Brief Definitive Report

Constitutive Expression of Pore-forming Protein in Peripheral Blood γ/δ T Cells: Implication for Their Cytotoxic Role In Vivo

By Motomi Nakata,* Mark J. Smyth,‡ Yoko Norihisa,‡
Akemi Kawasaki,* Yoichi Shinkai,* Ko Okumura,* and Hideo Yagita*

*From the *Department of Immunology, Juntendo University School of Medicine, Bunkyo-ku, Tokyo 113, Japan; and the ‡Laboratory of Experimental Immunology, Biological Response Modifiers Program, National Cancer Institute, Frederick Cancer Research and Development Center, Frederick, Maryland 21702*

Summary

The cytotoxic activity and pore-forming protein (PFP) expression of human peripheral blood (PB) γ/δ T cells were examined. Fresh γ/δ T cells isolated from PB lymphocytes by fluorescence-activated cell sorting exhibited a substantial natural killer–like cytotoxic activity against K562 target cells and had a high cytotoxic potential triggered by anti-CD3 monoclonal antibody (mAb) against P815 target cells bearing FcγR. Immunocytochemical staining with an anti-PFP mAb revealed that virtually all PB γ/δ T cells are granular lymphocytes with abundant PFP in their cytoplasmic granules. Constitutive expression of PFP in PB γ/δ T cells was also demonstrated by Northern blot analysis. These observations support the proposed role of γ/δ T cells in cytolytic immune surveillance in vivo.

Human peripheral blood (PB) contains T cells bearing two different sets of TCR chains: α/β and γ/δ (1). In contrast to the well-characterized regulatory and effector functions of α/β T cells, those of γ/δ T cells are largely unknown.

The γ/δ T cell lines maintained in conditioned medium containing IL-2 generally exhibit MHC-unrestricted cytotoxicity not only against NK-susceptible target cells but also against NK-resistant target cells (lymphokine-activated killer [LAK] cytotoxicity) (1). In conjunction with the recent discovery that some γ/δ T cells react with bacterial antigens and heat-shock proteins (2), it was proposed that γ/δ T cells may play a role in cytolytic immune surveillance for infected or transformed cells (3). However, cytolytic function of fresh γ/δ T cells remains to be determined.

We describe here that PB γ/δ T cells have a high cytolytic potential without IL-2 stimulation, and constitutively express pore-forming protein (PFP), which is a potent mediator of cell-mediated cytolysis (4).

Materials and Methods

mAbs. Unlabeled anti-Leu-4 (anti-CD3) mAb and hybridoma cells producing 3G8 (anti-CD16) mAb were provided by Dr. L.L. Lanier (Becton Dickinson Immunocytometry Systems, Mountain View, CA) and Dr. J.C. Unkeless (Mount Sinai Medical School, New York), respectively. The hybridoma cells producing OKT4 mAb and OKT8 mAb were obtained from the American Type Culture Collection (Rockville, MD). FITC- or PE-conjugated anti-Leu-2a (anti-CD8), anti-Leu-4, and anti-Leu-19 (anti-CD56) mAbs were purchased from Becton Dickinson Immunocytometry Systems. FITC-conjugated TCR-δ-1 (anti-TCR-γ/δ) was purchased from T Cell Sciences (Cambridge, MA).

Cell Preparation and Culture. PBMC were isolated from the blood of healthy donors using Ficoll-Hypaque. After depleting monocytes and B cells by adherence to plastic dishes and nylon wool, highly enriched populations of $CD3^+$ T cells (whole T cells) and $CD3^-56^+$ large granular lymphocytes (LGL) were obtained by discontinuous Percoll density gradient centrifugation as described previously (5). $CD8^+$ TCR-γ/δ^- T cells (>95%) and TCR-γ/δ^+ T cells (>95%) were isolated from the whole T cells by sorting on a FACStar (Becton Dickinson & Co.). $CD3^-56^+$ NK cells (>95%) were sorted from the LGL population. Thymocytes were obtained from children (< 5 yr old) undergoing corrective cardiac surgery. TCR-γ/δ^+ thymocytes were sorted from $CD4^-8^-$ thymocytes prepared by treatment with OKT4 mAb, OKT8 mAb, and C. Isolated NK and T cell subsets (1–2 × 10^6/ml) were stimulated with 100–1,000 U/ml of human rIL-2 (generously supplied by Shionogi Pharm. Co. Ltd., Osaka, Japan, and Cetus Corp., Emeryville, CA) for 6–18 h as previously described (5).

