NK)[1], but the suppressor cells have been difficult to characterize, and the mechanism of suppression is unknown (15, 17, 19). The regulation of NK is of particular interest because of evidence that NK may be important in host defense against malignancy; the growth of certain tumors in vivo is inversely correlated with levels of NK, and spontaneous malignancy is increased when NK is deficient (20–22). Levels of NK also correlate with resistance to metastasis of certain tumors (23).

Recently, we demonstrated that NK by human peripheral blood mononuclear cells (PBMC) is suppressed in vitro by phorbol diester tumor promoters (24). Suppression of NK by these agents correlated with their potency as tumor promoters, the most potent agent being 12-O-tetradecanoylphorbol-13-acetate (TPA). Suppression was reduced or absent when PBMC were depleted of adherent cells, implicating monocytes in the suppression of NK (24). Other investigators have shown that TPA stimulates monocytes and polymorphonuclear leukocytes (PMN) to release reactive metabolites of molecular oxygen (RO), including superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radical ($OH\cdot$), and singlet oxygen ($^1O_2$) (25–28).

In these studies, we demonstrate cellular suppression of NK by monocytes or PMN. Suppression can be stimulated by TPA or zymosan and requires the generation of RO by either monocytes or PMN. Suppression appears to be primarily due to the generation of H_2O_2. Our findings suggest that NK may be suppressed when monocytes or PMN are activated to release RO, and thus RO may play a significant role in the autoregulation of immunity.

METHODS

Reagents. The following were obtained from Sigma Chemical Co., St. Louis, MO: TPA, 3-aminotriazole, glucose oxidase (*Aspergillus niger*, type V, 320 U/mg), scopoletin, cytochrome C (horse heart, type VI), peroxidase (horseradish, type II, R.Z. 1.5, 152 purpurogallin U/mg), indomethacin, dexamethasone, catalase (bovine liver, 17,000 U/mg) and superoxide dismutase (SOD) (bovine erythrocyte, type I, 3,000 U/mg). The catalase had no detectable SOD activity at the concentrations used, as determined by the reduction of cytochrome C (see below). The SOD had no peroxidase or catalase activity, as determined by a lack of effect on H_2O_2 (quantified at A_{240}, using a molar extinction coefficient of 43.6). To inactivate SOD or catalase, the enzymes were first extensively dialyzed against 1 mM EDTA and then autoclaved for 30 min. The inactivated enzymes were then dialyzed against RPMI 1640. Inactivated enzymes had no detectable activity. Xanthine oxidase (0.45 U/mg) was obtained from Boehringer Mannheim Biochemicals, Indianapolis, IN. Zymosan was obtained from ICN K & K Laboratories Inc., Plainview, NY (lots 31543-A and 41419-A). Zymosan was opsonized immediately before use by incubation in fresh human serum (1 mg zymosan per milliliter serum). Except where indicated otherwise, "zymosan" refers to opsonized zymosan throughout the text. *N*-formylmethionyl-leucyl-phenylalanine (fMetLeuPhe) and *N*-formyl-norleucyl-leucyl-phenylalanine (fNleLeuPhe) were obtained from Vega-Fox Biochemicals Div, Newbery Energy Corp. Tucson, AZ. Human fibroblast interferon (IFN-β), produced by Dr. Rentschlar Arzneimittel, Lauthein, W. Germany, was the kind gift of Dr. Saul Schepartz, National Institutes of Health, Bethesda, MD. Tyrosine-copper (II) complex was synthesized and generously provided by Dr. Bruce Freeman, Duke University, Durham, NC. Certain reagents were dissolved in dimethyl sulfoxide or ethanol and diluted before use. The final concentration of dimethyl sulfoxide or ethanol was always <0.01%, which was shown not to alter natural killing or the suppression of natural killing.

Media. All experiments were performed in RPMI 1640 (Tissue Culture Facility, University of California, San Francisco, CA). For glucose-free medium, D-galactose was substituted for D-glucose. For cytotoxicity assays, medium was supplemented with 20% fetal bovine serum, HEPES buffer, penicillin, and streptomycin (24). Assays for O_2^- and H_2O_2 were performed in RPMI 1640 lacking phenol red.

Cells. PBMC from heparinized whole blood were obtained under a protocol approved by the University of California, San Francisco, and Veterans Administration Committees on Human Research and were sedimented onto Ficoll-Hypaque gradients as described (24). Nonadherent and adherent PBMC were separated by incubation on cell-conditioned plastic flasks by means of a modification (24) of the method of Ackerman and Douglas (29). In some experiments, phagocytes were removed from PBMC by ingestion of carbonyl iron and exposure to magnetism (30). PMN were collected by resuspending peripheral blood cells that penetrated Ficoll-Hypaque and removing erythrocytes by sedimentation in 2% dextran (M_r 70,000), a modification of the method of Hong (31). The remaining cells were >95% PMN, as judged by morphology and the presence of chloroacetate esterase (32). Staining for monocyte nonspecific α-naphthyl butyrate esterase was done according to the method of Yam et al. (32).

Cellular cytotoxicity. Natural killing against K562, a myelogenous leukemia, was assessed during a 3-h ^{51}Cr-release assay as described (24). Cytotoxicity was always tested at effector/target ratios ranging from 5:1 to 20:1 or higher. Because suppression of NK altered the slope of the killing curve, we chose not to express data in lytic units. For clarity, however, we have usually expressed killing at one effector/target ratio, a point on the slopes of the killing curves. In each case, similar results were obtained at other effector/target ratios. Cytotoxicity by PMN against P388, a DBA/2 lymphoma, was assessed by the same procedure. Unless otherwise indicated, test agents were added at the start of the

[1] *Abbreviations used in this paper*: NK, natural killing; PBMC, peripheral blood mononuclear cells; PMN, polymorphonuclear leukocytes; RO, reactive metabolites of oxygen; SOD, superoxide dismutase; TPA, 12-O-tetradecanoylphorbol-13-acetate.

cytotoxicity assay. All experiments were performed at least three times unless otherwise noted.

