

## INTERLEUKIN 2 INDUCTION OF PORE-FORMING PROTEIN GENE EXPRESSION IN HUMAN PERIPHERAL BLOOD CD8<sup>+</sup> T CELLS

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One of the major components found in the granules of CTL is pore-forming protein (PFP)<sup>1</sup> (perforin or cytolysin) (1-4), a C-9 like protein, which in the presence of Ca<sup>2+</sup> polymerizes to form transmembrane channels in target cells. Resting CTL that exhibit no cytolytic activity contain little or no PFP; however, synthesis of PFP and demonstrated cytotoxic activity can be induced in human PBMC by incubation with anti-CD3 mAb (OKT-3) or IL-2 for several days (5, 6).

The recent cloning of PFP cDNA and use of PFP cDNA probes have indicated that PFP mRNA is expressed specifically in cytolytic lymphocytes regardless of whether they were generated in vivo or cultured in vitro (3, 4, 7). In particular, human PBMC expressed low amounts of PFP mRNA; however, when cultured with IL-2, these levels increased fivefold within 24 h and preceded an increase in cytotoxicity by 12 h (3).

Despite these findings it was still unclear whether IL-2 or anti-CD3 mAb could directly induce PFP expression in T cells and large granular lymphocytes (LGL). Our studies have investigated PFP mRNA expression in purified human peripheral blood CD3<sup>-</sup> LGL, CD3<sup>+</sup> T cells and their subsets (CD4<sup>+</sup>/CD8<sup>+</sup>) stimulated with IL-2, OKT-3 mAb, PMA, and ionomycin.

### Materials and Methods

*Preparation of Lymphoid Cells.* PBMC were separated on Ficoll-Hypaque as described (8). Leukocyte suspensions were washed with HBSS and were resuspended in RPMI 1640 containing 10% heat-inactivated FCS. Adherent cells (monocytes and B cells) were removed by incubation on plastic dishes and nylon wool. To enable crosslinking of the OKT-3 mAb, monocytes were collected by treatment of the plastic dishes with versene-EDTA and mixed (8%) with purified CD3<sup>+</sup> lymphocytes. Highly enriched populations of CD3<sup>+</sup> T cells (>90%) and

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M. J. Smyth was a Fogarty Visiting Fellow of the National Institutes of Health and is currently supported by a C. J. Martin Traveling Fellowship, National Health and Medical Research Council of Australia.

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<sup>1</sup> *Abbreviations used in this paper:* ADCC, antibody-dependent cell-mediated cytotoxicity; LGL, large granular lymphocytes; PFP, pore-forming protein.

While this paper was in the process of revision, another article was published that reported data similar to that contained herein (Liu, C.-C., S. Rafii, A. Granelli-Piperno, J. A. Trapani, and J. D.-E. Young. 1989. Perforin and serine esterase gene expression in stimulated human T cells. Kinetics, mitogen requirements, and effects of cyclosporin A. *J. Exp. Med.* 170:2105.

CD3<sup>-</sup> LGL (>95%) from PBMC were obtained by centrifugation of nylon wool-passed cells on discontinuous density gradients of Percoll (Pharmacia Fine Chemicals, Uppsala, Sweden). Subsequent SRBC rosetting of CD56-enriched Percoll fractions was used to remove contaminating T cells to obtain the CD3<sup>-</sup>/LGL populations (9). T cell subsets (CD4<sup>+</sup>/CD8<sup>+</sup>) were negatively selected by adherence of mAb<sup>+</sup> (OKT-4 or OKT-8) cells to F(ab')<sub>2</sub> anti-mouse Ig (Cappel Laboratories, Malvern, PA)-coated petri dishes (Falcon Labware, Oxnard, CA) (10). Upon purification, the populations were analyzed by flow cytometry to determine the extent of contamination. Cells were phenotyped by using phycoerythrin- or FITC-conjugated mAbs: anti-Leu-3a (IgG1, anti-CD4); anti-Leu-2a (IgG1, anti-CD8); anti-Leu-4 (IgG1, anti-CD3); anti-Leu-19 (IgG1, anti-CD56); anti-Leu 11a (3G8, IgG1, anti-CD16); and anti-Leu 11b (Vep 13, IgM, anti-CD16) (Becton Dickinson Immunocytometry Systems, Mountain View, CA). Two color fluorescence measurements were performed on a FACScan IV flow cytometer (Becton Dickinson).

**Lymphocyte Stimulation.** Isolated T cells and LGL were cultured in RPMI 1640 medium supplemented with 2 mM L-glutamine, 2% heat-inactivated FCS, 100 U/ml penicillin; 100 µg/ml streptomycin, and 6 mM Hepes buffer (pH 7.3) (Gibco Laboratories, Grand Island, NY). Cultures (1–2 × 10<sup>6</sup>/ml) were stimulated with PMA (Sigma Chemical Co., St. Louis, MO) at 10 ng/ml, ionomycin (Calbiochem-Behring Diagnostics, La Jolla, CA) at 3 µg/ml, human rIL-2 (Cetus Corp., Emeryville, CA) at 0–6,000 U/ml or OKT-3 mAb at 0.5–10 µg/ml.

**Monoclonal Antibodies.** TU27 mAb reacts with the p75 subunit of the IL-2 receptor and inhibits the binding of IL-2 to this receptor (11). H-31 mAb reacts with the p55 IL-2 receptor and inhibits IL-2 binding to this subunit (12).

