Brief Definitive Report

Expression of Perforin and Serine Esterases by Human γ/δ T Cells

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Summary

γ/δ T cells have recently been described in association with a number of disorders, including autoimmune diseases. γ/δ T cells are thought to play a cytotoxic role, but their mechanism of action is not known. Several granule mediators of cytotoxicity, including a pore-forming protein (perforin), and a family of serine esterases, have been isolated from cytotoxic T lymphocytes (CTL), lymphokine-activated killer (LAK) cells, and natural killer (NK) cells. We demonstrate here that γ/δ T cells also express these mediators. Northern blots show that γ/δ T cells express perforin, serine esterase 1 (SE 1), and SE 2. Three polyclonal antisera—raised against murine perforin, a peptide composed of amino acids 1–34 of human perforin, and human perforin expressed in bacteria—all reacted with a 70-kD protein in γ/δ T cells on Western blots. Immunostaining with antiperforin antisera shows that primary γ/δ T cells also contain perforin. Electron microscopy reveals that the granules of γ/δ T cells resemble those of CTL, LAK, and NK cells. γ/δ T cells also resemble LAK cells in possessing inclusion bodies in their nuclei. These results imply that γ/δ T cells resemble other cytolytic lymphocytes in their mechanism of action.

Cells with γ/δ TCRs (γ/δ T cells) generally exhibit MHC-unrestricted cytotoxicity in vitro. Target cells recognized by γ/δ T cells are markedly different from those recognized by CTL and by NK cells (1), suggesting that γ/δ T cells are a previously unrecognized type of killer lymphocyte. γ/δ T cells react with both the mycobacterial and the mammalian 65-kD heat shock protein (HSP65); this crossreactivity may be important in the pathogenesis of autoimmune diseases, including rheumatoid arthritis (RA) (2–4). The cytotoxic mechanisms of γ/δ T cells are not known, although several mediators of cytotoxicity have been described in other cytolytic lymphocytes (5–8). These mediators, present in the granules of CTL, NK cells, and lymphokine-activated killer (LAK) cells, include a pore-forming protein (PFP; perforin, cytolysin), serine esterase 1 (SE 1; Hanukah Factor, granzyme A), and SE 2 (granzyme B). We used Northern blots, immunoblots, and immunostaining to determine whether γ/δ T cells also contain these cytotoxic mediators.

Materials and Methods

Cells. Human γ/δ T cell clones 1.2, 1.3, 1.4, and 4.1 (all TCR-α/β^-, -γ/δ^+, CD3$^+$, CD4$^-$, CD8$^-$) were derived from the synovial fluid of a patient with RA as described (2). γ chain cDNA sequencing data showed common use of $V_\gamma 9$-Jp rearrangements with junctional diversity among clones, suggesting that the four clones were distinct clonal populations (Loh, E., J. Holoshitz, and S. Strober, manuscript in preparation). Human LAK cells were prepared by stimulating plastic nonadherent PBMC for 10 d with 1,000 U/ml of rIL-2 (Cetus Corp., Emeryville, CA). Human peripheral blood NK cells stimulated with IL-2 were generously provided by Dr. Bice Perussia (Wistar Institute, Philadelphia, PA).

Cytotoxicity Assays. Standard 4-h ^{51}Cr release assays (9) were performed using tumor lines K562, Daudi, and autologous (JBEB) or heterologous (DSEB) EBV-transformed B cell lines as targets.

cDNA Probes and Northern Blot Analysis. ^{32}P-labeled cDNA probes specific for perforin, SE 1, SE 2, and γ-actin were prepared and hybridized sequentially with total RNA blots on nylon membranes as described (9).

Antibodies and Immunoblot Analysis. Rabbit polyclonal antiperforin antisera were generated against purified mouse perforin (10), human perforin expressed in bacteria (11), and against a synthetic peptide (pN1-34) composed of amino acids 1–34 of human perforin. The peptide was immobilized on nitrocellulose, mixed with Freund's adjuvant, and 10–15 μg was injected subcutaneously into rabbits; five booster injections were given. Immunoblotting was done exactly as described (9).