Measurement of O_2^-. The rate of O_2^- production was assayed by the SOD-inhibitable reduction of ferricytochrome C (33). The change in absorbance of cytochrome C at 550 nm was monitored continuously by a Gilford 250 spectrophotometer. Cells were suspended in 1 ml RPMI 1640 supplemented with 1% fetal bovine serum and 0.08 mM cytochrome C at 37°C. A base-line rate of cytochrome C reduction was established for 2–4 min. Release of RO was then stimulated by the addition of TPA, zymosan, or other agents, and the reduction of cytochrome C was followed for up to 30 min to establish an initial linear rate. In each experiment, SOD (2,000 U/ml) was added to some samples during the linear portion of the reaction to establish the SOD-suppressible rate. At the end of each assay the ferricytochrome C was totally reduced with sodium dithionite. The stimulated rate of cytochrome C reduction was corrected for the resting rate or the non-SOD-inhibitable rate. The increase in A_{550} was converted to nanomolar ferricytochrome C by using a molar extinction coefficient of 21,000. For experiments examining the time-course of RO release, cells were incubated at 37°C with TPA or zymosan or other agents for periods of up to 3 h. At varying times, samples were taken for assessment of O_2^-. Rates of O_2^- production were expressed as a percentage of the initial linear rate.

Measurement of H_2O_2. The rate of H_2O_2 production was assayed by the peroxidase-mediated extinction of scopoletin fluorescence (34). The 460-nm emission of scopoletin, when excited by 350-nm light, was monitored continuously at 37°C in a Turner model 430 spectrofluorometer (Turner Designs, Mountain View, CA). Cells were suspended in 1.0 ml RPMI 1640 containing 1% fetal bovine serum, 0.01 μM scopoletin, and 0.25 μM horseradish peroxidase in a quartz cuvette. The cell suspensions were brought to 37°C and a base-line rate of scopoletin quenching was established for 2–4 min. Reactions were initiated with TPA, zymosan, or other agents, then followed for up to 30 min to establish an initial linear rate. In each experiment catalase was added at 200 U/ml during the linear portion of the reaction to establish the catalase-suppressible rate. The stimulated rate of scopoletin quenching was corrected for base-line rate or for the noncatalase-suppressible rate. The scopoletin quenching was calibrated by adding known amounts of H_2O_2 (quantified at A_{240}, as above) to the assay buffer. Time-course experiments were performed as described for O_2^-.

RESULTS

NK is suppressed by opsonized zymosan. We have demonstrated (24) that NK by PBMC is suppressed by tumor-promoting phorbol diesters. Because these agents stimulate the release of RO by monocytes or PMN (25–28), we examined the effects of serum-opsonized zymosan, an unrelated agent that also stimulates the release of RO (27, 34). NK was reduced when opsonized zymosan was present during the cytotoxicity assay. Zymosan suppressed NK in a dose-dependent fashion and was as effective as TPA (Fig. 1). As with TPA, viability was not affected by concentrations of zymosan that suppressed NK, as assessed by trypan blue exclusion. Unopsonized zymosan also suppressed NK, but to a lesser extent (not shown). The dose–response curve for suppression of NK by opsonized zymosan varied with the source and with the lot of zymosan. In the experiments presented here, two lots of zymosan of similar but not identical potency were used.

Suppression of NK by zymosan or TPA requires the presence of monocytes or PMN. Previous experi-

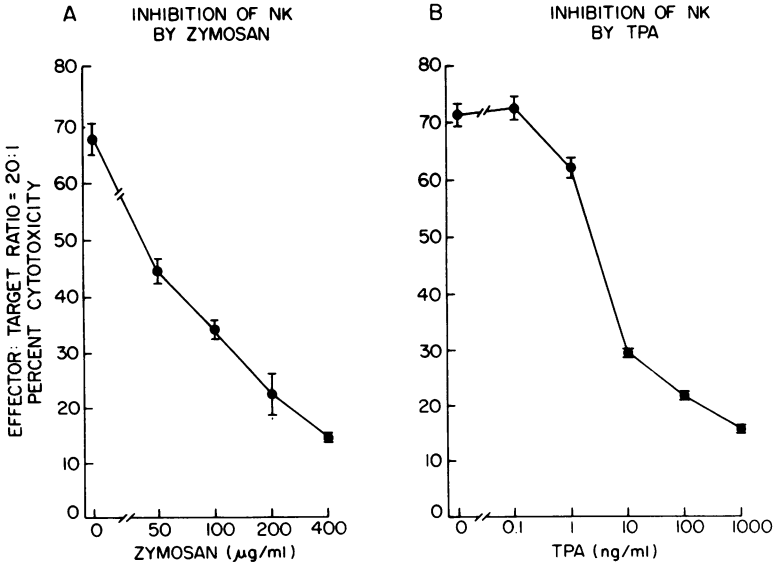

FIGURE 1 Suppression of NK in the presence of zymosan (A) or TPA (B). The graphs show killing at a PBMC/target cell ratio of 20:1. Similar results were obtained at ratios of 10:1 and 5:1.