**Proliferation Assay.** Triplicate cultures of 2 × 10<sup>5</sup> CD3<sup>+</sup> CD56<sup>-</sup> T cells in 200 µl of media (as above) were seeded in a 96-microwell plate and were stimulated with IL-2, OKT-3 mAb, PMA, and ionomycin as above. Cultures were incubated at 37°C in 5% CO<sub>2</sub> for 10 h. <sup>3</sup>[H]TdR (New England Nuclear, Boston, MA) (6.7 Ci/mmol) incorporation was determined during the last 4 h of culture (1 µCi/well).

**RNA Analyses.** Total cytoplasmic RNA was purified from T cells and LGL by the method of Chomczynski et al. (13). For Northern analysis RNA was subjected to electrophoresis on a 0.8% agarose formaldehyde gel, then transferred to Nytran (Schleicher & Schuell, Keene, NH). All blots were hybridized to <sup>32</sup>P-labeled human PFP cDNA, human IFN-γ cDNA or chicken β-actin cDNA as described previously (14). The blots were then exposed to Kodak X-OMAT AR film for 0–7 d at –70°C. Comparative levels of PFP mRNA expression were determined from the film using a gel scanner (ISCO, Lincoln, NE). The human PFP cDNA was isolated from a cDNA library constructed from the NK-like clone YTN-10 (15). The human IFN-γ cDNA was obtained from Dr. G. Ricca (Rorer Biotechnology, King of Prussia, PA) and the chicken β-actin cDNA was from Dr. D. W. Cleveland (Princeton University, Princeton, NJ).

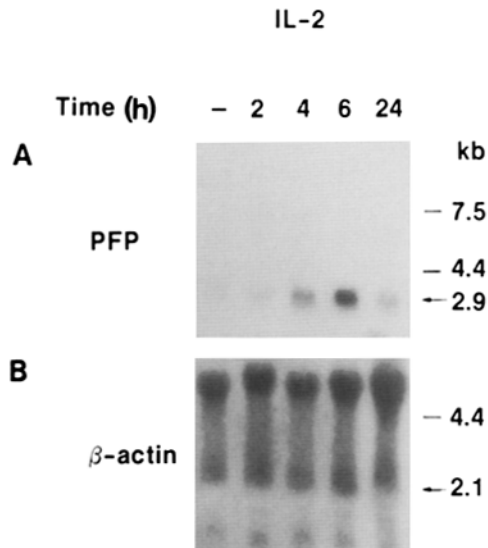
**IFN-γ Assay.** Human IFN-γ was assayed by RIA (Centocor, Malvern, PA) of culture supernatants obtained after treatment of cells with the indicated agents.

**IL-2 Assay.** Human IL-2 was assayed by an ELISA of culture supernatants (Collaborative Research Incorporated, Bedford, MA) obtained after treatment of cells with OKT-3 mAb.

**Cytotoxicity Assay.** Cytotoxicity was measured in a standard 4-h <sup>51</sup>Cr-release assay (7). The heteroconjugated antibody-dependent cytotoxicity assay was performed as described previously (16).

## Results

**IL-2 Induction of PFP mRNA Expression in T Cells.** Upon purification of CD3<sup>+</sup> lymphocytes from human peripheral blood, flow cytometry determined this population to exhibit >90% reactivity with the anti-Leu-4 mAb and <3% reactivity with the anti-Leu-19 mAb. The effect of IL-2 on PFP mRNA expression in these CD3<sup>+</sup> lymphocytes was examined using Northern blot analysis of their total cellular RNA. Induction of PFP mRNA expression occurred in T cells that were cultured with 1,000 U/ml of IL-2 (Fig. 1). With 4 h of IL-2 stimulation, levels of PFP mRNA



**FIGURE 1.** Kinetics of PFP mRNA expression in human peripheral blood CD3<sup>+</sup> CD56<sup>-</sup> lymphocytes. This population was cultured for various periods of time (2-24 h) in the presence of IL-2 (-, unstimulated for 24 h). Total cellular RNA was isolated from the cultured cells and Northern blot analysis was performed on 10  $\mu$ g of RNA. The filter was hybridized with a cDNA probe for (A) human PFP and (B) chicken  $\beta$ -actin.

were increased fourfold above those of the unstimulated T cell control. Maximal expression of PFP mRNA was observed after 6 h incubation with IL-2 (seven to eight times control levels), followed by a decline back to lower steady-state levels at later time points. Clearly induction of PFP mRNA in T cells was also observed with lower concentrations of IL-2 (>50 U/ml), although the increase was dose dependent and variable between donors (data not shown).

To monitor experiments for the contribution of <3% contaminating CD3<sup>-</sup> LGL or the possible preactivation of some of the donor's CD3<sup>+</sup> T cells, an RIA for human IFN- $\gamma$  production was performed on the supernatants of these IL-2-stimulated T cell cultures and the Northern blot (from Fig. 1) was hybridized with a human IFN- $\gamma$  cDNA probe. Consistent with T cells requiring two signals for IFN- $\gamma$  production (17), neither human IFN- $\gamma$  activity (<1 U/ml) nor 1.3-kb human IFN- $\gamma$  mRNA were detected from T cell cultures stimulated with IL-2 alone (data not shown). This indicated that the contribution of contaminating LGL or preactivated T cells was minimal in these T cell cultures.