Cytotoxicity Assay. Cytotoxicity was measured in a standard 4-h ^{51}Cr release assay (5). In all experiments, graded numbers of effector cells were added to 5 × 10^3 ^{51}Cr-labeled target cells. Lytic units (LU) were defined as the number of effector cells required to cause 30% lysis of 5 × 10^3 target cells. In some experiments, retargeting mAbs (anti-Leu-4 or 3G8) were added to a final concentration of 0.5 μg/ml.

Immunocytochemical Staining. A rat anti–mouse PFP mAb, P1-8

Table 1. *Cytotoxic Activity of Freshly Isolated or IL-2-activated PB γ/δ T Cells*

Exp.	Effector cells	LU*/10^6 Cells			
			P815		
		K562	(−)	Leu-4‡	3G8‡
1	Fresh NK cells	44.4	<0.1	ND	37.0
	IL-2-activated§ NK cells	70.2	11.2	ND	40.0
	Fresh $CD8^+$ T cells	<0.1	<0.1	7.1	ND
	IL-2 activated $CD8^+$ T cells	<0.1	<0.1	28.6	ND
	Fresh γ/δ T cells	32.2	<0.1	20.0	ND
	IL-2-activated γ/δ T cells	51.2	6.1	46.6	ND
2	Fresh γ/δ T cells	27.0	<0.1	33.4	ND
	IL-2-activated γ/δ T cells	55.6	10.6	64.6	ND
3	Fresh γ/δ T cells	25.6	<0.1	35.8	ND
	IL-2-activated γ/δ T cells	52.6	9.0	64.6	ND
4	Fresh γ/δ thymocytes	<0.1	<0.1	<0.1	ND

* LU was defined as the number of effector cells required to cause 30% lysis of 5 × 10^3 target cells.
‡ mAbs were added to 4-h ^{51}Cr release assay at a final concentration of 0.5 μg/ml.
§ Isolated lymphocytes were cultured with 100 U/ml of rIL-2 for 18 h.

(6), strongly crossreacts with human PFP (manuscript in preparation). Immunocytochemical staining of isolated human lymphocyte subsets with P1-8 was performed as described for murine lymphocytes (6).

RNA Analysis. Preparation of total cellular RNA, Northern blot analysis with ^{32}P-labeled human PFP cDNA (7) and murine 18S ribosomal RNA cDNA (Dr. L. Varesio, Frederick Cancer Research Facility, Frederick, MD) probes, and relative quantification of PFP mRNA were performed as described previously (8).

Results and Discussion

Cytotoxic Activity of Freshly Isolated or IL-2-activated PB γ/δ T Cells. We first tested the cytotoxic activity of freshly isolated γ/δ T cells from PBL or thymocytes using three criteria (Table 1). A NK-sensitive human target K562 was used for evaluating NK cytotoxicity. A NK-resistant murine target P815 bearing FcγR was used for evaluating LAK cytotoxicity and the cytolytic potential redirected by anti-CD3 mAb.

