Natural Killing of Tumor Cells by Human Peripheral Blood Cells

SUPPRESSION OF KILLING IN VITRO BY TUMOR-PROMOTING PHORBOL DIESTERS

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ABSTRACT Tumor-promoting phorbol diesters were shown to suppress natural killing in vitro by human peripheral blood mononuclear cells. The inhibitory effect of different phorbol diesters and their analogues correlated with their potency as tumor promoters, the most effective agent being 12-*O*-tetradecanoylphorbol-13-acetate (TPA). Both peripheral blood cells and targets specifically bound TPA, and natural killing could be inhibited by pretreatment of either cell population with TPA, though this was less effective than direct addition of TPA to the assay. Cells that had been pretreated with TPA released TPA and metabolites of TPA during subsequent incubation in fresh medium. This release of TPA was evidently responsible for the inhibition of natural killing by pretreated target cells; in experiments where labeled and unlabeled target cells were mixed, pretreatment of unlabeled targets with TPA inhibited killing of labeled targets. Suppression of natural killing by TPA was greatly reduced when adherent cells were removed from the peripheral blood cells, suggesting that monocytes mediate suppression. Inhibition of natural killing by TPA provides a model for examining the regulation of natural killing. Suppression of natural killing by phorbol diesters may contribute to their activity as tumor promoters.

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INTRODUCTION

Natural killing refers to the rapid cellular lysis of certain tumor cell lines (and even certain normal cells) without prior sensitization to the target (1–3). This spontaneous cytotoxicity is mediated by a distinct subgroup of mononuclear cells called natural killer (NK)[1] cells (1, 2). NK cells lack the features of mature T cells, B cells, or macrophages (1, 2, 4), though a portion of human NK cells binds weakly to sheep erythrocytes, suggesting an association with T cells (5). Most NK cells in human peripheral blood bear Fc receptors, but natural killing is not primarily dependent on antibody nor on the Fc receptor (6–8).

Natural killing has been implicated in host defense against malignancy. Enhancement of natural killing in mice protects against implanted tumor cells (9), and strain differences in natural killing correlate with resistance to tumors that are NK targets (10). Mice and humans that are congenitally deficient in natural killing are susceptible to certain tumors (11, 12).

Tumor promoters are agents that are not directly carcinogenic, but which facilitate the induction of tumors by other agents (13–15). An example is the promotion of benzo(a) pyrene-induced skin cancer in mice by the

[1] *Abbreviations used in this paper*: FBS, fetal bovine serum; NK, natural killer (cells); PBMC, peripheral blood mononuclear cells; PD, phorbol diesters; PDD, phorbol-12,13-didecanoate; R_F, mobility on chromatography relative to the solvent front; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; 4-*O*-methyl TPA, 12-*O*-tetradecanoylphorbol-13-acetate-4-*O*-methyl ether.

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topical application of croton oil (16). The tumor-promoting capacity of croton oil is due to phorbol diesters (PD), particularly 12-*O*-tetradecanoylphorbol-13-acetate (TPA) (17). There is evidence that PD promote malignant transformation directly, i.e., independent of effects on host defense. Thus, PD tumor promoters increase the transformation in culture of normal cells by benzo(a)pyrene, malignant viruses, or ultraviolet irradiation (18–20). They also alter the cytoskeletal structure of cultured cells (21, 22) and may inhibit or accelerate differentiation of certain cell lines (23, 24). In the absence of other agents, tumor promoters can induce neoplastic transformation of fibroblasts from humans with a genetic predisposition to cancer (hereditary adenomatosis of the colon and rectum) (25).

PD might also promote malignancy by reducing host defense. Until recently, however, evidence suggested that PD enhance tumor killing. Thus, PD tumor promoters increase killing by activated macrophages and by polymorphonuclear leukocytes through the generation of reactive forms of oxygen (26–28). PD tumor promoters are also mitogenic for a subpopulation of human T lymphocytes (29), but the activity of these cells in host defense is not known. Because of the potential role for natural killing in host defense against malignancy, we examined the effect of tumor promoters on natural killing by human peripheral blood mononuclear cells (PBMC).

This report demonstrates that natural killing by human PBMC is inhibited by PD tumor promoters. Inhibition of natural killing by PD correlated with the potency of PD as tumor promoters, TPA being the most potent inhibitor. The action of TPA involved the effector cell population (PBMC), suggesting that PD may, in part, promote malignancy by reducing host defense.