To examine whether IL-2 was inducing PFP mRNA expression that required simultaneous T cell proliferation, we compared IL-2 and other stimuli for their effect on [<sup>3</sup>H]TdR uptake in CD3<sup>+</sup> lymphocytes (Fig. 2). Previously determined positive control combinations of PMA and ionomycin or OKT-3 mAb and accessory monocytes (18) predictably induced T cell DNA synthesis. By comparison, IL-2-treated T cells did not synthesize DNA (Fig. 2). In addition, T cells stimulated by IL-2 for 6 h in the absence or presence of cycloheximide (CHX) indicated that IL-2-induced PFP mRNA expression in the absence of new protein synthesis (i.e., in the presence of CHX) (data not shown). Therefore, it appeared that human peripheral blood CD3<sup>+</sup> lymphocytes contained low but detectable amounts of PFP mRNA that could be directly and rapidly induced by IL-2 in the absence of cellular proliferation, or new protein synthesis.

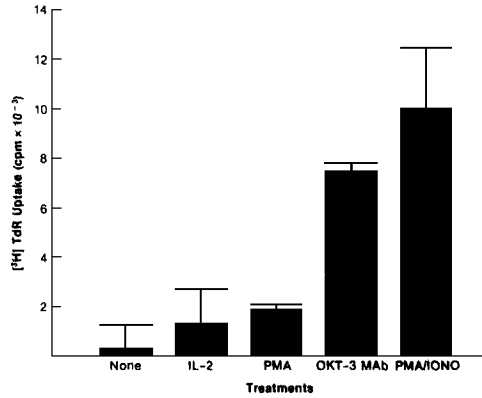


FIGURE 2. Proliferation of T cells after stimulation with IL-2, PMA, OKT-3 mAb or PMA and ionomycin (IONO). A total of  $2 \times 10^5$  purified  $CD3^+ CD56^-$  T cells were stimulated with IL-2 (1,000 U/ml), PMA (10 ng/ml), a combination of PMA (10 ng/ml) and ionomycin (3  $\mu$ g/ml), or OKT-3 mAb (10  $\mu$ g/ml) plus 8% monocytes. After 6 h, cultures were pulsed for 4 h with  $^3H$ TdR. Counts were calculated as the mean  $\pm$  SEM (triplicate determinations).

**PFP mRNA Expression in IL-2-stimulated CD4<sup>+</sup> and CD8<sup>+</sup> T Cell Subsets.** Having established that IL-2 directly induced PFP mRNA in whole unfractionated populations of  $CD3^+$  T cells, it was important to determine whether IL-2 induction was specifically occurring in both or either of the  $CD4^+$  and  $CD8^+$  subsets. Separated  $CD4^+$  and  $CD8^+$  subsets from  $CD3^+$  T cells of human peripheral blood were analyzed by flow cytometry for their reactivity with the anti- $CD4$  (anti-Leu-3a) mAb and the anti- $CD8$  (anti-Leu-2a) mAb. Three populations (unfractionated,  $CD4^+$ , and  $CD8^+$ ) were stimulated with 1,000 U/ml IL-2 for 6 h and their PFP mRNA expression was examined by Northern analysis (Fig. 3). Densitometric quantitation of the 2.9-kb PFP mRNA bands revealed that the population enriched for  $CD8^+$

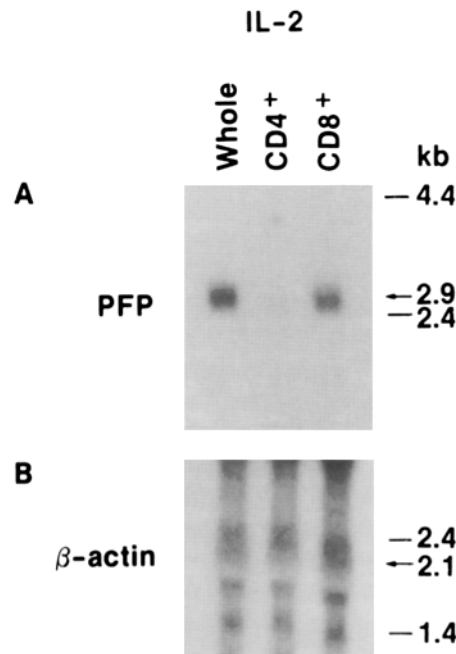


FIGURE 3. Expression of PFP mRNA in human peripheral blood T cell subsets stimulated with IL-2. Fractionated  $CD4^+$  (81%  $CD4^+$ , 6%  $CD8^+$ ),  $CD8^+$  (68%  $CD8^+$ , 20%  $CD4^+$ ), and *whole* unfractionated (55%  $CD4^+$ , 34%  $CD8^+$ ) T cells were cultured for 6 h in the presence of IL-2 (1,000 U/ml). Total cellular RNA was isolated and Northern blot analysis was performed on 10  $\mu$ g of RNA. The filter was hybridized with a cDNA probe for (A) human PFP and (B) chicken  $\beta$ -actin.

cells by CD4<sup>+</sup> depletion (68% CD8<sup>+</sup>, 20% CD4<sup>+</sup>) had a 10-fold greater expression than the CD4<sup>+</sup> population (6% CD8<sup>+</sup>, 81% CD4<sup>+</sup>), and 1-2-fold greater PFP mRNA expression than the unfractionated control population (55% CD4<sup>+</sup>, 34% CD8<sup>+</sup>) (Table I). These data demonstrate that IL-2 predominantly induces PFP mRNA expression in CD3<sup>+</sup> CD8<sup>+</sup> lymphocytes.