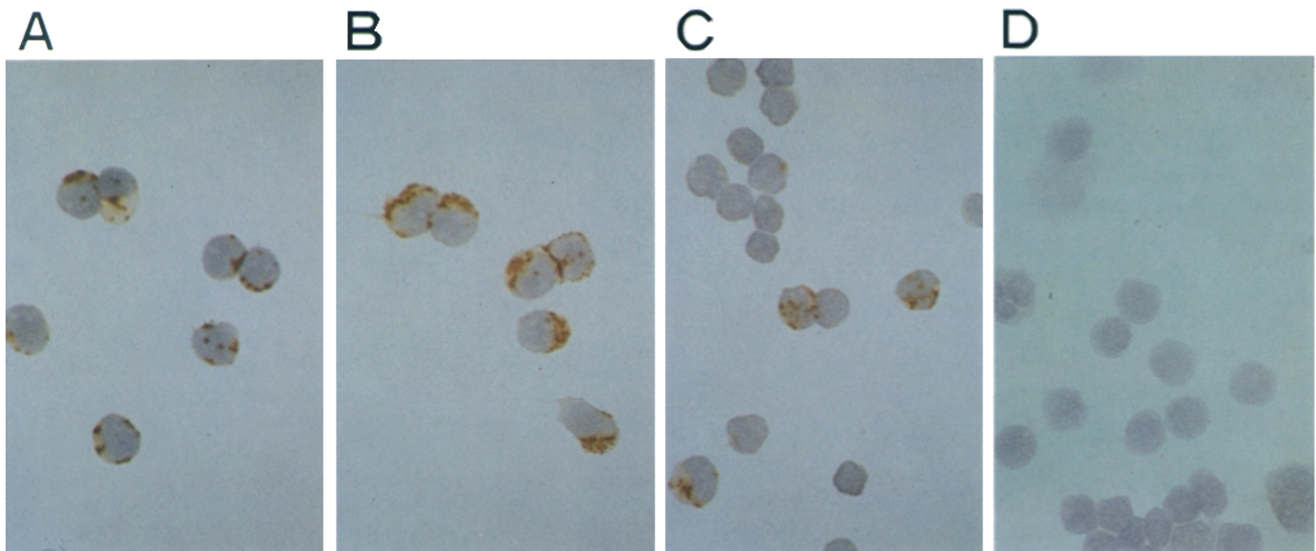

Figure 1. Immunocytochemical staining of PB γ/δ T cells (*A*), PB NK cells (*B*), PB $CD8^+$ T cells (*C*), and γ/δ thymocytes (*D*) with an anti-PFP mAb (×200).

Freshly isolated PB γ/δ T cells from all three donors exhibited substantial NK cytotoxicity but no LAK cytotoxicity. The possibility that the cytotoxicity against K562 resulted from retargeting by the TCR-δ-1 mAb used and the FcγR on K562 could be eliminated because the same positively sorted γ/δ T cells did not lyse FcγR$^+$ P815 target cells. In addition, freshly isolated γ/δ T cells had a greater cytolytic potential than CD8$^+$ T cells when triggered by anti-CD3 mAb, and their cytotoxic potential was sometimes comparable with that of freshly isolated NK cells triggered by anti-CD16 mAb. These results clearly indicate that PB γ/δ T cells have a potent cytotoxic activity comparable with PB NK cells in vivo. In contrast, γ/δ T cells in the thymus (γ/δ thymocytes) had neither NK activity nor cytolytic potential, suggesting that γ/δ T cells acquire cytotoxic activity post- or extra-thymically.

We next examined the IL-2 induction of cytotoxic activity of PB γ/δ T cells. In a previous study, we demonstrated that NK and LAK cytotoxicity, but not the cytolytic potential of PB NK cells, were upregulated by a brief exposure to IL-2, while, in contrast, CD8$^+$ T cells developed a high cytolytic potential, but not NK or LAK cytotoxicity by the same treatment (5). Similar results were obtained again herein with NK and CD8$^+$ T cells as controls (Table 1). NK cytotoxicity and cytolytic potential of PB γ/δ T cells were augmented by a brief exposure to IL-2 by about twofold. In addition, PB γ/δ T cells exhibited LAK cytotoxicity after IL-2 stimulation like NK cells.

Constitutive Expression of PFP in PB γ/δ T Cells. To further define the cytolytic function of PB γ/δ T cells, we examined the expression of PFP, which has been proposed to be a potent mediator of cell-mediated cytolysis (4), in these cells by immunocytochemical staining with an anti-PFP mAb and Northern blot analysis. Previously, we demonstrated a strong correlation between cytolytic potential and PFP mRNA expression in CD8$^+$ T and NK cells (5).

Immunocytochemical staining with an anti-PFP mAb revealed a dispersely granular pattern of cytoplasmic staining in 95–100% of the freshly isolated γ/δ T cells (Fig. 1 *A*), 95–100% of CD3-56$^+$ NK cells (Fig. 1 *B*), and 13–30% of CD8$^+$ T cells (Fig. 1 *C*) among eight individuals. Consistent with previous observations (9, 10), the granular morphology of γ/δ T cells was also observed under electron microscopy, demonstrating that the majority of the cells had electron-dense granules scattered in the cytoplasm (data not shown). In contrast, γ/δ thymocytes exhibited virtually no staining (Fig. 1 *D*). These results indicate that γ/δ T cells, like NK cells, in PB had abundant PFP in their cytoplasmic granules, but those in the thymus did not, thereby suggesting that the cytolytic machinery involving PFP develops post- or extra-thymically.

Constitutive expression of PFP in γ/δ T cells was also demonstrated by Northern blot analysis (Fig. 2). PB γ/δ T cells expressed abundant PFP mRNA without IL-2 stimulation at about half the level of PB NK cells and ~20-fold more than CD8$^+$ T cells (Table 2). A brief exposure to IL-2 led to a striking induction (~13-fold) of PFP gene expression in CD8$^+$ T cells but not significantly in γ/δ T cells or NK cells (Fig. 2, Table 2). These results indicate that PB γ/δ T cells constitutively express PFP.