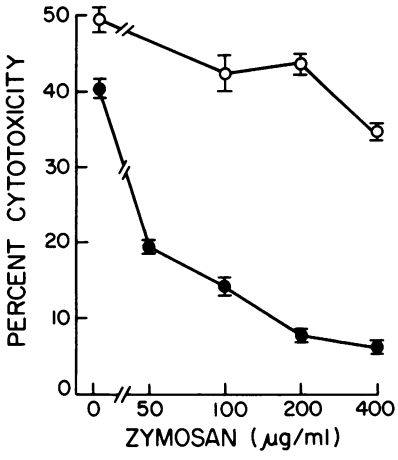

FIGURE 2 Suppression of NK by zymosan is reduced by the removal of adherent cells. Shown are percent cytotoxicity by PBMC at an effector/target cell ratio of 20:1 (●) and by nonadherent cells at an effector/target cell ratio of 15:1 (○), which approximates the contribution of nonadherent cells to unseparated PBMC. Similar results were obtained at other effector/target cell ratios.

ments with TPA demonstrated that suppression of NK was markedly reduced when PBMC were depleted of adherent cells (24). To probe the cellular requirements for suppression of NK, we removed adherent cells from PBMC and then replaced adherent cells or PMN before testing for suppression by zymosan or TPA. As previously shown for TPA (24), suppression of NK by zymosan was greatly reduced in the absence of adherent cells (Fig. 2). Suppression by TPA or zymosan could be restored by either adherent cells or PMN (Fig. 3). Restoration of suppression was dependent on cell number. Maximum suppression of NK required fewer PMN than adherent cells (Fig. 3). In most experiments, high concentrations of zymosan (≥200 μg/ml) or of TPA (100–1,000 ng/ml) produced partial suppression of NK by nonadherent cells alone (Fig. 3). It is likely that this represents a suppressive effect of small numbers of residual monocytes (see below).

By esterase staining and morphology, 20–30% of PBMC were monocytes, whereas <5% (usually <2%) of nonadherent cells were monocytes, and >95% of the adherent cells were esterase positive. Similarly, >95% of the adherent cells expressed Fc-receptors, as monitored by binding of IgG-coated erythrocytes, >95% were phagocytes, and the population secreted lysozyme for at least 5 d in culture. Thus, most or all of the adherent cells were monocytes. When PBMC were depleted of phagocytic cells by ingestion of carbonyl iron and exposure to magnetism, suppression of NK by TPA was again reduced, providing additional evidence that suppression of NK by unseparated PBMC was dependent on the monocyte population (data not shown).

Suppression of NK by TPA or zymosan is accompanied by the release of RO. We next examined the relationship between the release of RO and the suppression of NK in response to TPA or zymosan. We

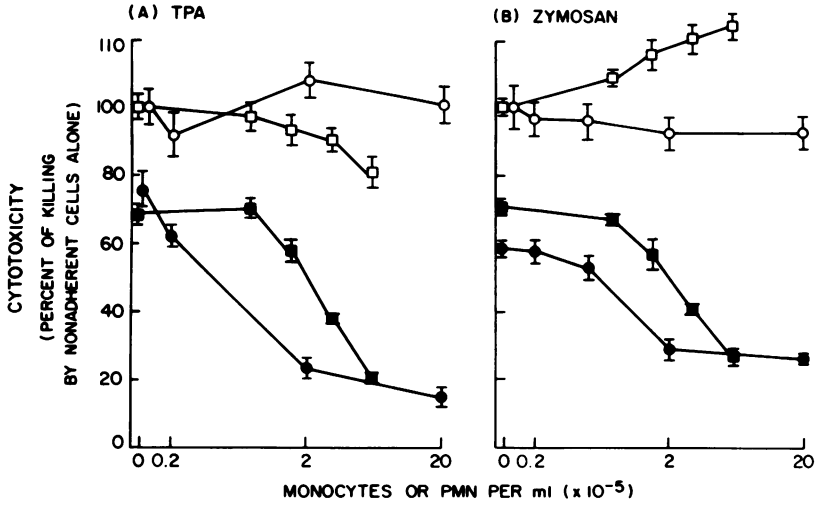

FIGURE 3 Requirement for monocytes or PMN in suppression of NK. Nonadherent cells (2×10^6/ml) were tested for NK in the presence of increasing concentrations of monocytes or PMN. Closed symbols show killing in the presence of monocytes (■) or PMN (●) with TPA (200 ng/ml, Fig. 3A) or zymosan (200 μg/ml, Fig. 3B). Open symbols show killing in the presence of monocytes (□) or PMN (○) without TPA or zymosan. In these experiments, high concentrations of TPA and zymosan were used, which partially suppressed killing by nonadherent cells alone.

found that PBMC released O_2^- and H_2O_2 in response to amounts of TPA or zymosan that suppressed NK (Table I). In studies of dose response, the onset of RO production in the presence of TPA occurred abruptly at concentrations in the range of 1 to 10 ng/ml, reaching maximum stimulation in the same range with little or no further increase in rate at 100–1,000 ng/ml (not shown). Production of O_2^- and H_2O_2 in the presence of opsonized zymosan was first detected at 50-100 μg/ml, and levels of RO production increased with increasing concentrations of zymosan to 400 μg/ml (the maximum concentration used to suppress NK). This pattern of RO production paralleled the dose response for suppression of NK (Fig. 1).

The stimulation of RO over time differed between TPA and zymosan. TPA rapidly produced a high rate of O_2^- and H_2O_2 release, which began to decline after 10–15 min, reaching levels near base line by 3 h. The addition of fresh TPA at this point elicited no further response. In contrast, zymosan stimulated the release of both O_2^- and H_2O_2 over the entire 3 h of incubation. The addition of TPA after 3 h increased the rate of RO release (not shown). Although 20 ng/ml of TPA produced a greater initial rate of RO release than did 400 μg/ml of zymosan, the total RO released over a 3-h period in response to zymosan was comparable to that released in response to TPA (Table I). Similar results were obtained with PMN, which produced RO at a greater rate than PBMC in response to either TPA or zymosan (Table I).

The chemotactic peptides, fMetLeuPhe and fNleLeuPhe, stimulated the release of RO by PMN and, weakly, by PBMC. However, the reaction was complete at 1 min for PMN and at 15 min for PBMC so that the total RO production was small (Table I). The subsequent addition of TPA (but neither chemotactic peptide) led to restimulation. This transient response is in accord with previous studies (35). Neither fMetLeuPhe nor fNleLeuPhe suppressed NK in concentrations from 10 nM to 10 μM (data not shown).