**IL-2 Induction of T Cell PFP mRNA via the p75 IL-2 Receptor.** The expression of functional IL-2 receptor on peripheral blood T cell populations, has previously been confirmed by TU27 (anti-p75 IL-2 receptor) and H-31 (anti-p55 IL-2 receptor) mAbs in flow cytometric analysis. CD8<sup>+</sup> T cells express the p75, but not the p55, IL-2 receptor (16). To determine the involvement of p55 and p75 IL-2 receptors in the rapid and direct induction of PFP mRNA in human peripheral blood T cells, PFP mRNA expression was examined after culturing CD3<sup>+</sup> T cells with IL-2 (6 h, 1,000 U/ml) in the presence/absence of 100 µg/ml H-31 and/or TU27 mAbs. As shown in Fig. 4 TU27 mAb alone or in combination with H-31 mAb dramatically inhibited IL-2 induction of PFP mRNA in T cells. By contrast, the H-31 mAb alone had no effect on IL-2 induction of PFP mRNA in T cells (Fig. 4). A cytotoxicity assay performed in parallel with the same T cells after a 16-h culture indicated that the induction of cytolytic activity was indeed abrogated by the TU27 (anti-p75 IL-2 receptor mAb) but not by the H-31 (anti-p55 IL-2 receptor mAb) (data not shown). In conclusion, these data indicate that the p75 IL-2 receptor is directly involved in IL-2 induction of T cell PFP mRNA and cytotoxicity.

**OKT-3 mAb Induction of PFP mRNA Expression in T Cells.** The OKT-3 mAb (10 µg/ml) could induce PFP mRNA expression in CD3<sup>+</sup> lymphocytes in the presence of 8% adherent monocytes (Fig. 5 A). Elevated levels of PFP mRNA were observed in T cells after 9 h of OKT-3 stimulation (greater than five times control levels), while peak PFP mRNA expression occurred after more than 24 h of culture in OKT-3 mAb (~13 times control levels) (Fig. 5 A). The OKT-3 mAb was also used at doses of 0.5, 1, and 5 µg/ml and was found to induce T cell PFP mRNA expression (data not shown). Therefore it appeared that induction of PFP mRNA by OKT-3 mAb was direct in CD3<sup>+</sup> lymphocytes, although in contrast to IL-2 stimulation (Fig. 1), no increase in PFP mRNA expression was detected within 4 h of the addition of OKT-3 mAb (Fig. 5 A). An ELISA for human IL-2 revealed that no detectable IL-2 was observed in cultures of T cells stimulated with OKT-3 mAb for 9 h or less; however, the supernatants of 24-h OKT-3 mAb-treated CD3<sup>+</sup> T cells contained 30 U/ml IL-2. The absence of detectable IL-2 in OKT-3 mAb-treated T cell cultures after 9 h suggests that induction of PFP mRNA by OKT-3 mAb precedes any possible

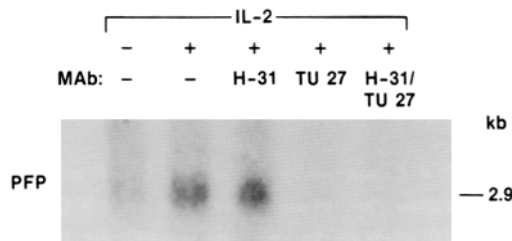


FIGURE 4. The effect of anti-IL-2-R mAbs on IL-2 induced PFP mRNA expression in human peripheral blood CD3<sup>+</sup> CD56<sup>-</sup> T cells. T cells were cultured with (+) or without (-) IL-2 (1,000 U/ml) in the presence or absence (-) of 100 µg/ml of anti-p55 IL-2-R (H-31) mAb and/or anti-p75 IL-2 receptor (TU27) mAb for 6 h at 37°C. Total cellular RNA was isolated from the cultured cells and Northern blot analysis was performed on 10 µg of RNA. The filter was hybridized with <sup>32</sup>P-labeled human PFP cDNA.