We recently reported that abnormally expanded PB γ/δ T cells, which were prepared from a granular lymphocyte proliferative disorder (GLPD) patient, exhibited strong NK activity and constitutively expressed PFP mRNA (11). The present results indicate that this previous observation did not represent a special case of GLPD but a common feature of normal PB γ/δ T cells.

It has been demonstrated that human PB γ/δ T cells are mainly composed of two mutually exclusive subsets distinguished by their reactivity with anti-TCR-γ/δ mAbs, BB3,

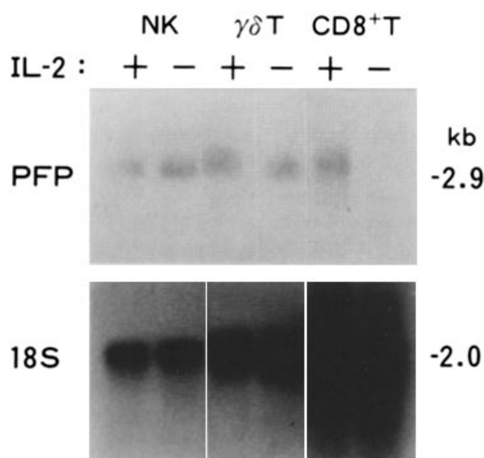

Figure 2. Expression of PFP mRNA in PBL subsets. CD3$^-$56$^+$ NK cells, γ/δ$^+$ T cells, and γ/δ$^-$CD8$^+$ T cells were cultured for 6 h in the presence (+) or absence (−) of rIL-2 (1,000 U/ml). Total cellular RNA was isolated from the cultured cells, and Northern blot analysis was performed on 1–3 μg of RNA. The filter was hybridized with a cDNA probe for human PFP, then stripped by washing with boiling 0.01× SSC/0.01% SDS, and rehybridized with a cDNA probe for murine 18S RNA.

Table 2. *Induction of PFP mRNA Expression in Lymphocyte Subsets by Brief Exposure to IL-2*

Subset	Treatment*	Relative PFP mRNA expression‡
NK cells	–	38.8
	IL-2	34.1
CD8$^+$ T cells	–	1.0
	IL-2	12.9
γ/δ T cells	–	19.9
	IL-2	23.0

* Isolated lymphocyte subsets were cultured with or without 1,000 U/ml of rIL-2 for 6 h.

‡ Expression of PFP mRNA relative to untreated CD8$^+$ T cells equal to unity was determined by quantitative densitometry of Northern blots indicated in Fig. 2 (ratio of PFP/18S RNA expression).

and δTCS-1 (12). These two subsets exhibited differential tissue distribution (13), and the cell lines established from these two subsets exhibited differential morphology (14) and capacity to spontaneously lyse tumor target cells (12). However, since virtually all PB γ/δ T cells containing 4–22% δTCS-1$^+$ cells (among eight individuals) were granularly stained with anti-PFP mAb, there appears to be no difference in PFP expression between these two subsets.

Studies on the distribution of γ/δ T cells in murine tissues suggested that these T cells may exert their functions preferentially at the mucosal level (15). Freshly isolated murine intestinal intraepithelial lymphocytes (IEL) have been demonstrated to have a high cytolytic potential triggered by anti-TCR/CD3 mAbs (16). We recently observed that some freshly isolated murine Thy-1$^+$ dendritic epidermal cells and IEL express PFP as estimated by immunocytochemical staining with anti-PFP mAb (manuscript in preparation). Although the cytolytic activity and expression of PFP in human tissue γ/δ T cells remain to be determined, the present data support the proposed role of γ/δ T cells in immune surveillance, possibly exerting cytolytic functions against virus-infected, transformed, or stressed cells.

We thank Drs. L. L. Lanier, J. C. Unkeless, and L. Varesio for providing anti-Leu-4 mAb, 3G8 hybridoma, and murine 18S ribosomal RNA cDNA probe, respectively, and Flow Cytometry, Clinical Immunology Services, Frederick Cancer Research Development Center (FCRDC), for their help in isolating PBL subsets. We also thank Drs. H. A. Young and J. R. Ortaldo, National Cancer Institute-FCRDC, for providing support, and Ms. M. Wakita and Mr. F. Maruta for secretarial assistance.