METHODS

Agents. The following were obtained from Sigma Chemical Co. (St. Louis, Mo.): TPA, 4-*O*-methyl-TPA (phorbol-12-myristate-13-acetate 4-*O*-methyl ether) and 4α-PDD (4α-phorbol-12,13-didecanoate). PDD (phorbol-12,13-didecanoate) was obtained from Vega Biochemicals (Tucson, Ariz.). Phorbol-12,13,20-triacetate was obtained from Polysciences, Inc. (Warrington, Pa.). Mezerein was kindly supplied by Dr. Nancy Colburn, Bethesda, Md. Anthralin was obtained from Phaltz Baer (Flushing, N. Y .) Ethyl phenylpropiolate was kindly supplied by Dr. Stuart Yuspa, Bethesda, Md. [20-^{3}H(*n*)]TPA was obtained from New England Nuclear (Boston, Mass.). Analysis of this reagent by high pressure liquid chromatography on a reverse phase column eluted with methanol/water (85:15, vol/vol) demonstrated that it was >95% homogeneous radiochemically. [^{3}H]TPA was concentrated by evaporation under nitrogen, dissolved in ethanol, and further diluted in medium for use. All other reagents were stored in ethanol or dimethylsulfoxide (DMSO) at −20°C and diluted at least 1:10^4 in medium prior to use. Neither ethanol nor DMSO at this dilution altered natural killing.

Media. All cell preparations were performed in RPMI 1640 (Gibco Laboratories, Grand Island Biological Co., Grand Island, N. Y.); this was supplemented with 1% fetal bovine serum (FBS, Sterile Systems, Inc., Logan, Utah). For assessment of natural killing, medium was supplemented with 20% FBS, 2 mM glutamine, 5 mM HEPES-buffer (*N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid), 100 μg/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml fungizone ("complete" medium).

Peripheral blood mononuclear cells. Effector cells were obtained from heparinized whole blood by sedimentation onto Ficoll-Hypaque. The cells were washed thrice and viability was then determined by exclusion of trypan blue.

Natural killing was assessed against K562, a myelogenous leukemia cell line grown in suspension culture in RPMI 1640 supplemented with 10% FBS (30). K562 target cells were labeled by incubation in $Na_2[^{51}Cr]O_4$ (^{51}Cr, carrier free; New England Nuclear) for 1 h, using 100 μCi/10^7 cells in a volume of 0.5 ml. The targets were washed thrice and resuspended to 2×10^5 live cells/ml. Target cells (0.1 ml) were mixed with PBMC (0.1 ml) in round-bottom microtiter plates (tissue culture grade; Linbro Chemical Co., New Haven, Conn.), with effector cells in varying concentration to give effector:target cell ratios as noted. Triplicate samples were prepared for each ratio. The plates were centrifuged at 100 *g* for 3 min, incubated in 5% CO_2 at 37°C for 3 h, and then centrifuged at 500 *g* for 10 min at 4°C. One-half the supernate was withdrawn for determination of released ^{51}Cr by use of a gamma spectrometer. Spontaneous release was determined by the use of unlabeled K562 target cells as effectors. Maximum release was determined by incubation of target cells in saponin (7 mg/ml) and EDTA (0.1 mg/ml). Percent cytotoxicity was determined by:

$$\left[\frac{\text{CPM}_{\text{effector cells}} - \text{CPM}_{\text{spontaneous release}}}{\text{CPM}_{\text{maximum release}} - \text{CPM}_{\text{spontaneous release}}}\right] \times 100.$$

We have either presented the entire killing curve for an experiment or, for comparing many curves, have presented cytotoxicity at a fixed effector/target ratio.

The cells responsible for the rapid in vitro lysis were not adherent to nylon wool or to plastic, and the majority did not adhere rapidly to sheep erythrocytes.

Pretreatment of cells with [^{3}H]TPA. PBMC or K562 (2×10^7/ml) were pretreated with [^{3}H]TPA (200 ng/ml) for 30 min at 20°C in the absence of FBS. Samples of suspended cells were taken to determine total radioactivity. The cells were then pelleted at 500 *g* for 10 min and the supernate was sampled to determine free (i.e., not cell-associated) TPA. The cells were then washed thrice, and resuspended to 10^7/ml in complete medium. Aliquots of 0.2 ml were incubated at 37°C in round-bottom microtiter plates. After varying time periods, triplicate samples were centrifuged at 500 *g* for 10 min. The supernate and the cells were collected separately to determine free and cell-associated TPA, respectively. All sampling was in triplicate. In separate studies to analyze the specificity of TPA binding, [^{3}H]TPA was incubated with PBMC (at 0.3 ng/ml) or with K562 (at 3 ng/ml) for 1 h at 37°C in the presence of varying amounts of unlabeled TPA. After incubation, the cells were washed twice in cold medium and radioactivity in the cell pellet was determined.