Immunostaining. CD4$^-$, CD8$^-$ plastic nonadherent PBMC selected by panning were cultured for 3 d with 500 U/ml of rIL-2 (12), stained with TCR-δ1 mAb (T Cell Sciences, Cambridge, MA) and sorted using a FACStar® (Becton Dickinson & Co., Mountain

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View, CA). Sorted cells (referred to as primary γ/δ T cells) were 97% pure on FACS analysis. Primary γ/δ T cells were cytocentrifuged, and stained sequentially with antiperforin antiserum and FITC-conjugated goat anti-rabbit IgG $(Fab')_2$ (Tago Inc., Burlingame, CA).

Electron Microscopy. Clone 1.4 cells were fixed with an equal volume of 4% glutaraldehyde and 2% paraformaldehyde in 0.1 M cacodylate buffer, pH 7.2, postfixed with 2% OsO_4, dehydrated in a graded ethanol series, and embedded in Epon 812. Ultra-thin sections stained with uranyl acetate and lead citrate were examined in an electron microscope.

Results and Discussion

Cytotoxicity of γ/δ T Cells. γ/δ T cell clones displayed MHC-unrestricted cytotoxicity against both NK-resistant (Daudi) and NK-sensitive (K562) target cell lines (Fig. 1). However, no cytolytic activity could be found with autologous or heterologous EBV-transformed B cell lines (Fig. 1), suggesting that the cytolytic activity of these cells was antigen specific and MHC independent.

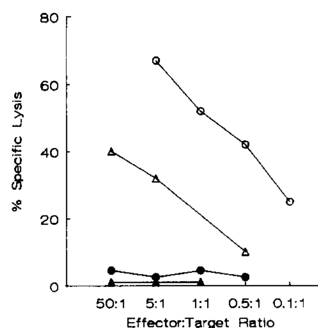

Figure 1. Cytotoxicity of γ/δ T cells. γ/δ T cells were tested for cytolytic activity on ^{51}Cr-labeled tumor lines Daudi (*open circles*), K562 (*open triangles*), and on two EBV-transformed B cell lines: JBEB (autologous; *closed circles*) and DSEB (heterologous; *closed triangles*).

γ/δ T Cells Express Perforin and Serine Esterases. Northern blots of γ/δ T cell clones (Fig. 2 *a*; data shown for clone 1.4) showed that they expressed mRNAs for perforin, SE 1, SE 2, and actin. The size of the expressed messages was the same as that seen in human LAK cells used as a control (12). Daudi cells (Fig. 2 *a*) and a panel of tumor cell lines (not shown; see reference 12) did not express mRNAs for perforin or SEs.

These results were further confirmed by immunoblot analysis (Fig. 2 *b*) using three perforin-specific antisera raised against human perforin expressed in *Escherichia coli*, a peptide composed of amino acids 1–34 of human perforin, and purified murine perforin (not shown). All three antisera reacted specifically with human NK cells, CTLL-R8, and all four clones of γ/δ T cells (data shown only for clone 1.4); but not with K562 cells, or YAC-1 cells (not shown). Pre-immune antisera did not react with any of the cell lysates tested. To establish the presence of perforin in primary γ/δ T cells, we used immunostaining of a FACS-sorted population that was 97% pure. As seen in Fig. 3, primary γ/δ T cells stained with antiperforin antisera showed a granular pattern similar to that seen with CTL and NK cells. Three independent lines of evidence (Northern blots, Western blots with three antisera raised against different forms of perforin, and immunostaining of primary γ/δ T cells with anti-perforin antisera) all suggest that γ/δ T cells express perforin. Perforin has now been shown to be produced by all cytotoxic effector lymphocytes. Our results, obtained with clonal and primary cell populations, are the first definitive study reporting perforin expression in γ/δ T cells.