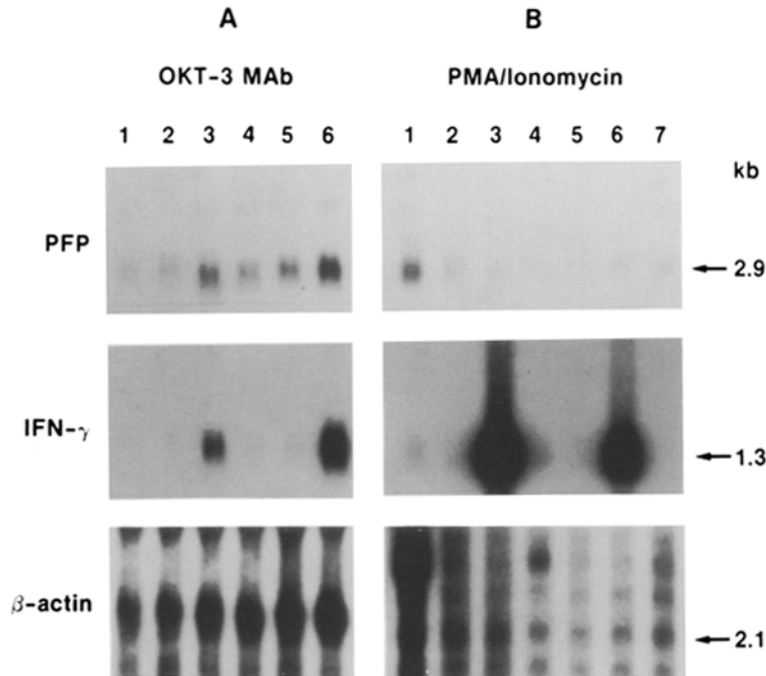


FIGURE 5. Kinetics of expression of PFP, IFN- $\gamma$ , and  $\beta$ -actin mRNA in human peripheral blood CD3<sup>+</sup> CD56<sup>-</sup> T cells. T cells were treated: (A) lane 1, 24 h unstimulated; lane 2, 4 h OKT-3 mAb; lane 3, 9 h OKT-3 mAb; lane 4, 9 h IL-2; lane 5, 9 h OKT-3 mAb and IL-2; lane 6, 24 h OKT-3 mAb. (B) Lane 1, 6 h PMA; lane 2, 6 h ionomycin; lane 3, 6 h PMA and ionomycin; lane 4, 24 h PMA; lane 5, 24 h ionomycin; lane 6, 24 h PMA and ionomycin; lane 7, 24 h unstimulated. All the treatments in panel A included 8% monocytes and doses were as follows: OKT-3 mAb (10  $\mu$ g/ml), IL-2 (1,000 U/ml), PMA (10 ng/ml), and ionomycin (3  $\mu$ g/ml). Total cellular RNA was isolated from the cultured cells and Northern blot analysis was performed. Both filters A and B were hybridized with <sup>32</sup>P-labeled human PFP cDNA; then the filters were stripped by washing with boiling 0.01  $\times$  SSC, 0.01% SDS and rehybridized with human IFN- $\gamma$  cDNA and chicken  $\beta$ -actin cDNA, respectively. The IL-2 levels in the supernatants of OKT-3 mAb-stimulated T cells were (A) lane 1, 0 U/ml; lane 2, 0 U/ml; lane 3 0 U/ml; and lane 6, 30 U/ml. It should be noted that the increased level of PFP cDNA hybridized in B (PMA, 6 h; lane 1) could be accounted for by a proportional increase in the chicken  $\beta$ -actin cDNA that hybridized in this lane.

indirect induction of PFP mRNA by OKT-3 mAb-stimulated IL-2. OKT-3 mAb and IL-2 were also tested in combination to determine whether their effects on PFP mRNA expression were synergistic, additive, or antagonistic. After 9 h of treatment, IL-2 increased expression 3.2-fold and OKT-3 mAb increased expression 5.2-fold, while their combination enhanced PFP mRNA expression 5.4-fold over unstimulated control CD3<sup>+</sup> lymphocytes (Fig. 5 A). Similarly, after 24 h a combination of IL-2 and OKT-3 mAb was less than additive in stimulating PFP mRNA in CD3<sup>+</sup> lymphocytes (data not shown).

*The Effect of PMA and Ionomycin on PFP mRNA Expression in T Cells.* T cell activation can be studied under conditions that bypass the requirement for crosslinking of the TCR/CD3 complex. Extracellular stimuli such as the calcium ionophore, ionomycin, and the phorbol ester/protein kinase C (PKC) activator, PMA, have been

used to identify important phosphatidylinositol-PKC signaling pathways in CTL activation (19), granule release (20, 21) and cytolysis (22). To define if these pathways were important in the induction of PFP mRNA expression in CD3<sup>+</sup> T cells, the effects of PMA, ionomycin, or their combination were examined. It was clear that PMA and ionomycin, either alone or in combination, were not capable of effecting PFP mRNA expression in CD3<sup>+</sup> T cells (Fig. 5 B), yet in combination they did induce IFN- $\gamma$  expression (Fig. 5 B) and promote <sup>3</sup>[H]TdR incorporation (Fig. 2). These data indicate that nonspecifically increasing intracellular calcium, either alone or in combination with PKC activation by PMA, has no effect upon PFP mRNA expression in CD3<sup>+</sup> T cells.

**PFP mRNA Expression in CD3<sup>-</sup> LGL.** LGL are CD3<sup>-</sup> CD56<sup>+</sup> cells that contain PFP (1, 5) and exhibit NK cell activity (23). The majority of LGL are also CD16<sup>+</sup> cells capable of mediating antibody-dependent cellular cytotoxicity (ADCC) (24). On purification of CD3<sup>-</sup> CD56<sup>+</sup> LGL, this population was analyzed by flow cytometry to be <2% reactive with anti-CD3 mAb, 80% reactive with anti-CD56 mAb, and 75% reactive with the anti-CD16 mAb. These purified CD3<sup>-</sup> lymphocytes were then cultured with or without 1,000 U/ml IL-2 for 6 h and their total cellular RNA was examined by Northern analysis (Fig. 6, A and B). Unlike CD3<sup>+</sup> lymphocytes, unstimulated CD3<sup>-</sup> CD56<sup>+</sup> LGL had a moderate to high PFP mRNA expression (Table I), consistent with their known high content of PFP (5). Furthermore, PFP mRNA levels were not increased after 6 h of stimulation by IL-2 (Fig. 6 A) (Table I). It should be noted that stimulation of CD3<sup>-</sup> LGL with IL-2 for 24 and 72 h (different donors) also did not increase the high resting PFP mRNA levels of these cells (Table I).