Chromatography of [^{3}H]TPA. To assess the cellular metabolism of TPA, effectors or targets were preincubated with [^{3}H]TPA, washed, and incubated in fresh medium as in the previous section. Samples were taken for thin-layer chromatography, including: (*a*) [^{3}H]TPA prior to use, (*b*)

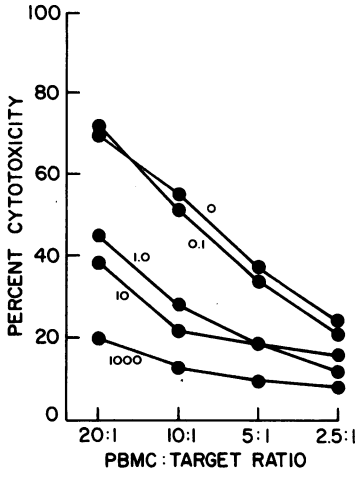

FIGURE 1 Effect of TPA on natural killing by PBMC. The values adjacent to the killing curves denote the concentration of TPA in nanograms per milliliter.

supernate from preincubated cells, (*c*) preincubated cells after washing, (*d*) supernate after incubation of pretreated cells for 1 h at 37°C, (*e*) pretreated cells (washed) after incubation for 1 h at 37°C, and (*f*) [^{3}H]TPA incubated in complete medium without cells.

The samples were lyophilized and extracted into 100% methanol. The methanol extract was analyzed on 20 × 20-cm silica G-coated thin-layer plates (Polygram SilG/UV$_{254}$, Brinkmann Instruments, Inc., Westbury, N. Y.), developed with methylene chloride:acetone (3:1 vol/vol) added to the tank 10 min before chromatography (31). The chromatograms were cut into 1-cm strips and radioactivity determined. TPA migrated with mobility on chromatography relative to the solvent front (R_F) = 0.7–0.8, phorbol monoesters with R_F = 0.1–0.4, and phorbol with R_F = 0.0, as described by O'Brien and Diamond (31).

Inhibition of natural killing by supernates from cells pretreated with TPA. PBMC or K562 were pretreated with TPA at varying concentrations as above, washed thrice, and resuspended to 10^7 cells/ml in complete medium. Aliquots of 0.2 ml (2 × 10^6 cells) were incubated at 37°C for 1 h. The cells were then pelleted at 500 *g* for 10 min and one-half the supernate was withdrawn to test for suppression of natural killing by addition to a mixture of fresh PBMC and targets.

Removal of adherent cells. Adherent and nonadherent PBMC were separated by the method of Ackerman and Douglas (32). Briefly P388Dl (murine macrophage) cells were grown to confluence in 75-cm^2 tissue culture flasks (Costar 3075, Costar Packaging, Cambridge, Mass.) and then removed by exposure to 10 mM EDTA for 15 min at 20°C. PBMC were incubated on these conditioned flasks (2 × 10^7 cells per flask) for 1 h at 37°C in 5% CO_2. Nonadherent cells were removed by decanting. Cell recovery was 50–60%. Adherent cells were recovered by incubation in EDTA (32). Monocytes were identified by morphology and esterase staining, according to the method of Yam et al. (33).

TABLE II

*Suppression by TPA of Natural Killing: Effect of Adding TPA after the Start of the Assay**

Time from start of assay	Percent killing remaining	Percent suppression observed	Percent suppression predicted‡
min			
0	100	58	—
30	75	45	44
60	49	27	28
90	30	24	17
120	17	19	10
180	0	0	0

* Natural killing was assessed over 3 h at a PBMC/target ratio of 20:1. In the absence of TPA, 25% of targets were killed (here considered as "100%" killing). Suppression by TPA was assessed by adding TPA to a final concentration of 100 ng/ml at varying times after the start of the assay.

‡ Calculated as 58% of the killing remaining, based on 58% suppression of killing when TPA was added at the start of the assay.

RESULTS

TPA reduces natural killing by PBMC. As little as 1 ng/ml of TPA was often sufficient to reduce natural kill-

TABLE I

Release of ^{51}Cr by Labeled PBMC, Showing No Effect of TPA on the Viability of PBMC

Number of cells per well (*0.2 ml*)		^{51}Cr released over 3 h (as percentage of maximum)					
		TPA (*ng/ml*)					
PBMC (^{51}Cr-labeled)	K562 (unlabeled)	0	0.1	1.0	10	100	1,000
4 × 10^5*	0	3.9	4.3	3.8	4.6	4.3	4.5
4 × 10^5*	2 × 10^4	4.4	4.8	4.2	4.7	4.3	4.2
2 × 10^5‡	0	4.5	5.1	4.0	4.8	4.8	4.9
2 × 10^5‡	2 × 10^4	5.4	5.2	4.5	4.8	4.5	4.6

* Maximum release, 40,109 dpm.

‡ Maximum release, 19,820 dpm.

The assay was carried out exactly as for natural killing except that PBMC instead of K562 target cells were labeled.