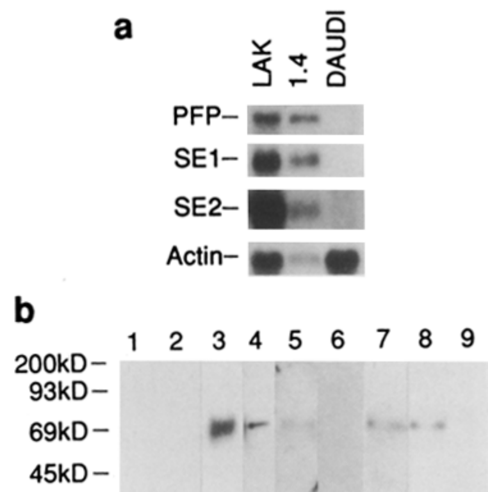

Figure 2. Expression of perforin, SE 1, and SE 2 by γ/δ T cells. (*a*) Northern blot analysis of total RNA from human LAK cells (lane *1*), γ/δ T cells (lane *2*), and Daudi cells (lane *3*) with cDNA probes specific for perforin, SE 1, SE 2, and actin. (*b*) Immunoblot analysis of human NK cells (lanes *1*, *4*, and *8*), γ/δ T cells (lanes *2*, *5*, and *7*), CTLL-R8 (lane *3*), and K562 (lanes *6* and *9*), with preimmune serum (lanes *1* and *2*), antiserum raised against perforin expressed in bacteria (lanes *3–6*), and antiserum raised against an NH_2-terminal peptide of human perforin (lanes *7–9*).

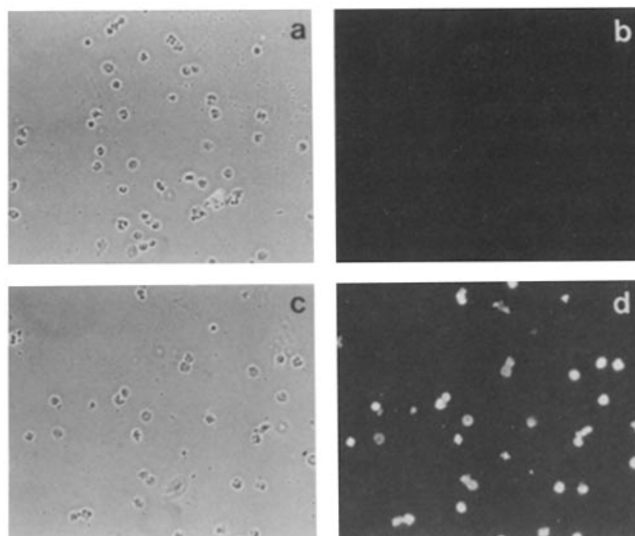

Figure 3. Immunofluorescence staining of primary γ/δ T cells using antiperforin antibody. Primary γ/δ T cells, enriched by cell sorting (97% pure), were stained with preimmune serum (*b*) or with antiperforin antiserum (*d*). Corresponding phase contrast micrographs are shown in *a* and *c*.