Stimulation of O_2^- and H_2O_2 production by cell subpopulations exposed to TPA was studied in more detail. At an optimal concentration of TPA (20 ng/ml), nonadherent PBMC produced little or no detectable O_2^- or H_2O_2 (Table II), confirming the histologic evidence that <2% of the tested cells were monocytes. Similarly, K562 target cells, although derived from a myelogenous leukemia, did not produce detectable RO in response to TPA (Table II).

Suppression of NK is reduced in glucose-free medium. The production of RO requires metabolism of glucose (36) and Nathan et al. (37) have shown that mouse macrophages fail to produce H_2O_2 after incubation in glucose-free medium. In our studies, when human PBMC were preincubated in glucose-free medium for 2 h and then exposed to TPA, the production of O_2^- was reduced by >95% and production of H_2O_2 could not be detected (Table II). When PBMC were tested for NK in glucose-free medium after a 2-h preincubation without glucose, suppression by TPA or zymosan was greatly reduced (Fig. 4, for results with TPA). NK was fully suppressed by TPA or zymosan if glucose was restored at the start of the cytotoxicity assay. Notably, NK itself was not impeded by the ab-

TABLE I

*Stimulation of H_2O_2 and O_2^- Production by PBMC and PMN: Comparison of Initial Rates to Total Production over 3 h**

Cell preparation	Stimulus	Concentration	H_2O_2 generated		O_2^- generated	
			Maximum rate	Total	Maximum rate	Total
			nmol/min	*nmol/3 h*	*nmol/min*	*nmol/3 h*
PBMC	None	0	<0.001	<0.01	<0.002	<0.4
	TPA	20 ng/ml	0.103	5.9	0.30	18.4
	Zymosan	400 μg/ml	0.050	11.3	0.18	28.9
	fMetLeuPhe	1 μM	0.002	<0.02	0.05	<0.5
	fNleLeuPhe	1 μM	0.002	<0.02	0.03	<0.5
PMN	None	0	<0.01	<0.5	<0.01	<1
	TPA	20 ng/ml	0.98	24.9	2.2	95.9
	Zymosan	400 μg/ml	0.13	17.3	0.27	30.6
	fMetLeuPhe	1 μM	0.70	0.7	2.1	2.1
	fNleLeuPhe	1 μM	0.30	0.3	1.2	1.2

* Cells (5×10^5) were incubated with stimuli at 37°C for 0–3 h. At 30-min intervals the cells were assayed for rate of RO production. The total amount of RO was estimated from these rates, summed over each 30-min intervals. The data shown are the means of two experiments.

TABLE II

*Release of H_2O_2 and O_2^- by Different Cell Populations Stimulated with TPA: Comparison with Enzymatic Generation**

Cell type or enzyme	H_2O_2	O_2^-
	% PBMC	% PBMC
PBMC, unseparated, 2×10^6/ml	100	100
PBMC, unseparated, 2×10^6/ml, glucose-free medium	<1	6
PBMC, adherent, 5×10^5/ml	75	67
PBMC, nonadherent, 1.5×10^6/ml	<1	3
PMN, 5×10^5/ml	359	374
K562, 5×10^5/ml	<1	<1
Xanthine, 1.0 mM/xanthine oxidase, 11.25 mU/ml	‡	347
Glucose, 5 mM/glucose oxidase, 0.25 U/ml	3,144	<1

* Cell assay mixtures contained 20 ng/ml TPA. Ethanol controls showed no SOD- or catalase-suppressible response. The values are expressed as the percentage of the H_2O_2 or O_2^- released by unseparated PBMC (2×10^6/ml) under the assay conditions described in Methods; the 100% values for PBMC (0.16±0.01 nmol H_2O_2/min, and 1.4±1.1 nmol O_2^-/min) were determined from at least two different cell preparations. Cell concentrations for adherent and nonadherent PBMC were chosen to reflect their contribution to total PBMC in the cytotoxic assay. PMN and K562 were tested at the same concentrations as adherent cells, and enzymes were used at suppressive concentrations.

‡ No H_2O_2 could be detected in this system. However, the presence of xanthine oxidase appears to affect the scopoletin assay (Goldstein, I., personal communication).

sence of glucose, suggesting that RO are not required for the mediation of NK.

Suppression of NK is not mediated by monocytes or PMN with the defect of chronic granulomatous disease. Patients with chronic granulomatous disease are unable to initiate a respiratory burst in response to agents that normally stimulate RO (38–41). We had the opportunity to examine peripheral blood cells from one such patient for their ability to mediate suppression of NK. Neither PMN nor monocytes from this patient permitted TPA-induced suppression of NK by nonadherent cells from the patient or from a normal donor (Fig. 5). However, PMN or monocytes from a normal donor suppressed NK by nonadherent cells from either the patient or the normal donor (Fig. 5). In this experiment, TPA actually stimulated NK by nonadherent cells, especially those of the patient with chronic granulomatous disease. To a varying degree, we have observed TPA-mediated stimulation of killing by nonadherent cells from normal donors in three of eight experiments, but we are as yet uncertain whether this represents NK or other cellular cytotoxicity.

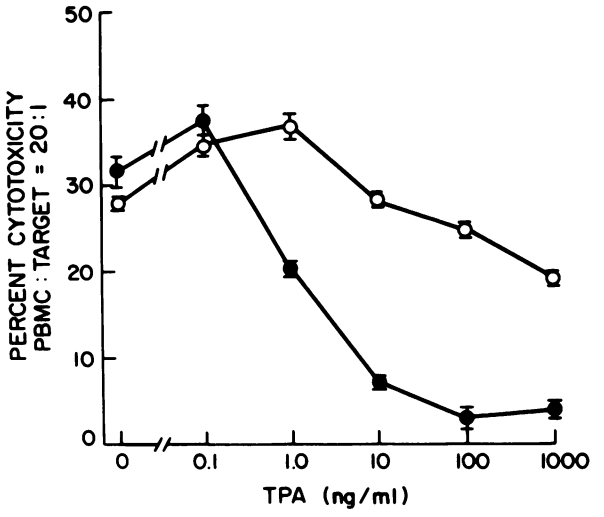

FIGURE 4 Loss of NK suppression by TPA in glucose-free medium. PBMC were incubated in glucose-free medium for 2 h at 37°C and then resuspended in the presence (●) or absence (○) of glucose for the NK assay. The spontaneous release of ^{51}Cr by K562 was not affected by the lack of glucose (7.9% of maximum release without glucose, 9.4% with glucose).