An RIA for human IFN- $\gamma$  production was performed on the supernatants of these IL-2-stimulated CD3<sup>-</sup> LGL cultures and the Northern blot was also hybridized

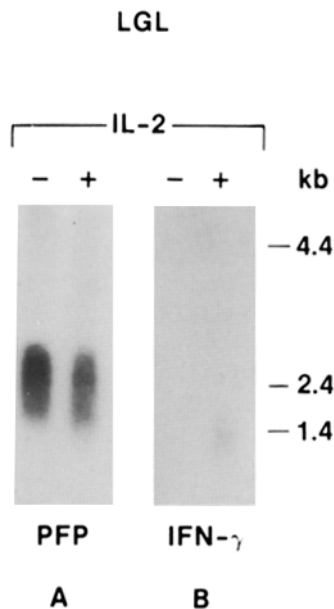


FIGURE 6. Expression of PFP mRNA in human peripheral blood CD3<sup>-</sup> CD56<sup>+</sup> LGL. This population was cultured for 6 h in the presence (+) or absence (-) of IL-2 (1,000 U/ml). Total cellular RNA was isolated from the cultured cells and Northern blot analysis was performed on 10  $\mu$ g of RNA. The filter was hybridized with a cDNA probe for (A) human PFP and (B) human IFN- $\gamma$ .

TABLE I  
*Induction of PFP mRNA Expression in Lymphocyte Subsets*

Exp.	Subset	Treatment	Dose	Time	Relative PFP mRNA expression
			U/ml	h	
1*	CD3 <sup>+</sup> CD56 <sup>-</sup>	—	—	0	1.0
	CD3 <sup>-</sup> CD56 <sup>+</sup>	—	—	0	12.8
2	CD3 <sup>+</sup> CD56 <sup>-</sup>	—	—	6	1.0
		IL-2	1,000	6	6.6
	CD3 <sup>-</sup> CD56 <sup>+</sup>	—	—	6	18.4
		IL-2	1,000	6	13.7
3	CD3 <sup>+</sup> CD56 <sup>-</sup>	—	—	24	1.0
	CD3 <sup>-</sup> CD56 <sup>+</sup>	—	—	24	15.4
		IL-2	1,000	24	14.6
4	CD3 <sup>+</sup> CD56 <sup>-</sup>	—	—	72	1.0
	CD3 <sup>-</sup> CD56 <sup>+</sup>	—	—	72	12.9
		IL-2	1,000	72	14.0
5 <sup>†</sup>	CD3 <sup>+</sup> CD56 <sup>-</sup>	IL-2	1,000	6	1.0
	CD3 <sup>+</sup> CD8 <sup>+</sup>	IL-2	1,000	6	1.3
	CD3 <sup>+</sup> CD4 <sup>+</sup>	IL-2	1,000	6	0.1

\* Each experiment represents a different donor.

<sup>†</sup> Expression of PFP mRNA relative to untreated CD3<sup>+</sup> T cells equal to unity was determined by quantitative densitometry of Northern blots (ratio of PFP/ $\beta$ -actin expression, 10  $\mu$ g total cellular RNA/lane). In Exp. 5 the PFP mRNA expression of IL-2-treated CD8<sup>+</sup> and CD4<sup>+</sup> T cell subsets was calculated relative to IL-2-treated whole unfractionated CD3<sup>+</sup> T cells rather than untreated CD3<sup>+</sup> T cells.

with the human IFN- $\gamma$  probe. In accordance with LGL requiring a single signal for IFN- $\gamma$  production (17), human IFN- $\gamma$  activity (2.6 U/ml) and human IFN- $\gamma$  mRNA (Fig. 6 B) were detected from LGL cultures stimulated for 6 h with IL-2 alone. Human IFN- $\gamma$  mRNA was not observed in unstimulated CD3<sup>-</sup> LGL nor was there human IFN- $\gamma$  activity in the supernatant. Therefore, human peripheral blood CD3<sup>-</sup> LGL contain elevated levels of PFP mRNA that cannot be further induced by stimulation with a 1,000 U/ml dose of IL-2.

*IL-2 Induction of Cytotoxic Activity in CD3<sup>+</sup> T Cells and CD3<sup>-</sup> CD16<sup>+</sup> NK Cells.* The observed inability of IL-2 to augment PFP expression in peripheral blood CD3<sup>-</sup> LGL (Fig. 6 A) was somewhat unexpected, since it has been demonstrated that CD3<sup>-</sup> CD16<sup>+</sup> NK cells are predominantly responsible for IL-2-stimulated spontaneous cytotoxicity in human PBMC (23). However, all the previously described results on augmentation of NK cell cytotoxicity by IL-2 were derived by simply estimating the direct killing against NK-sensitive or NK-resistant target cells. The efficiency of such direct killing will be affected by processes other than the cytolytic potential of the killer cell, such as cellular binding, recognition, and triggering events (25, 26). Indeed unfractionated human PBMC exhibited an augmented cytotoxicity against the human myeloid leukemia cell line K562 after a 16-h exposure to IL-2