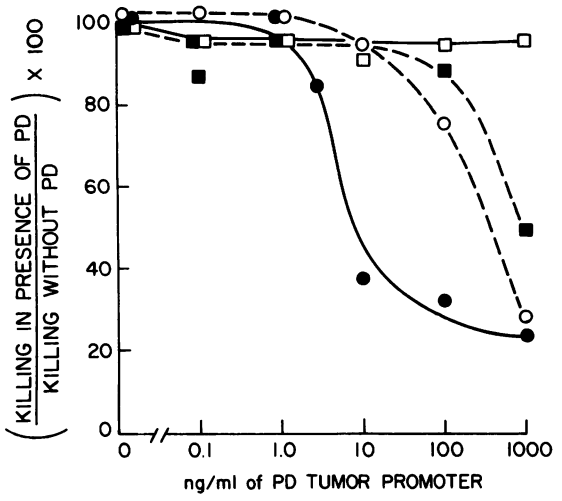

FIGURE 2 Inhibition of natural killing by phorbol diesters: (●) TPA, (○) PDD, (■) 4-*O*-methyl TPA, (□) 4α-PDD. Killing was assessed at a PBMC/target ratio of 10:1. Cytotoxicity in the absence of PD was 43%.

ing when added to the incubation mixture at the start of the assay, and a marked reduction was regularly seen with concentrations of 10 ng/ml or more (Fig. 1). The effect of TPA was demonstrated on PBMC from each of 12 individuals. TPA, in concentrations up to 1,000 ng/ml, was not toxic to PBMC during the assay as determined either by ^{51}Cr-labeling of the PBMC rather than the targets (Table I) or by exclusion of trypan blue (not shown). When PBMC were incubated overnight in TPA (up to 100 ng/ml) there was still no loss of viable cells when compared to cultures without TPA as assessed by exclusion of trypan blue.

When TPA was added after the start of the assay, the remaining natural killing was inhibited (Table II). When TPA was added at the end of the assay, no inhibition was seen, demonstrating that TPA does not interfere with the recovery of ^{51}Cr.

Correlation between inhibition of natural killing and potency of tumor promotion by phorbol diesters. We next examined the inhibition of natural killing by other PD and related compounds, by other tumor promoters, and by nonpromoting substances with inflammatory properties. PDD, a tumor promoter of moderate potency, inhibited natural killing at concentrations ~100-fold higher than TPA (Fig. 2). 4α-PDD, an analogue of PDD that is inactive as a tumor promoter, had no effect on natural killing. 4-*O*-methyl TPA, an analogue of TPA that is relatively weak as a tumor promoter, was inhibitory only at 1,000 times the inhibitory concentration of TPA. Table III summarizes the potency of these agents and several other (relatively weak) tumor promoters with regard to (*a*) tumor promo-

TABLE III
Potency of Phorbols as Tumor Promoters Compared with Suppression of Natural Killing and Skin Irritation

Compound	Suppression of natural killing (human)*	Tumor promotion (mouse)‡	Skin irritation (mouse)‡
TPA	++++	++++	++++
PDD	++	+++	++++
Mezerein	+	++	++++
Phorbol-12,13-dibenzoate	++	+	+++
4-*O*-methyl TPA	+	+	++
Phorbol-12,13,diacetate	+	+	+
4α-PDD	0	0	0
Phorbol-12,13,20-triacetate	0	+	—
Phorbol	0	0	0
Anthralin§	+	++	++
Ethyl phenylpropiolate§	0	0	++++
Saccharin§	0	+	0

* Graded by 50% inhibition of cytotoxicity at a PBMC/target ratio of 10:1. ++++, 1–10 ng/ml; +++, 10–100 ng/ml; ++, 100–1,000 ng/ml; +, <50% inhibition at 1,000 ng/ml; 0, no inhibition at 1,000 ng/ml.

‡ From Kinzel et al. (45), and, for ethyl phenylpropiolate, from Raick and Burdzy (46). Skin irritation is graded as the dose producing inflammation of the ear at 24 h: ++++, 0.01–0.1 nmol/ear; +++, 0.1–1.0 nmol/ear; ++, 1.0–10 nmol/ear; +, 10–100 nmol/ear; 0, no inflammation at 100 nmol/ear (—), not tested.

§ Not phorbols. Anthralin and ethyl phenylpropiolate were tested for effect on natural killing in amounts equimolar to TPA, up to 1,700 nM. Saccharin was without effect up to 5 mg/ml.

tion, (*b*) inhibition of natural killing, and (*c*) skin irritation. There was a correlation between the suppression of human natural killing by PD and tumor promotion in the mouse. Inhibition of natural killing correlated less well with the potency of PD as skin irritants. Mezerein, a diterpene ester tumor promoter related to the phorbol derivatives, is a potent skin irritant but had little effect on natural killing. No inhibition of natural killing was seen with 5 mg/ml saccharin, a tumor promoter that is not a PD, nor with ethyl phenylpropiolate, a powerful skin irritant that is not a tumor promoter.