Killing by cells from the patient with chronic granulomatous disease was not inhibited even by high concentrations of TPA. This suggests that the partial suppression of normal nonadherent cells by high concentrations of TPA (or zymosan) may be due to RO production by low numbers of residual monocytes. This possibility was further supported by the observation that suppression of normal nonadherent cells could be further reduced by a second removal of adherent cells (not shown). Alternatively, nonadherent cells may contain cells other than monocytes that can generate small amounts of RO in response to zymosan or TPA.

Suppression of NK by PBMC is reversed by catalase. Suppression of NK by TPA was inhibited in the presence of catalase, which inactivates H_2O_2 (Table III). Catalase completely reversed suppression by 10 ng/ml TPA in 5 of 14 experiments. Heat-inactivated catalase had no effect on suppression of NK in each of four experiments (Table III). The concentrations of catalase required to overcome suppression of NK were similar to those required to scavenge H_2O_2; 200 U/ml of catalase reduced recoverable H_2O_2 by 50% compared with cell preparations incubated with TPA alone, and 2,000 U/ml reduced H_2O_2 to undetectable levels (not shown).

Scavengers of O_2^- were less effective than catalase in reversing the suppression of NK. Bovine erythrocyte SOD inhibited suppression only partially, but the effect was significant (Table III). Heat-inactivated SOD

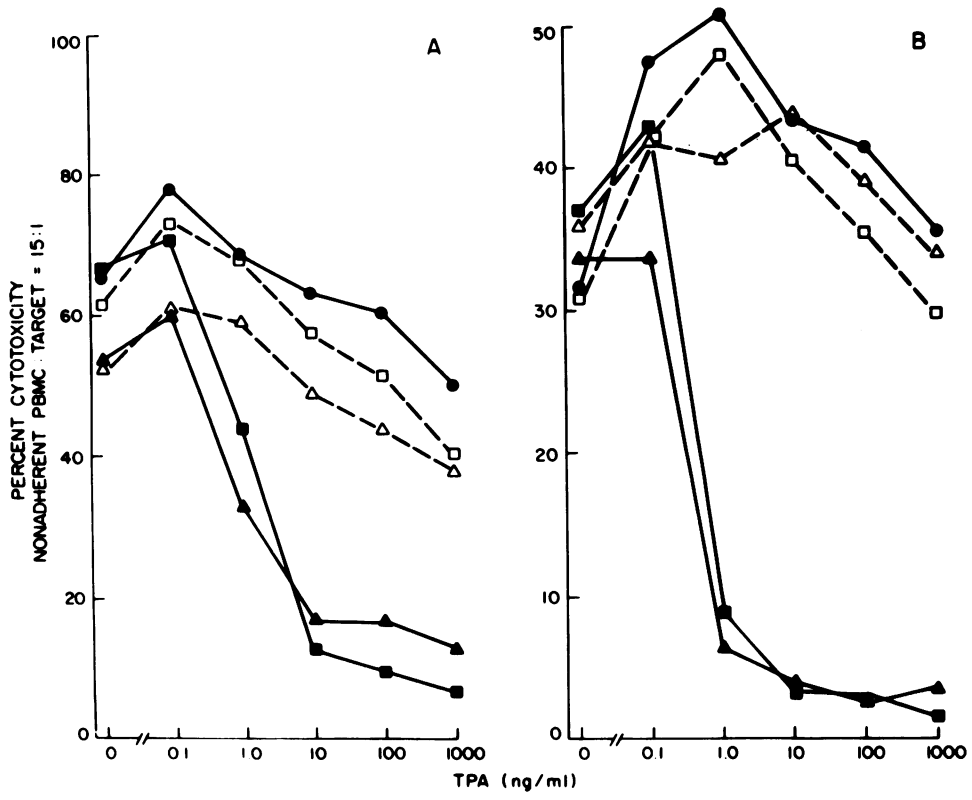

FIGURE 5 Monocytes or PMN from a patient with chronic granulomatous disease fail to mediate TPA-induced suppression of NK. NK by nonadherent cells alone (●), 15 × 10^5/ml, is shown for a normal volunteer (A) and for the patient (B). The nonadherent cells were mixed with 5 × 10^5/ml normal monocytes (■) or normal PMN (▲), or with the patient's monocytes (□), or PMN (△). The normal monocytes or PMN permitted suppression of NK by nonadherent cells from either the patient or the volunteer, whereas the chronic granulomatous disease monocytes or PMN did not.

was without effect (Table III). The partial effect of SOD occurred at concentrations that scavenged O_2^-; the recovery of O_2^- in response to TPA was reduced to 50% by 20-100 U/ml and to <5% by 400 U/ml. Lower levels of SOD had a slight stimulatory effect on unsuppressed NK, but this was not sufficient to account for its reversal of suppression by TPA (Table III). The combination of SOD and catalase was more effective in reversing suppression than catalase alone, but the difference was not significant (not shown). Tyrosine-copper (II), a compound that promotes the dismutation of O_2^- to H_2O_2 (42), had little effect on the suppression of NK at concentrations up to 320 μM (Table III). This agent, at 1 μM, reduced detectable O_2^- production by PBMC in response to TPA to <1%.