in a dose-dependent manner (Fig. 7). However, no significant increase in NK cell cytotoxic potential in these samples was observed in the parallel estimation of redirected cytotoxicity using the anti-CD16 mAb (3G8)  $\times$  antinitrophenyl (NP) hapten mAb heteroconjugate against a NP-modified murine T lymphoma EL-4. The direct cytotoxicity of resting or IL-2-stimulated PBMC against K562 was mediated by CD16<sup>+</sup> LGL, but not by CD3<sup>+</sup> T cells, since cytotoxicity was abolished by depletion of CD16<sup>+</sup> cells from PBMC using anti-Leu-11b and complement (data not shown). As described in our previous study (16), peripheral blood T cell cytotoxicity against NP-modified EL-4, using the heteroconjugate containing anti-CD3 (OKT-3 mAb), could be rapidly augmented by exposure to IL-2 in a dose-dependent manner (Fig. 7). Therefore, the cytotoxic potential of peripheral blood T cells and NK cells induced in response to IL-2 was in good correlation with IL-2-induced PFP mRNA levels in these cells.

### Discussion

By fractionating human peripheral blood lymphocytes into CD3<sup>+</sup> CD8<sup>+</sup> and CD3<sup>+</sup> CD4<sup>+</sup> T cells and CD3<sup>-</sup> LGL, we have studied how PFP gene expression is regulated. Our studies at the mRNA level were consistent with peripheral T cells treated with IL-2-producing PFP (5, 27). The PFP mRNA expression in CD3<sup>+</sup> T cells was directly induced by IL-2 in a dose-dependent manner, independently of DNA synthesis (Fig. 2) and new protein synthesis. The kinetics of IL-2 stimulation of PFP mRNA synthesis in these cells were very rapid (Fig. 1), and furthermore, it was demonstrated that IL-2 induced PFP mRNA expression in CD8<sup>+</sup>, but not in CD4<sup>+</sup> T cells (Fig. 3). In addition, the expression of PFP mRNA in untreated T cells (Fig. 1, 4, 5) was likely to be representative of  $\sim$ 20% of resting CD8<sup>+</sup> T cells that have been shown to be reactive with anti-PFP mAbs (Okumura, K., unpublished observations). This parallels the previous finding that all the detectable PFP activity resides in the Lyt-2<sup>+</sup> (CD8<sup>+</sup>) subset of activated murine or human peripheral T lymphocytes cultured in IL-2 (5, 27) and is consistent with our previous observations that the IL-2-induced cytolytic activity in peripheral blood T cells was confined to the CD8<sup>+</sup> subset (16). As the cytolytic activity of cloned or primary CTL

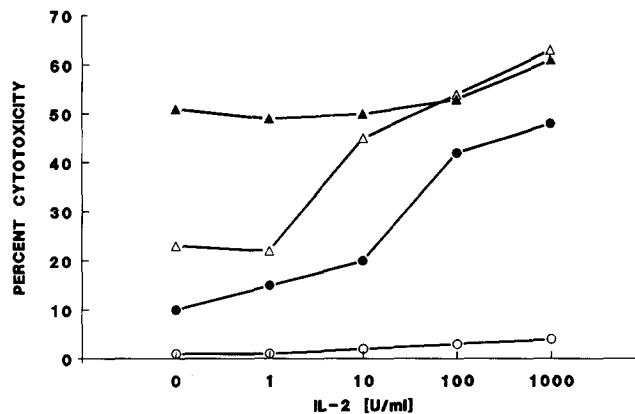


FIGURE 7. Cytotoxic activity of human peripheral blood CD3<sup>+</sup> T cells and CD16<sup>+</sup> NK cells stimulated with IL-2. Unfractionated PBMC were cultured with various doses of IL-2. After 16 h, cytotoxicity was tested against NP-modified EL-4 in the presence of OKT-3  $\times$  anti-NP (●) or 3G8  $\times$  anti-NP (▲) heteroconjugated antibody at an E/T ratio of 5:1. Open circles indicate basal cytotoxicity in the absence of heteroconjugates. Open triangles indicate the direct cytotoxicity of IL-2 stimulated NK cells against the NK-sensitive K562 target at an E/T ratio of 5:1.

is generally due to CD8<sup>+</sup> T cells (28), the IL-2-inducible expression of PFP mRNA in this subset further suggests that PFP has an essential function in CTL-mediated target cell lysis. Although recently we have demonstrated that low levels of the p75 IL-2 receptor are preferentially expressed on peripheral blood CD8<sup>+</sup> T cells (16), these studies herein have indicated that an anti-IL-2 receptor p75 mAb can completely abrogate IL-2 induction of peripheral blood T cell PFP mRNA (Fig. 5) and T cell cytotoxic potential. This suggests that IL-2 signaling via the p75 IL-2 receptor is directly implicated in PFP gene expression in T cells.