This work was supported by grants from the Naito Foundation, Uehara Memorial Foundation, and Sankyo Foundation of Life Science. M. J. Smyth is currently supported by a C. J. Martin Traveling Fellowship, National Health and Medical Research Council of Australia. Y. Norihisa is a Fogarty Visiting Fellow of the National Institutes of Health.

Address correspondence to Hideo Yagita, Department of Immunology, Juntendo University School of Medicine, 2-1-1 Hongo, Bunkyo-ku, Tokyo 113, Japan.

Received for publication 20 August 1990.

References

1. Brenner, M.B., J.L. Strominger, and M.S. Krangel. 1988. The γδ T cell receptor. *Adv. Immunol.* 43:133.
2. Raulet, D.H. 1989. Antigen for γ/δ T cells. *Nature (Lond.).* 339:342.
3. Janeway, C.A. 1988. Frontiers of the immune system. *Nature (Lond.).* 330:804.
4. Tschopp, J. 1990. Perforin-mediated target cell lysis by cytolytic T lymphocytes. *Annu. Rev. Immunol.* 8:279.
5. Smyth, M.J., J.R. Ortaldo, Y. Shinkai, H. Yagita, M. Nakata, K. Okumura, and H.A. Young. 1990. Interleukin 2 induction of pore-forming protein gene expression in human peripheral blood CD8$^+$ T cells. *J. Exp. Med.* 171:1269.
6. Kawasaki, A., Y. Shinkai, Y. Kuwana, A. Furuya, Y. Iigo, N. Hanai, S. Itoh, H. Yagita, and K. Okumura. 1990. Perforin, a pore-forming protein detectable by monoclonal antibodies, is a functional marker for killer cells. *Int. Immunol.* 2:677.
7. Shinkai, Y., M.C. Yoshida, K. Maeda, T. Kobata, K. Maruyama, J. Yodoi, H. Yagita, and K. Okumura. 1989. Molecular cloning and chromosomal assignment of a human perforin (*PFP*) gene. *Immunogenetics.* 30:452.
8. Smyth, M.J., J.R. Ortaldo, W. Bere, H. Yagita, K. Okumura, and H.A. Young. 1990. Interleukin-2 and interleukin-6 synergize to augment the pore-forming protein gene expression and cytotoxic potential of human peripheral blood T cells. *J. Immunol.* 145:1159.
9. Faure, F., S. Jitsukawa, F. Triebel, and T. Hercend. 1988. CD3/TiγA: a functional γ-receptor complex expressed on human peripheral lymphocytes. *J. Immunol.* 140:1372.
10. Ferrini, S., D. Zarcone, M. Viale, G. Cerruti, R. Millo, A. Moretta, and C.E. Grossi. 1989. Morphologic and functional characterization of human peripheral blood T cells expressing the T cell receptor γ/δ. *Eur. J. Immunol.* 19:1183.
11. Oshimi, K., Y. Shinkai, K. Okumura, Y. Oshimi, and H. Mizoguchi. 1990. Perforin gene expression in granular lymphocyte proliferative disorders. *Blood.* 75:704.
12. Triebel, F., and T. Hercend. 1989. Subpopulations of human peripheral T gamma delta lymphocytes. *Immunol. Today.* 10:186.
13. Falini, B., L. Flenghi, S. Pileri, P. Pelicci, M. Fagioli, M.F. Martelli, L. Moretta, and E. Ciccone. 1989. Distribution of T cells bearing different forms of the T cell receptor γ/δ in normal and pathological human tissues. *J. Immunol.* 143:2480.
14. Grossi, C.E., E. Ciccone, N. Migone, C. Bottino, D. Zarcone, M. Mingari, S. Ferrini, G. Tambussi, O. Viale, G. Casorati, R. Millo, L. Moretta, and A. Moretta. 1989. Human T cells expressing the γ/δ T-cell receptor (TcR-1): Cγ1- and Cγ2-encoded forms of the receptor correlate with distinctive morphology, cytoskeletal organization, and growth characteristics. *Proc. Natl. Acad. Sci. USA.* 86:1619.
15. Itohara, S., A.G. Farr, J.J. Lafaille, M. Bonneville, Y. Takagaki, W. Haas, and S. Tonegawa. 1990. Homing of a γδ thymocyte subset with homogeneous T-cell receptors to mucosal epithelia. *Nature (Lond.).* 343:754.
16. Goodman, T., and L. Lefrancois. 1989. Intraepithelial lymphocytes. Anatomic site, not T cell receptor form, dictates phenotype and function. *J. Exp. Med.* 170:1569.