Natural killing is reduced by pretreatment of either PBMC or K562 with TPA. Natural killing was reduced by pretreatment of PBMC or K562 with TPA for 30 min. The inhibitory effect increased over 2 h of pretreatment, although much of the effect was seen within 30 min (Fig. 3). For pretreatment of PBMC, the concentration of TPA required for 50% inhibition was 10-to-100-fold higher than required when TPA was added directly to the assay, whereas for K562 target cells, the concentration required was 1,000-fold higher (Fig. 4). Pretreatment of PBMC with TPA thus inhibited natural killing more efficiently than pretreatment of targets. However, pretreatment of either PBMC or K562 might result in cellular binding of TPA. The TPA could then be released during the assay for natural killing and act on the untreated cells. This possibility was examined using labeled TPA.

TPA binds to both PBMC and K562 and is released when TPA-treated cells are incubated in fresh medium. When PBMC or K562 were pretreated with 200 ng/ml of [^{3}H]TPA for 30 min at 20°C, both cell populations bound an average of 10^6 molecules of TPA per cell,

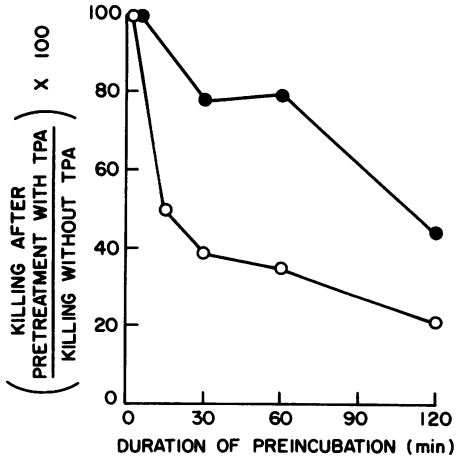

FIGURE 3 Time-course of inhibition of natural killing by pretreatment of effector PBMC (○) or target cells (●) with 1,000 ng/ml TPA at 20°C, showing the effect of duration of preincubation. Killing was assessed at a PBMC/target ratio of 20:1. Cytotoxicity in the absence of TPA was 35%.

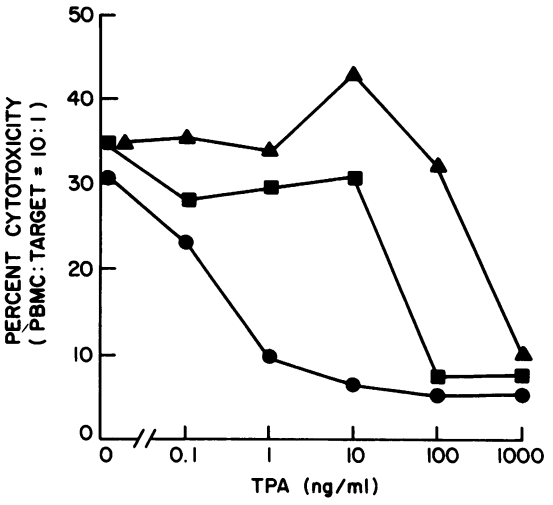

FIGURE 4 Inhibition of natural killing by TPA, comparing the dose-response for (*a*) direct addition of TPA to the killing assay (●), (*b*) pretreatment of the effector PBMC with TPA (■), or (*c*) pretreatment of the target cells with TPA (▲). Cells were pretreated with TPA for 30 min at 20°C and washed thrice before use in the assay.

representing about one-third of the total TPA. Almost all of the radioactivity associated with PBMC or K562 was still associated with TPA as determined by chromatographic analysis (Fig. 5, Table IV). [^{3}H]TPA bound to PBMC or to K562 both specifically and nonspecifically, either at 20° or 37°C. As shown in Fig. 6, binding was partially inhibited by unlabeled TPA, and the specific inhibition was similar for PBMC and K562. Binding of [^{3}H]TPA was unaffected by phorbol (not shown).

Both PBMC and targets pretreated with [^{3}H]TPA released radioactivity upon subsequent incubation in complete medium at 37°C (Fig. 7). There was rapid release during the first hour, followed by continued, slow release. During the first hour, PBMC released 25% of the total cell-associated radioactivity, whereas targets released ~12%. Most of the radioactivity was still associated with TPA, but radioactive metabolites of TPA were found both in cells and in the medium (Fig. 5, Table IV). PBMC were more active than K562 in metabolizing TPA. In different experiments, metabolites represented 10–25% of the radioactivity released by PBMC in 1 h. The metabolites were more polar than TPA and are presumably the result of deacylation of TPA, yielding phorbol and phorbol monoesters, which are inactive as tumor promoters.