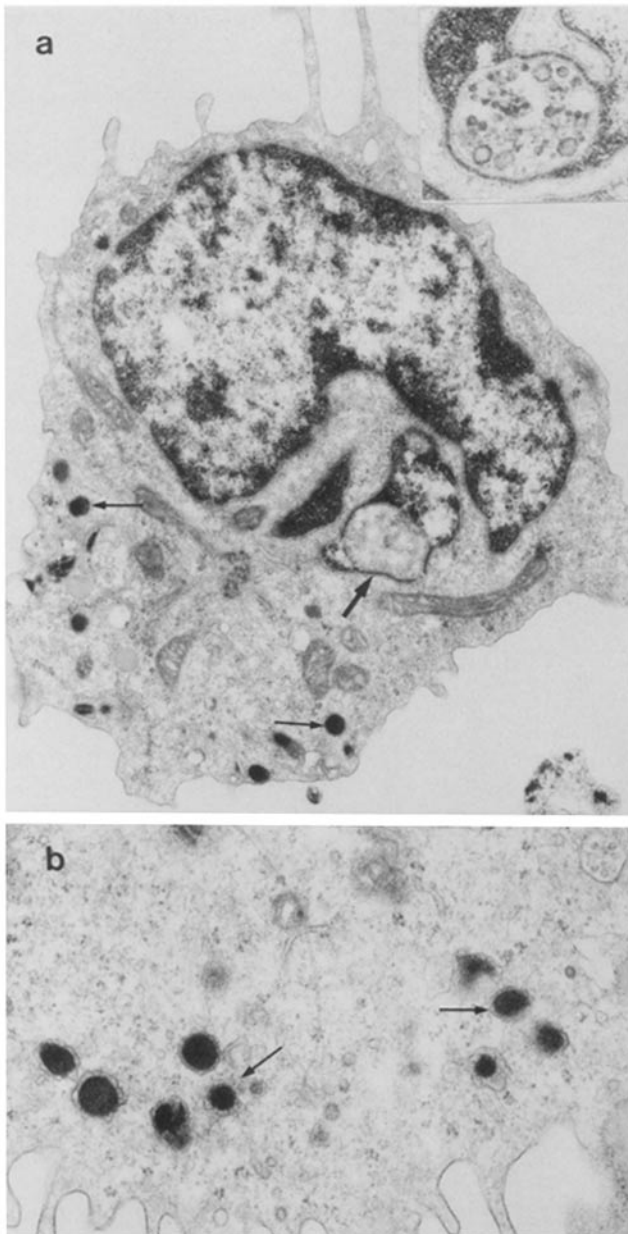

Figure 4. (*a*) Ultrastructure of a γ/δ T cell. The large arrow indicates an inclusion body in the nucleus. Small arrows show cytoplasmic granules (×10,000). Inset demonstrates a typical inclusion body, composed of membrane-like structures and electron-dense particles. (*b*) High magnification of cytoplasmic granules. The granules are bound by a single membrane and contain the dense cores with numerous surrounding vesicles (×23,000).

Ultrastructure of γ/δ T Cells. γ/δ T cells possess long microvilli, well-developed mitochondria, elongated rough endoplasmic reticulum, and 100–160-nm granules surrounded by a single membrane (Fig. 4, *a* and *b*). The granules are composed of a central core of electron-dense fine material surrounded by numerous membrane-bound vesicles and resemble those seen in CTL and NK cells. The nuclei are irregular and are rich in chromatin concentrated near the nuclear membrane. Many nuclei contain one or two inclusion bodies (IB) that consist of membrane-like structures, electron-dense particles, and free ribosomes bound by a double membrane complex (Fig. 3 *a*, inset). Similar IB were found recently in human LAK cells by Groscurth et al. (13). They suggest that the IB are derived from endoplasmic reticulum, contain glycogen, and represent overstimulation of LAK cells with IL-2. As LAK cells are a heterogenous population that includes γ/δ T cells (14), it is possible that IB are specifically associated with γ/δ T cells. We are attempting to resolve this issue using immunoelectron microscopy of LAK cells with anti-TCR-γ/δ antibodies.

γ/δ T cells may be implicated in RA and other autoimmune diseases. In RA patients, large numbers of γ/δ T cells are found in the synovial fluid of affected joints (2, 4). This observation, together with our finding that γ/δ T cells contain perforin and SEs, suggests the possibility that γ/δ T cell–mediated destruction of joint tissue is of importance in the pathology of RA. Increased numbers of γ/δ T cells are also seen in pulmonary sarcoidosis (15), ataxia-telangiectasia (16), leprosy and leishmaniasis (17), atopic dermatitis (18), and Chediak-Higashi syndrome (19), suggesting that γ/δ T cells play a role in a number of immunologic disorders.

A significant role for granule mediators in cell killing is supported by the fact that antigenic or mitogenic signals known to induce cytotoxic effectors also induce mRNAs for perforin and SEs in T cells (12, 20). The present results therefore strongly support the inclusion of γ/δ T cells along with CTL, NK, and LAK cells as cytolytic lymphocytes.

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