

PROSTAGLANDINS FROM HUMAN  
T SUPPRESSOR/CYTOTOXIC CELLS MODULATE  
NATURAL KILLER ANTIBACTERIAL ACTIVITY

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The activity of the immune system is regulated by a precise balance between positive and negative signals (1-2). NK cells appear to be involved in a variety of biological processes, including anticancer activity, defense against viral, bacterial, and fungal infections, and resistance to parasites, among others (3-4). Although some descriptions of suppressor cells and substances for NK antitumor activity have been reported (5-12), the phenotype and the mechanism involved in the NK suppression process remain unclear (9-12). Recently, we have described that the purification of human NK cells by negative selection to  $\geq 98\%$  CD16<sup>+</sup> cells led to an increase in antibacterial activity that was out of proportion to the enrichment in NK cells (13). We hypothesized that a suppressor population could have been removed during the cell purification procedure. The aim of the present paper is to determine the cell population that inhibits the antibacterial activity and study the mechanism involved in the suppression process.

Materials and Methods

*Separation of Cell Populations.* Suppressor and effector cells were prepared by negative selection as described (13). Briefly, PBMC from healthy donors were separated on a Ficoll-Hypaque (Pharmacia Fine Chemicals, Piscataway, NJ) cushion, followed by successive adherence for 60 min to a glass petri plate and passage through wool nylon column. Monocyte-depleted (MDC) cells were incubated for 30 min at 4°C with the mAbs (20  $\mu$ l/10<sup>6</sup> cells) Leu-2 (anti CD8), Leu-3 (anti CD4), Leu-4 (anti CD3) (Becton Dickinson & Co., Mountain View, CA) for negative selection of NK cells, or with the mAbs Leu-3 (anti CD4) and Leu11 (anti CD16) for negative selection of T cytotoxic/suppressor cells. The cells were placed on a polystyrene petri dish coated with goat anti-mouse IgG (Tago, Burlingame, CA) for 60 min at 4°C. The nonadherent lymphoid cells were aspirated from the plates. The cells were cultured overnight in a serum antibiotic-free media HB104 (Dupont Co., Wilmington, DE) before use. For each experiment the phenotype of nonadherent (NK or CD8 enriched) cells was tested by direct immunofluorescence staining with FITC-conjugated Leu-1, Leu-19, Leu-2, Leu-3, Leu-4, and LeuM1, and enumerated by either fluorescence microscopy or by FACScan (Becton Dickinson & Co.). The surface phenotype (%) of lymphocyte populations after selection for NK cells was: Leu-2 (0), Leu-3 (0.1), Leu-4 (0), Leu-11 (98.5  $\pm$  2), Leu-19 (98  $\pm$  2),

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and Leu-M<sub>1</sub> (0); the surface phenotype (%) after selection for T cytotoxic/suppressor cells was: Leu-2 (98 ± 2), Leu-3 (0), Leu-4 (97 ± 2), Leu-11 (0), Leu-19 (0), and LeuM<sub>1</sub> (0).

**Antibacterial Assay.** *Escherichia coli* B/rA was obtained from T. Kagoma (UNM). The antibacterial assay was performed as described (13). The percentage of bacteria killed was determined from the expression percent killing = 100 × [(CFU experimental - CFU control)/(CFU control)]. The data are graphed as percent killing compared to varying E/T ratios, expressed as the log<sub>10</sub> of NK cell number mixed with a constant number of bacteria (2.5 × 10<sup>5</sup> CFU/ml).

**PGE Radioimmunoassay.** Supernatants from CD8<sup>+</sup> cultured cells, from CD8<sup>+</sup> cells treated overnight with indomethacin (1 μg/ml; Sigma Chemical Co., St. Louis, MO), and from CD8<sup>+</sup> cells treated with piroxicam (50 mM; Sigma Chemical Co.), were harvested. The concentration of PGE<sub>2</sub> in these supernatants was determined by RIA with PGE<sub>2</sub> <sup>125</sup>I RIA kit from Dupont Inc., Boston, MA.