Despite this partial metabolism of TPA, most of the TPA released by preincubated PBMC or by K562 cells was intact. It might therefore act on untreated cells during the assay for natural killing. To examine this point the supernates from PBMC or K562 pretreated with TPA were added to fresh effectors and targets to test

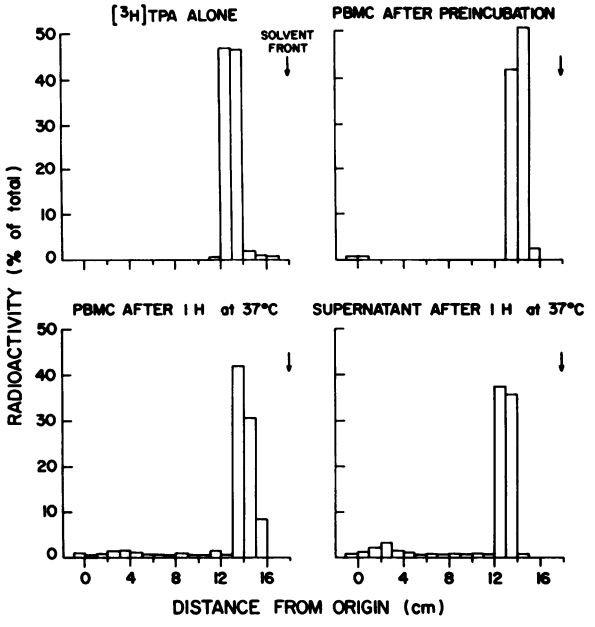

FIGURE 5 Chromatographic analysis of [^{3}H]TPA incubated with PBMC. The upper left panel shows TPA incubated in the absence of cells. The upper right panel shows material retained by PBMC after incubation in [^{3}H]TPA for 30 min at 20°C ("preincubation"). The PBMC were then washed and incubated in fresh medium at 37°C for 1 h. The lower left panel shows material retained by the cells. The lower right panel shows material released into the medium. Despite partial degradation, most of the TPA associated with the PBMC and in the supernate is intact (Table III).

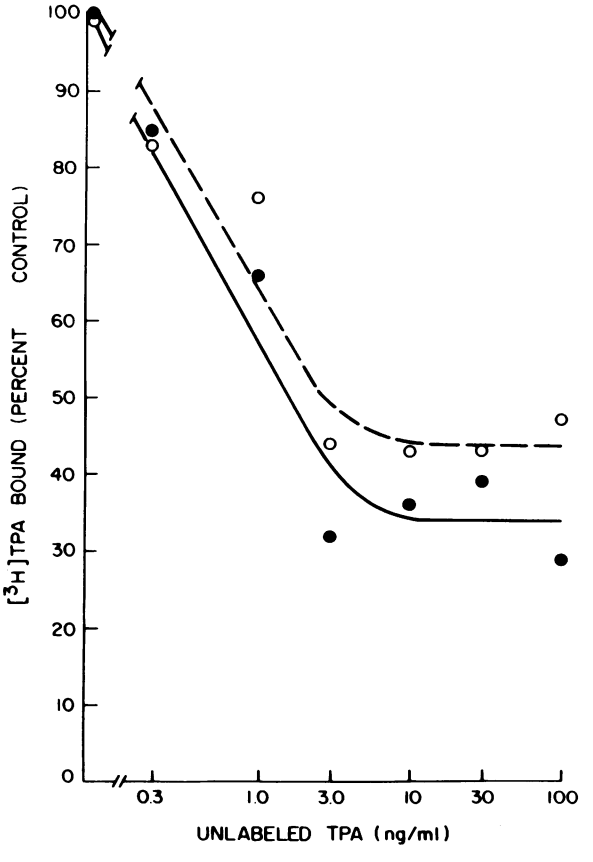

FIGURE 6 Inhibition of binding of [^{3}H]TPA to PBMC (○) or K562 (●) by unlabeled TPA, demonstrating both specific (inhibitable) and nonspecific binding. For conditions of the assay, see Methods.

for suppression of natural killing. The supernates from PBMC were more suppressive than the supernates from K652 (Table V), consistent with the demonstration that PBMC released more TPA.

Killing of labeled targets is inhibited in the presence of unlabeled cells pretreated with TPA. If pretreatment of K562 target cells with TPA inhibited natural killing indirectly, by the release of TPA during the assay, then killing of labeled ("hot") targets should be suppressed in the presence of unlabeled ("cold") targets that had been pretreated with TPA. To test this possibility killing was assessed against a 1:1 mixture of hot and cold targets, with TPA pretreatment of either target. When neither target population was treated with TPA, cold targets blocked natural killing of labeled targets by ~50%, as expected. Natural killing was then further reduced equally by pretreatment of

TABLE IV
*Metabolism of TPA by PBMC and K562**

Material assayed	Percentage of TPA intact	Percentage of TPA metabolized	Percentage of TPA metabolized to phorbol
PBMC	90.9	9.1	1.1
Supernate from PBMC	86.8	13.2	1.4
K562	97.0	3.0	0.4
Supernate from K562	96.2	3.8	0.4

* PBMC or K562 were pretreated with [^{3}H]TPA (200 ng/ml, cells at 2 × 10^7/ml) for 30 min at 20°C. The cells were then washed, resuspended to 10^7/ml, and incubated for 1 h at 37°C. The cells were pelleted and samples of the supernate and of the pelleted cells were analyzed by thin-layer chromatography.