The mechanisms of induction of PFP mRNA in CD3<sup>+</sup> T cells were also examined using OKT-3 mAb or an activator of PKC (PMA) alone or in combination with a calcium ionophore (ionomycin). OKT-3 mAb induced PFP mRNA expression in T cells (Fig. 5 A), although less rapidly than IL-2; however, bypassing cross-linking of the TCR/CD3 complex with the extracellular stimuli, PMA and ionomycin, did not stimulate PFP mRNA induction in CD3<sup>+</sup> lymphocytes (Fig. 5 B). Thus, although PKC activation has been reported to be required for CTL granule release and triggering (19, 21), PKC activation by PMA and ionomycin does not induce PFP mRNA expression in T cells. It is possible that IL-2 or OKT-3 mAbs do not induce PFP mRNA by activating the same PKC pathway as PMA or that other protein kinases are involved in induction of PFP mRNA in T cells. Indeed, the zeta-zeta homodimer of the T cell-CD3 complex has been demonstrated to have tyrosine kinase activity (29), and recent studies have implicated tyrosine protein kinase activity in the stimulation of T cells by IL-2 via the  $\beta$  subunit of the IL-2 receptor (30, 31). Future studies will be used to determine which protein kinases are involved in PFP mRNA induction in T cells.

In contrast to CD3<sup>+</sup> T cells, human peripheral blood CD3<sup>-</sup> CD56<sup>+</sup> LGL had a high constitutive expression of PFP mRNA (Fig. 6 A) with the levels of PFP mRNA in the CD3<sup>-</sup> LGL 13–19 times that found in the CD3<sup>+</sup> T cells of the same donors (Table I). By comparing and extrapolating data from a number of different donors and calculating the expression of PFP mRNA relative to untreated CD3<sup>+</sup> T cell populations, the levels of PFP mRNA in CD3<sup>-</sup> LGL were two to three times greater than those in unfractionated CD3<sup>+</sup> T cells optimally stimulated with IL-2 (Table I). This difference becomes less significant when comparing CD3<sup>-</sup> LGL with purified CD8<sup>+</sup> T cells.

Unlike T cells, LGL PFP mRNA levels were not increased after 6 h of stimulation by IL-2 (Fig. 6 A). In addition, the cytolytic potential of peripheral blood CD3<sup>-</sup> CD16<sup>+</sup> NK cells was not augmented by a brief exposure to IL-2 (Fig. 7). We believe this data is not in conflict with previous studies, which demonstrated the enhanced direct cytotoxicity of NK cells responding to IL-2 (23, 32). Our studies have measured the redirected cytotoxic potential of NK cells after a brief exposure to IL-2 in addition to simply determining direct killing against an NK-sensitive target cell. We and others have also previously reported difficulty in enhancing the ADCC of NK cells (33, 34). A high constitutive expression of PFP mRNA in peripheral blood LGL was implicated in its spontaneous cytotoxicity; however, an increase in PFP mRNA expression was not involved in the enhanced spontaneous cytotoxicity of LGL responding to IL-2. Therefore, although peripheral blood CD8<sup>+</sup> T cells and CD3<sup>-</sup> LGL are similarly activated by IL-2 via its interaction with the IL-2 recep-

tor  $\beta$  chain (16, 35), the mechanisms responsible for the IL-2-induced cytotoxicity of these cells appear to be quite different.

### Summary

Our studies have analyzed pore-forming protein (PFP) mRNA expression in resting and stimulated human peripheral blood CD3<sup>-</sup> large granular lymphocytes (LGL), CD3<sup>+</sup> T cells, and their CD4<sup>+</sup> or CD8<sup>+</sup> subsets. Signals that stimulate T cells to develop cytotoxic activity (i.e., IL-2 or OKT-3 mAb) led to the induction of PFP mRNA in T cells. The data indicated that IL-2 directly increased PFP mRNA in the CD8<sup>+</sup> subset of T cells, in the absence of new DNA or protein synthesis. Abrogation of IL-2-induced PFP mRNA expression and cytotoxic potential of T cells by the anti-p75 IL-2 receptor mAb suggested that low numbers of p75 IL-2 receptors on CD8<sup>+</sup> T cells were capable of transducing signals responsible for these IL-2-induced effects. The induction of T cell PFP mRNA via CD3, using OKT-3 mAb, was less rapid but greater than that caused by IL-2; however, a combination of PMA and ionomycin, which bypasses crosslinking of the TCR/CD3 complex, could not mimic this increase in PFP mRNA levels in T cells. The role of second messenger systems in regulating PFP mRNA expression remains to be determined. In contrast, high constitutive PFP mRNA expression was observed in CD3<sup>-</sup> LGL and these mRNA levels could not be enhanced by stimulation with IL-2. The cytotoxic potential of peripheral blood T cells and LGL induced in response to IL-2 correlated with IL-2-induced PFP mRNA levels in these cells and was consistent with PFP being one of several important molecules involved in the effector function of cytotoxic lymphocytes.

We acknowledge the kind gift of H-31 and TU27 mAbs provided by Dr. Kazuo Sugamura, Department of Microbiology, Tohoku University School of Medicine, Sendai, Japan. We thank Mr. William Bere for expert technical assistance; Clinical Immunology Services, PRI, FCRF, Frederick, MD for providing the IFN- $\gamma$  RIA and IL-2 ELISA; Drs. Craig Reynolds, John O'Shea, and Dan Longo for helpful comments concerning this manuscript; and Ms. Susan Charbonneau and Amy Wenner for secretarial assistance.

*Received for publication 16 October 1989 and in revised form 28 December 1989.*

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