Suppression of NK is not dependent on peroxidase. In the presence of peroxidase, H_2O_2 can react with halides (X^-) to form highly reactive oxyhalides (OX^-) that may be bactericidal (36, 43). The reaction can be inhibited by sodium azide, cyanide, or aminotriazole (43). Each of these agents can alone inhibit NK (44 and unpublished observations). However, at 0.3 mM, sodium azide had little effect on NK but completely inhibited human myeloperoxidase activity in the H_2O_2-mediated quenching of scopoletin fluorescence. This concentration of sodium azide did not block suppression of NK by TPA or zymosan. In fact, suppression by zymosan was enhanced in each of three experiments. Cyanide or aminotriazole, in concentrations that inhibit peroxidase (1 mM and 10 mM, respectively [43]) partially reduced NK but did not block further suppression by TPA (not shown). These results indicate that peroxidase is not required for the suppression of NK and that H_2O_2 does not exert its suppressive activity through interaction with halides.

Suppression of NK is not affected by scavengers of OH· or $^1O_2$. The formation of O_2^- and H_2O_2 may lead to the production of OH· and $^1O_2$, highly active oxygen metabolites with short half-lives (36, 45, 46). To examine the role of these agents in the suppression

TABLE III

Effect of Scavengers of H_2O_2 and O_2^- on Suppression of NK by TPA

Experiment	Scavenging agent		No. of experiments	Cytotoxicity at effector/target = 20:1	
				TPA added	
				0	10 ng/ml
				% control*	
A	Catalase, *U/ml*	0	14	100	50‡
		50		100±5§	66±3 ($P < 0.0002$)§
		250		96±8	71±4 ($P < 0.0002$)
		1000		100±3	80±6 ($P < 0.0001$)
		4000		103±3	85±6 ($P < 0.0005$)
B	Catalase (heat inactivated), *U/ml*	0	4	100	48‡
		50		97±1	54±4
		250		98±3	51±5
		1000		91±4	51±4
		4000		93±4	41±7
C	Bovine SOD, *U/ml*	0	7	100	54‡
		5		104±2 ($P < 0.05$)	62±4
		25		110±4	69±4 ($P < 0.01$)
		100		104±5	70±4 ($P < 0.02$)
		400		101±4	67±7
D	Bovine SOD (heat inactivated), *U/ml*	0	3	100	51‡
		5		90±5	52±7
		25		95±5	53±8
		100		94±1 ($P < 0.05$)	59±6
		400		98±1	52±9
E	Tyrosine-copper (II) complex, μg/ml	0	5	100	47‡
		40		114±14	55±4
		80		96±8	51±1 ($P < 0.02$)
		320		97±17	51±2

* Cytotoxicity in the absence of TPA or scavengers was normalized to 100%. The actual values for percent cytotoxicity in each experiment were: (A) 52±4, (B) 55±6, (C) 52±8, (D) 59±7, and (E) 40±7.

‡ Suppression by TPA in each set of experiments was highly significant ($P < 0.0001$).

§ Cytotoxicity in the presence of scavengers was compared to cytotoxicity in the absence of scavengers by using *t* testing. Values are means±SEM. In parentheses are *P* values (two-tailed); *P* values that are not significant ($P > 0.05$) are not shown.

of NK, we tested the effect of scavengers of OH· (mannitol, ethanol) or $^1O_2$ (histidine) on TPA-mediated suppression. No effect was seen with 50 mM mannitol, 10 mM histidine, or 20 mM ethanol, concentrations that were not toxic in the assay. It should be noted that the medium itself contains scavengers of these oxygen species (46). Thus, it is unlikely that either OH· or $^1O_2$ plays a major role in suppression.

Natural killing is suppressed by H_2O_2 generated enzymatically. NK was reduced when H_2O_2 was generated during the cytotoxicity assay by glucose oxidase and glucose (Fig. 6a). The rate of production of H_2O_2 in this system (Table II) was stable throughout the 3-h incubation. Suppression was reversed by catalase but not by heat-inactivated catalase (Fig. 6a). Loss of NK was dependent on the concentration of glucose oxidase. Suppressive concentrations of enzyme (≥0.1 U/ml) produced more H_2O_2 than could be detected during cellular suppression of NK. This may reflect a difference in local concentrations of H_2O_2 that may be higher at the interface between cells than in the medium (37). The enzymatic generation of H_2O_2 suppressed killing by nonadherent PBMC as well as unseparated PBMC, indicating that monocytes were not required for the suppressive action of H_2O_2 (not shown).

NK was also reduced when O_2^- and its product, H_2O_2, were generated during the cytotoxicity assay by xanthine oxidase and xanthine (Fig. 6b). Production of O_2^- by xanthine oxidase and xanthine (Table II) declined over the first hour to <20% of the maximum rate but was still detectable at 3 h. Suppression was

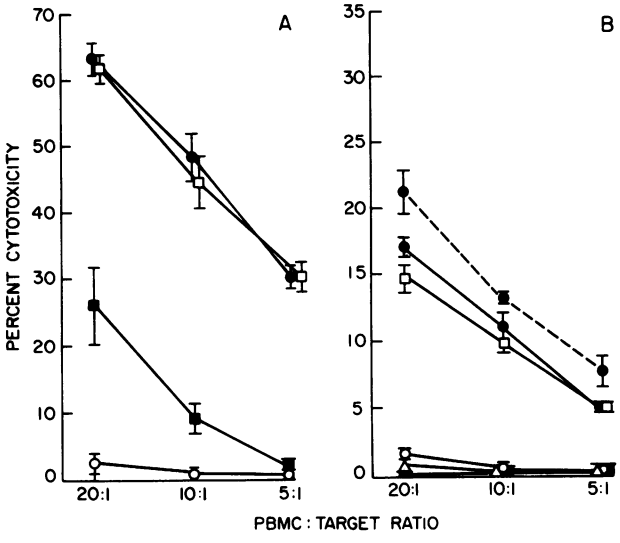

FIGURE 6 Effects on NK of RO generated enzymatically. (A) Suppression of NK by glucose oxidase and glucose. NK by untreated PBMC (● —— ●) was abrogated in the presence of 0.25 U/ml glucose oxidase and 5.0 mM glucose (○ —— ○). The effect was reversed by 2,000 U/ml catalase (□ —— □) but heat-inactivated catalase (■ —— ■) was relatively ineffective. (B) NK by PBMC (● —— ●) was also abrogated in the presence of 11 mU/ml xanthine oxidase and 1.0 mM xanthine (○). Suppression was again reversed by 2,000 U/ml catalase (□ —— □), or by 2,000 U/ml catalase and 200 U/ml SOD (●---●), but not by SOD alone (△ —— △). Heat-inactivated catalase was without effect (■ —— ■).