### Results and Discussion

To test the hypothesis that a suppressor population is involved in the downregulation of NK cell antibacterial activity, we isolated Leu-3 (CD4<sup>+</sup>) and Leu-2 (CD8<sup>+</sup>) T cells that were removed during the negative panning NK cell purification process. Purified CD4<sup>+</sup> or CD8<sup>+</sup> cells were co-incubated with purified autologous NK cells. No inhibitory effect was observed when NK cells were cultured overnight in the presence of CD4<sup>+</sup> cells (data not shown). In contrast, the antibacterial activity of NK cells was significantly reduced after coincubation with CD8<sup>+</sup> cells at CD8<sup>+</sup>/NK ratios of 1:9 and 1:4, and this effect was dramatically enhanced at ratios larger than 1:3 (Fig. 1 A). This appears to indicate that there is a threshold number of CD8<sup>+</sup> cells necessary to achieve significant suppression of NK cell activity. In addition, the threshold number of CD8<sup>+</sup> cells could also be related to the number or activity of suppressor cells required to produce one or more suppressive factors. CD8<sup>+</sup> cell free supernatants (CD8-SN) were harvested from 18-h cultures of purified T cyto-

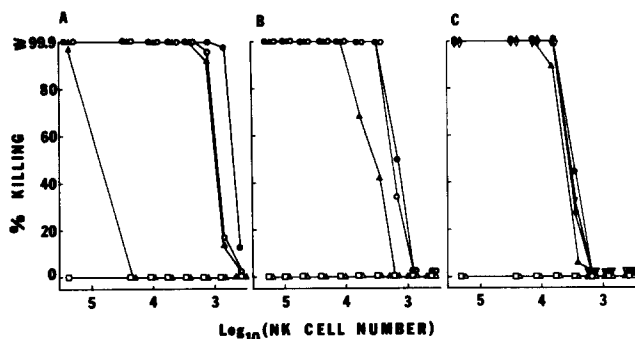


FIGURE 1. (A) Dependence of CD8-induced suppression of the NK antibacterial activity against *E. coli* on the CD8<sup>+</sup>/NK ratio. (●) NK cells control; (○) CD8<sup>+</sup>/NK cells at 1:9 ratio; (▲) CD8<sup>+</sup>/NK cells at 1:4 ratio; (△) CD8<sup>+</sup>/NK cells at 1:3 ratio; (□) CD8<sup>+</sup>/NK cells at 3:7 ratio. (B) Inhibition of the NK antibacterial activity by exogenous PGE<sub>2</sub>. (●) NK cells control; (○) NK treated with PGE<sub>2</sub> (10<sup>-9</sup> M); (▲) NK treated with PGE<sub>2</sub> (10<sup>-8</sup> M); (△) NK treated with PGE<sub>2</sub> (10<sup>-7</sup> M); (□) NK treated with PGE<sub>2</sub> (10<sup>-6</sup> M); (C) Effect of the arachidonic acid metabolism inhibitors, indomethacin and piroxicam (Sigma Chemical Co.), on the CD8<sup>+</sup>-mediated suppression of NK activity. The CD8<sup>+</sup> cell population was incubated overnight in the presence of indomethacin (1 μg/ml) or piroxicam (50 mM) (both from Sigma Chemical Co.), washed, and coincubated for 3 h with autologous NK cells at the CD8<sup>+</sup>/NK ratio of 3:7 in the absence and presence of exogenous PGE<sub>2</sub>. (●) NK cells control; (□) NK<sup>+</sup>CD8; (▽) NK plus Indomethacin-treated CD8<sup>+</sup> cells; (▼) NK plus piroxicam-treated CD8<sup>+</sup> cells; (△) NK plus indomethacin-treated CD8<sup>+</sup> cells plus PGE<sub>2</sub> (10<sup>-7</sup> M); (▲) NK plus Indomethacin-treated CD8<sup>+</sup> cells plus PGE<sub>2</sub> (10<sup>-8</sup> M). The experiments shown are representative of five that were performed with similar results.

ed with PGE<sub>2</sub> (10<sup>-7</sup> M); (□) NK treated with PGE<sub>2</sub> (10<sup>-6</sup> M); (C) Effect of the arachidonic acid metabolism inhibitors, indomethacin and piroxicam (Sigma Chemical Co.), on the CD8<sup>+</sup>-mediated suppression of NK activity. The CD8<sup>+</sup> cell population was incubated overnight in the presence of indomethacin (1 μg/ml) or piroxicam (50 mM) (both from Sigma Chemical Co.), washed, and coincubated for 3 h with autologous NK cells at the CD8<sup>+</sup>/NK ratio of 3:7 in the absence and presence of exogenous PGE<sub>2</sub>. (●) NK cells control; (□) NK<sup>+</sup>CD8; (▽) NK plus Indomethacin-treated CD8<sup>+</sup> cells; (▼) NK plus piroxicam-treated CD8<sup>+</sup> cells; (△) NK plus indomethacin-treated CD8<sup>+</sup> cells plus PGE<sub>2</sub> (10<sup>-7</sup> M); (▲) NK plus Indomethacin-treated CD8<sup>+</sup> cells plus PGE<sub>2</sub> (10<sup>-8</sup> M). The experiments shown are representative of five that were performed with similar results.