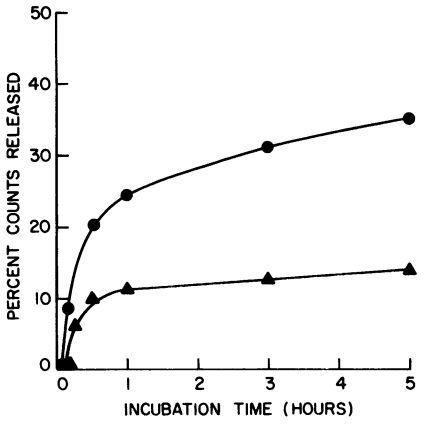

FIGURE 7 Release of radioactivity during incubation of [^{3}H]TPA-pretreated PBMC (●) or K562 cells (▲) for 1 h at 37°C. There is rapid release during the first 30–60 min followed by gradual release over the next 4 h.

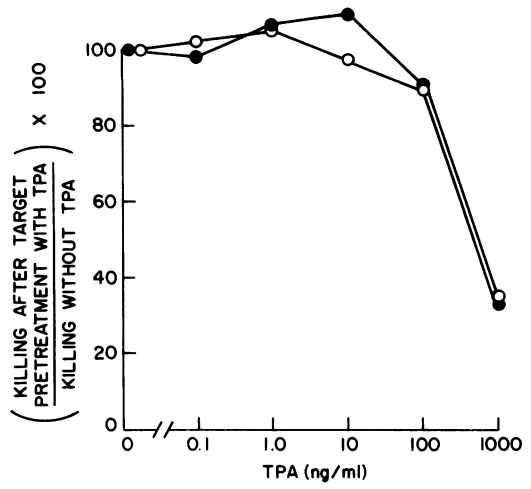

FIGURE 8 Inhibition of natural killing by pretreatment of "cold" (unlabeled) targets with TPA (○) is as effective as pretreatment of labeled targets with TPA (●). Killing of labeled targets was assessed in the presence of an equal number of unlabeled targets (each at 2×10^4 per well). The PBMC/target ratio was 10:1. Cytotoxicity without pretreatment of either target population was 17.2±1.5%.

either the hot or the cold targets with TPA (Fig. 8). We conclude that pretreatment of target cells with TPA inhibits natural killing indirectly, i.e., the TPA does not simply make the targets resistant to killing.

Suppression of natural killing by TPA requires adherent cells. PBMC depleted of adherent cells contained <2% monocytes compared with 20–30% in unseparated PBMC, as determined by esterase staining. Natural killing by untreated nonadherent cells was enriched 20–50% on a per cell basis compared to PBMC. In each of five experiments, natural killing by nonadherent cells was resistant to suppression by TPA (Fig. 9), even at 1,000 ng/ml. In two experiments, some stimulation of killing by nonadherent cells occurred at 0.01–1.0 ng/ml TPA. We do not yet know if this represents natural killing or stimulation of unrelated cytotoxic cells. Adherent cells alone did not usually kill K562 (Fig. 9), although in some experiments low levels of killing were seen when TPA was present at 10–100 ng/ml. In separate experiments, suppression was re-

TABLE V
*Inhibition of Natural Killing by Supernates from PBMC or from K562 Previously Exposed to TPA**

Concentration of TPA used for preincubation of PBMC or K562	Dilution of supernate from TPA-preincubated cells tested for inhibition of natural killing	Percent cytotoxicity by fresh cells in the presence of supernate from preincubated cells‡	
		Supernate from PBMC	Supernate from K562
ng/ml			
0	1:2	33.7±3.5	35.2±2.9
0	1:20	36.8±12.8	35.3±2.3
100	1:2	23.1±3.1§	38.9±4.3
100	1:20	39.0±3.0	35.7±5.5
1,000	1:2	19.6±2.4§	22.8±4.6§
1,000	1:20	21.4±3.5	34.4±2.3

* PBMC or K562 were exposed to TPA for 30 min at 20°C, then incubated in fresh medium for 1 h at 37°C. Supernates from the 1-h incubation were added to fresh effectors and targets to test for inhibition of natural killing. See Methods.

‡ Percent cytotoxicity at an effector/target ratio of 10:1, a point on the slope of the killing curve. Percent cytotoxicity in the absence of supernate was 33.8±3.3%. Similar results were obtained at effector/target ratios of 20:1 and 5:1.

§ Significantly different from control value (supernate from cells not exposed to TPA), $P < 0.025$ by Student's t test.