reversed by catalase but not by SOD, indicating that suppression was largely, if not entirely, mediated by H_2O_2.

Suppression of NK is not inhibited by indomethacin or dexamethasone. Prostaglandins have been implicated in the suppression of NK (18, 47). Moreover, the generation of RO may contribute to the production of bioactive metabolites of arachidonic acid such as prostaglandins (48, 49), and TPA stimulates the release of arachidonic acid from phospholipid (50). We therefore examined TPA-induced suppression of NK in the presence of indomethacin, at concentrations that inhibit cyclooxygenase and thus the production of prostaglandins. We also examined the effect of pretreating PBMC for 0–120 min with dexamethasone, which induces an inhibitor of the release of arachidonic acid from the cell membrane (51). Neither indomethacin (10 nM–10 μM) nor dexamethasone (0.1–10 μM) altered the suppression of NK by PBMC (data not shown).

NK recovers after suppression by zymosan. To determine if the RO-dependent suppression of NK was reversible, PBMC were incubated with 200 μg/ml zymosan for 3 h at 37°C, and then examined for NK. After this treatment, suppression of NK was then partially reversed either by the addition of catalase and SOD or by removal of zymosan by centrifugation through Ficoll-Hypaque (Table IV). When cells that had been recovered from Ficoll-Hypaque were incubated overnight, there was a further increase in NK, which then approached or equaled killing by cells that had not been exposed to zymosan (Table IV). Similar results were obtained in two additional experiments; in one, zymosan-suppressed NK was again fully recovered 24 h after recovery of cells on Ficoll-Hypaque, and in the other recovery was seen at 48 h. In other experiments, cells that were recovered by Ficoll-Hypaque after exposure to zymosan were also depleted of adherent cells. Although NK was enriched by this procedure, the regeneration of NK in the absence of adherent cells was variable and was not complete even after 96 h, indicating a requirement for adherent cells in the recovery of NK.

The ability to recover NK after exposure to zymosan suggests either that natural killer cells can recover their cytotoxic activity after exposure to zymosan or that they are rapidly replaced by the maturation of precursor cells that are resistant to RO. This is in accord with our findings that TPA and zymosan suppress NK without toxicity to PBMC. As further evidence that TPA is not generally toxic at concentrations that suppress NK, similar concentrations have been shown to stimulate, rather than suppress, cytotoxicity by monocytes or PMN (36, 52). We confirmed that TPA, in amounts that suppress NK, stimulates the killing of P388 lymphoma cells by PMN (not shown).

Interferon-stimulated NK is also suppressible by TPA. Interferon stimulates NK in vivo and in vitro (11, 12). To test the possibility that interferon might render NK cells resistant to suppression, PBMC were incubated in human fibroblast interferon for 24 h before the assay for NK. Interferon-treated cells showed at least a twofold increase in cytotoxicity on a per cell basis. Killing by both interferon-treated and untreated cells was suppressed in the presence of TPA (Table V).

DISCUSSION

Our studies demonstrate cellular regulation of human NK. We have demonstrated that both monocytes and PMN suppress NK in vitro when stimulated to release reactive metabolites of molecular oxygen. NK itself was not dependent on RO. The importance of RO in suppression of NK was established by (*a*) a correlation between the suppression of NK and the generation of RO, (*b*) loss of suppression in the absence of RO-producing cells (monocytes or PMN), (*c*) reduced suppression in glucose-free medium that did not support the generation of RO, and (*d*) lack of suppression in the

TABLE IV
Recovery of Natural Killing following Suppression by Zymosan

Treatment of PBMC before use in NK assay*	Effector/target cell ratio 20:1	10:1	5:1
	% cytotoxicity‡		
3-h incubation without zymosan followed by:			
No further treatment	35±3	22±2	14±2
Addition of catalase and SOD§	32±3	21±2	14±1
Recovery of cells on Ficoll-Hypaque	34±3	27±3	13±1
Recovery of cells on Ficoll-Hypaque, then 24-h incubation	33±1	18±1	10±1
3-h incubation with zymosan (200 μg/ml) followed by:			
No further treatment	12±2	8±1	5±1
Addition of catalase and SOD§	20±2	14±2	8±1
Recovery of cells on Ficoll-Hypaque	24±2	13±2	8±1
Recovery of cells on Ficoll-Hypaque, then 24-h incubation	31±3	22±2	14±1

* PBMC, 2×10^7/ml, were incubated with or without zymosan and washed before further treatment.
‡ Mean of triplicate samples±SEM.
§ SOD, 400 U/ml, and catalase, 4000 U/ml, were added at the start of the NK assay.

presence of monocytes or PMN from a patient with chronic granulomatous disease. Suppression of NK was particularly dependent on the generation of H_2O_2, as shown by (*a*) reversal of suppression in the presence of catalase, and (*b*) the demonstration that suppression could be mediated by the enzymatic production of H_2O_2 by glucose oxidase and glucose, or by xanthine oxidase and xanthine via dismutation from O_2^-. However, a role for O_2^- could not be excluded in that SOD partially reversed cell-mediated suppression of NK induced by TPA. Suppression was not blocked by agents that inhibited myeloperoxidase or by scavengers of OH· or $^1O_2$. Suppression of NK was not accompanied by a detectable loss of viable cells and was selective in that cytotoxicity by PMN was enhanced by concentrations of TPA that suppressed NK.