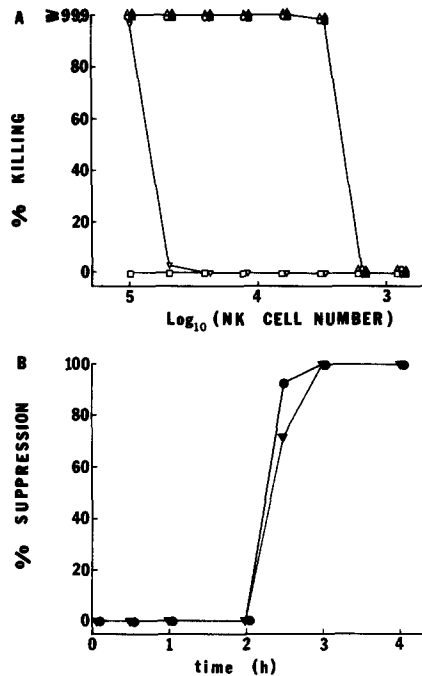


FIGURE 2. (A) Kinetics of CD8<sup>+</sup>-mediated suppression of NK cell antibacterial activity. Varying numbers of NK and CD8<sup>+</sup> enriched populations were mixed in medium at the CD8<sup>+</sup>/NK ratio of 3:7 and incubated at 37°C for different point times: (Δ) 30 min; (▲) 60 min; (■) 120 min; (▽) 150 min; (□) 180 min. After each incubation the bactericidal assay was performed as described in Fig. 1. Furthermore, the NK antibacterial activity of overnight cultured NK enriched cells, in the absence (○) and presence (□) of CD8<sup>+</sup> cells at the CD8<sup>+</sup>/NK ratio of 3:7, was tested. (B) Time of onset of (●) CD8<sup>+</sup> cell-mediated and (▼) CD8SN-mediated suppression of NK antibacterial activity for a fixed number of NK cells ( $5 \times 10^4$ ) at CD8<sup>+</sup>/NK ratio of 3:7, and 30% vol/vol CD8SN. The experiment shown is representative of three that were performed with similar results.

toxic/suppressor cells as described (13). Effector cells were incubated overnight, then washed and treated with different amounts of CD8-SN. The results (Fig. 2 B) showed that the CD8<sup>+</sup> supernatants contain one or more factors that abrogate both the antibacterial activity and the expression of the activation markers IL2-R and Tf-R (data not shown). CD8<sup>+</sup> supernatants were suppressive at up to 1:4 dilution. Similar results were obtained in cytotoxicity assays performed against K562 cells. Thus, at a NK/K562 ratio of 25:1, the percentage of cytotoxicity decreased from  $91 \pm 3\%$  to  $16 \pm 1\%$  in the presence of CD8<sup>+</sup> cells (CD8<sup>+</sup>/NK ratio, 3:7).

NK antitumor activity is suppressed *in vitro* by exogenous prostaglandins (14-17). To test whether prostaglandins were involved in the suppression of the NK antibacterial activity, the NK cell population was cultured overnight in the presence of different concentrations of exogenous PGE<sub>2</sub>, and then tested for antibacterial activity. Fig. 1 B shows that PGE<sub>2</sub> at concentrations of  $10^{-7}$  M completely abrogated the antibacterial activity of purified NK cells. This hypothesis was also tested by culturing the CD8<sup>+</sup> population in the presence of two different cyclooxygenase inhibitors, indomethacin, or piroxicam. Fig. 1 C shows that inhibition of prostaglandin synthesis abrogated the suppressor activity of the CD8<sup>+</sup> cells. These results suggested that purified T cytotoxic/suppressor cells are able to produce prostaglandins that inhibit activity of NK cells. We also measured by specific RIA the endogenous production of PGE<sub>2</sub> in overnight cultures of purified CD8<sup>+</sup> cells, and CD8<sup>+</sup> cells plus indomethacin or piroxicam, without bacterial or cytokine stimulation. CD8<sup>+</sup> cells secreted PGE<sub>2</sub> into the culture medium (Table I) in amounts ( $10^{-7}$  M) shown to inhibit NK activity when the PGE<sub>2</sub> is added exogenously (Fig. 1 B and references 15-17). In-

TABLE I  
PGE<sub>2</sub> Concentrations in 16–24 h Cultured CD8<sup>+</sup> Cell Free Supernatants