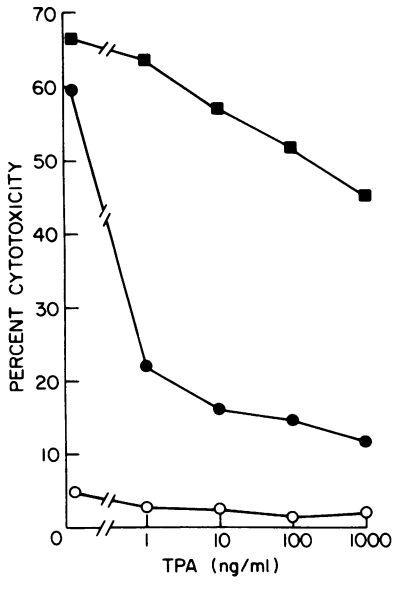

FIGURE 9 Natural killing by unseparated PBMC (●), by nonadherent PBMC (■), or by adherent PBMC (○) in the presence of TPA. The effector/target cell ratio was 20:1.

stored when adherent and nonadherent cells were recombined in a ratio of 1:3 (not shown).

DISCUSSION

We have demonstrated that phorbol diester tumor promoters reduce natural killing by human PBMC. PD were not toxic to PBMC, and inhibition by individual agents correlated with their potency as tumor promoters. Preincubation of PBMC or target cells with TPA also inhibited natural killing but only at higher concentrations.

TPA bound both to PBMC and target cells and was released during subsequent incubation in fresh medium. TPA-containing culture supernates from either PBMC or target cells inhibited natural killing by fresh PBMC. It is likely that the release of TPA accounts for the inhibitory effect of pretreating target cells with TPA, since preincubation of unlabeled targets inhibited killing of labeled targets.

In addition to promoting malignant transformation, PD induce metabolic and morphologic changes in normal cells that may be significant in considering the effect of PD on natural killing. Thus, PD interrupt metabolic cooperation (34), stimulate the release of glycosyltransferases from lymphocytes (35), alter the course of cell differentiation (23, 24), and stimulate the release of reactive oxygen groups from macrophages and polymorphonuclear cells (36–38). From studies in mice, Cudkowicz and Hochman (39) have proposed that NK cells are regulated by other cells, including macrophages (39). In our studies, suppression of natural killing was markedly reduced in the absence of adherent cells. This suggests that adherent cells may regulate natural killing in response to PD.

PD stimulate macrophages and polymorphonuclear cells to release reactive oxygen species, including superoxide anion, hydrogen peroxide, singlet oxygen, and hydroxyl radical (36–38). Metzger et al. (40) have recently demonstrated that hydrogen peroxide may play a role in macrophage-mediated immune suppression, and Goodman and Weigle (41) have shown that hydroperoxidation of arachidonic acid produces a nontoxic suppressor of lymphocyte proliferation and maturation. These findings suggest a role for reactive oxygen species in immune regulation. We are currently investigating the role of reactive oxygen species in the suppression of natural killing by PD.

In reducing natural killing, PD may promote malignancy by reducing host defense. While these studies were in progress, Keller reported that PD tumor promoters reduce natural killing in rats (42), and we have found the same effect in mice (unpublished observations). The in vivo administration of TPA to rats increased their susceptibility to injected tumor cells. These data provide evidence that promotion of malignancy by PD may involve effects on host defense in addition to effects on malignant transformation (42).

Keller also found that TPA suppressed killing by rat adherent peritoneal cells in a 36-h cytotoxicity assay (42). This is in contrast to the ability of TPA to stimulate cytotoxicity by macrophages in a "short-term" (4.5-h) assay, in which killing is mediated by the production of reactive oxygen species (27, 28). Although TPA stimulates macrophage cytotoxicity in short-term culture, it inhibits natural killing. This suggests that NK cells differ from macrophages in their mechanism of killing or in their susceptibility to the effects of TPA.

We have shown that PBMC and K562 demonstrate both saturable and nonsaturable binding of TPA; for PBMC the saturable binding is complete within 5 min at 37°C and for K562 it is complete at 20 min (Gindhart, T.D., B. Dalal, and W. E. Seaman. Unpublished data). A receptor for TPA has recently been demonstrated in human PBMC (43). A PD receptor has also been demonstrated in membrane preparations from chick embryo fibroblasts (44). It is possible that inhibition of natural killing is initiated by binding of PD to a specific cell receptor.

A notable observation in our studies was the ability of PBMC to catabolize TPA to metabolites that are inactive as tumor promoters. PBMC metabolized 10–25% of TPA in 1 h at 37°C. Thus, despite the effects of TPA on natural killing, PBMC are able to detoxify this tumor promoter. The rate of detoxification by PBMC is considerably faster than previously reported for human fibroblasts (31). Catabolism of TPA may therefore account for the observation that tumor promoters are most

effective when given with or after oncogens and that repeated administration of tumor promoters is usually required for maximum effect.

Studies of tumor promoters have primarily focused on their role in malignant transformation. It now appears that PD tumor promoters may also potentiate malignancy by reducing host defense. Inhibition of natural killing by these agents provides a new model for studying the regulation of NK cells. In particular, our findings suggest that NK cells are regulated by monocytes. Abrogation of the effects of tumor promoters on natural killing has a potential role in protection against malignancy.

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