TABLE V
Suppression of NK by TPA after Pretreatment of PBMC with Interferon

Pretreatment with interferon*	TPA in assay‡	Percent cytotoxicity at PBMC/target ratio shown 20:1§	10:1
U/ml	ng/ml		
0	0	39±5	24±3
0	10	33±3	22±3
0	100	27±3	18±2
0	1000	23±3	9±1
500	0	59±8	43±4
500	10	19±3	12±2
500	100	23±2	13±2
500	1000	17±4	11±2

* PBMC, at concentrations used in the assay, were preincubated in microtiter plates, with or without interferon, for 24 h at 37°C.
‡ Added at start of NK assay.
§ Mean of 3 replicates±SD. Similar results were obtained in two other experiments.

The generation of reactive metabolites of oxygen has, until recently, been considered primarily a mechanism for host defense against pathogens or tumors (36). Our findings suggest that the generation of RO plays a significant role in autoregulation. Metzger et al. (53) recently found that the production of H_2O_2 by macrophages was synergistic with the production of prostaglandins in suppressing lymphocyte proliferation; catalase and indomethacin were together more effective in blocking suppression than either agent alone. Suppression of NK in our system was not altered by indomethacin, indicating that the cyclooxygenase pathway does not play a role in suppression. Moreover, PMN were more effective than monocytes in suppressing NK, evidence that suppression does not require a cell product that is unique to monocytes. Under other conditions, indomethacin has been found to enhance NK, suggesting that prostaglandins may, under certain circumstances, suppress NK (18, 47).

Although suppression of NK was particularly dependent on the generation of H_2O_2, we could not exclude a role for O_2^- because suppression was partially blocked by high concentrations of SOD, but not heat-inactivated SOD. In contrast, another scavenger of

O_2^-, tyrosine-copper (II), had little effect on NK suppression. Moreover, when O_2^- was generated enzymatically, the consequent suppression of NK was entirely dependent on the formation of H_2O_2. The effects of RO generated enzymatically may differ from the effects of RO generated by cells in direct apposition to the NK cells, where local concentrations of RO may be high and metabolic pathways may be unique (37, 54). Thus, although H_2O_2 is of major importance in the suppression of NK, an additional (or separate) role for O_2^- remains possible. In the presence of peroxidases, H_2O_2 can interact with halides to form highly reactive oxyhalides (36, 43). These do not appear to play a role in the suppression of NK by TPA or zymosan. In fact, zymosan-induced suppression was enhanced by 0.3 mM sodium azide, which inhibited peroxidase activity >99%.

We do not know that RO directly mediate suppression of NK; the generation of RO may activate other cells or may lead to the oxidation of other substances, which in turn suppress NK. Goodman and Weigle (55) recently demonstrated that 15-hydroperoxidation of arachidonic acid produces a potent inhibitor of lymphocyte activation and proliferation. Moreover, in the presence of an O_2^--generating system, arachidonic acid is converted to a potent chemoattractant (56, 57). Similarly, exposure of plasma to O_2^- results in the formation of a potent chemotactic factor (58). Thus, O_2^- and perhaps other RO may alter cell constituents and/or plasma factors that in turn serve as cell regulators. We are currently investigating the possibility that induction of the respiratory burst leads to production of a soluble suppressor of NK. Zembala et al. (59) found a soluble suppressor of the mitogenic response to PHA in the supernatant from monocytes that had been incubated with zymosan for 24 h.

It may be possible to examine the direct effects of RO on NK cells by the use of purified NK cell populations (10, 61–63) or of cloned natural killer cells (64, 65). Goldfarb and Herberman (66) reported that TPA could suppress unstimulated and interferon-stimulated NK by cells that had been enriched for NK by Percoll density gradient centrifugation. The effect on unstimulated cells was less than in our studies with PBMC and was inconstant. In light of our studies, it seems likely that the enriched natural killer cell population includes a variable number of cells capable of producing RO. Goldfarb and Herberman observed a biphasic suppression of NK in response to TPA, with maximum suppression both at low concentration (1 ng/ml) and high concentration (100 ng/ml). We have not seen suppression of NK at low TPA concentrations in our system. However, the effector cell population in our studies contained other potentially cytotoxic cells, such as T cells, which may have been stimulated by low concentrations of TPA to kill K562.

After suppression of NK by zymosan, cytotoxicity was partially recovered after the addition of catalase and SOD or by removal of zymosan, and NK was further restored after incubation of cells in fresh medium. Similarly, Mastro et al. (60) found that the mixed lymphocyte response by bovine lymphocytes could be suppressed by TPA, but the response recovered after 3–4 d of culture in fresh medium. Our results indicate that RO do not abrogate cell function; either NK cells regain their cytotoxic capacity, or they are replaced by the maturation of precursor cells.

The effect of RO on NK may have significance in a variety of diseases in which macrophages and PMN are activated to release RO. For example, patients with systemic lupus erythematosus or Sjogren's syndrome have decreased levels of NK and increased lymphoreticular malignancy (67, 68). It is tempting to speculate that the loss of NK in patients with these illnesses may reflect the chronic stimulation of RO by circulating immune complexes or opsonized cells. We are currently examining this hypothesis.

ACKNOWLEDGMENTS

We thank Ms. E. Clark and Ms. M. Leung for excellent technical assistance and Dr. I. Goldstein for stimulating and helpful discussions. Dr. Diane Wara kindly assisted us in obtaining cells from the patient with chronic granulomatous disease.

This work was supported by the Veterans Administration, the National Institutes of Health, and the U. S. Department of Energy.

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