Treatment	PGE <sub>2</sub>			
	Exp. 1	Exp. 2	Exp. 3	Exp. 4
	<i>M</i>			
None	1.87 × 10 <sup>-7</sup>	1.81 × 10 <sup>-7</sup>	1.98 × 10 <sup>-7</sup>	2.32 × 10 <sup>-7</sup>
Indomethacin (1 μg/ml)	<7 × 10 <sup>-12</sup>	<7 × 10 <sup>-12</sup>	<7 × 10 <sup>-12</sup>	ND
Piroxicam (50 mM)	<7 × 10 <sup>-12</sup>	<7 × 10 <sup>-12</sup>	<7 × 10 <sup>-12</sup>	ND

<7 × 10<sup>-12</sup> M represents the limit of detection of the assay.

domethacin (1 μg/ml) and piroxicam (50 mM) blocked both the PGE<sub>2</sub> synthesis and suppression of NK activity (Table I and Fig. 1 C). Again, the addition of PGE<sub>2</sub> at 10<sup>-7</sup> M restored the suppression of NK antibacterial activity (Fig. 1 C).

We studied the kinetics of CD8<sup>+</sup>-mediated NK cell suppression by the optimal ratio of CD8<sup>+</sup>/NK cells, or by the optimal concentration of CD8<sup>+</sup> supernatants. There was no suppression in NK antibacterial activity after a 1-h incubation (Fig. 2, A and B). After a 2.5-h coincubation suppression was marked, and after 3–16 h suppression of NK activity was complete. The kinetics of suppression mediated by CD8<sup>+</sup> cell free supernatants (Fig. 2 B) was similar to that mediated by CD8<sup>+</sup> cells. Incubation of NK cells for 2.5 h in the presence of CD8<sup>+</sup> supernatants prevented and reversed the expression of the activation markers (data not shown).

From the data shown above the following conclusions can be drawn. First, the T cytotoxic/suppressor cell population (≥98% CD8<sup>+</sup> cells) produce PGE<sub>2</sub> at concentrations that suppress most in vitro T and NK cell functions (15–17). Second, the amount of PGE<sub>2</sub> produced by resting CD8<sup>+</sup> cells is even higher than that reported for unstimulated monocytes and macrophages (8.5 × 10<sup>-10</sup> to 4.3 × 10<sup>-8</sup> M) (18–21). Third, PGE<sub>2</sub> produced by CD8<sup>+</sup> cells downregulates both antibacterial activity and expression of activation markers of activated NK cells to a baseline level in less than 3 h. Fourth, inhibition of prostaglandin synthesis abrogates both the suppressive activity of the CD8<sup>+</sup> cells and their PGE<sub>2</sub> production.

The presence of CD8<sup>+</sup> suppressor cells explains why removal of relatively small numbers of T cells by panning resulted in large increases in antibacterial and antitumor NK activity (13). It is notable that an in vitro suppressor cell/NK cell ratio of 1:3 is sufficient to suppress all NK activity, in comparison to the higher physiologic ratio for these populations in blood of ~2:1. Other cells, including CD4<sup>+</sup> cells, may play a role in the modulation of NK cell activity. A previous study (22) had demonstrated PGE<sub>2</sub> production (1.1–1.7 × 10<sup>-7</sup> M) by CD8<sup>+</sup> cells stimulated by bacterial antigens, but concomitant production of PGE<sub>2</sub> by contaminating monocytes was not excluded. The results presented here establish that T cytotoxic/suppressor cells bearing the CD8 phenotype are potent inhibitors of NK cell activity and that this mechanism is mediated by PGE<sub>2</sub> production. The in vivo relevance of this mechanism can now be tested using cyclooxygenase inhibitors.

### Summary

We have recently described potent antibacterial activity of purified human NK

cells. Here we show that this function is regulated by T cytotoxic/suppressor CD8<sup>+</sup> cells. Thus, coculture of NK and CD8<sup>+</sup> cells for 3 h or longer times abrogated the expression of the NK antibacterial activity, and of two activation markers IL-2R and transferrin receptor (Tf-R). The suppressive activity was mediated by PGE<sub>2</sub> as demonstrated by direct PGE<sub>2</sub> determination in CD8<sup>+</sup> cell free supernatants, and by inhibition of CD8<sup>+</sup> cell suppression with indomethacin or piroxicam in vitro. We also found that resting T cytotoxic/suppressor cells purified by negative selection produce higher amounts of PGE<sub>2</sub> than adherent cells like monocytes and macrophages, and that these concentration levels are in the range of concentrations known to suppress a significant number of in vitro immunologic functions.

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