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Brief Definitive Report

HUMAN CD3$^+$ T LYMPHOCYTES THAT EXPRESS NEITHER CD4 NOR CD8 ANTIGENS

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Differential expression of the CD4 and CD8 antigens on T lymphocytes has been used as a basis to construct models of thymic differentiation, and to identify functionally distinct subsets of T cells (1, 2). In the thymus, most cells coexpress both CD4 and CD8, while minor subpopulations express the phenotypes CD4$^-$,8$^-$; CD4$^+$,8$^-$; and CD4$^-$,8$^+$ (1, 2). It has been proposed that during thymic ontogeny CD4$^-$,8$^-$ precursor cells differentiate into CD4$^+$,8$^+$ thymocytes. Upon maturation, CD4$^+$,8$^+$ thymocytes acquire the CD3/Ti complex and presumably lose either CD4 or CD8, then CD3$^+$,4$^+$,8$^-$ and CD3$^+$,4$^-$,8$^+$ T lymphocytes migrate into peripheral tissues (1, 2). Mature CD3$^+$,4$^+$,8$^-$ T cells augment Ig secretion by B lymphocytes and facilitate the generation of cytotoxic T cells, whereas CD3$^+$,4$^-$,8$^+$ T cells predominately mediate cytotoxicity and immune suppression (3, 4). In general, antigenic recognition by CD4$^+$ T cells is restricted by class II MHC gene products, whereas antigenic recognition by CD8$^+$ cells is restricted by class I MHC antigens (5, 6). It has been generally accepted that all peripheral T cells express either CD4 or CD8, with a minor population expressing both antigens. However, we have recently observed that a small subset of human peripheral blood CD3$^+$ T lymphocytes express neither CD4 nor CD8. Herein, we describe the characteristics of this unique subset of T cells.

Materials and Methods

Isolation of Peripheral Blood CD3$^+$,4$^-$,8$^-$ T Lymphocytes. Mononuclear cells from normal peripheral blood (Stanford Blood Center, Palo Alto, CA) were isolated using Ficoll/Hypaque. Monocytes were depleted by plastic adherence. Nonadherent cells were stained with a mixture of FITC-conjugated anti-Leu-2 (CD8), FITC-conjugated anti-Leu-3 (CD4), and phycoerythrin (PE)-conjugated anti-Leu-4 (CD3) (7). After extensive washing in ice cold PBS, the cells were resuspended in PBS containing 1% FCS. Lymphocytes expressing CD3, but lacking both CD4 and CD8, would therefore show specific red (PE) fluorescence, but not green (FITC) fluorescence, CD3$^+$,4$^-$,8$^-$ cells were isolated to >95% purity using a FACS 440 (Becton Dickinson Immunocytometry Systems, Mountain View, CA) (7). Fluorochrome-conjugated, isotype-matched control antibodies were used to establish the specificity of antibody binding (7).

Antibodies. Anti-Leu-1, -Leu-2, -Leu-3, -Leu-4, -Leu-5, -Leu-6, -Leu-11, and -Leu-12 react with the CD5, CD8, CD4, CD3, CD2, CD1, CD16, and CD19 differentiation antigens, respectively. Anti-Leu-19 reacts with the Leu-19 (NKH-1) antigen, which is present on NK cells and a subset of T lymphocytes (8).

Immunofluorescence and Flow Cytometry. Methods of immunofluorescence, flow cytometry, and data analysis have been presented elsewhere (7, 9).

Cytotoxicity Assays. ^{51}Cr-labeled tumor cells were used as targets in a 4-h radioisotope release assay (7).

Cell Culture. Cells were cultured in RPMI 1640 supplemented with 10% FCS (KC

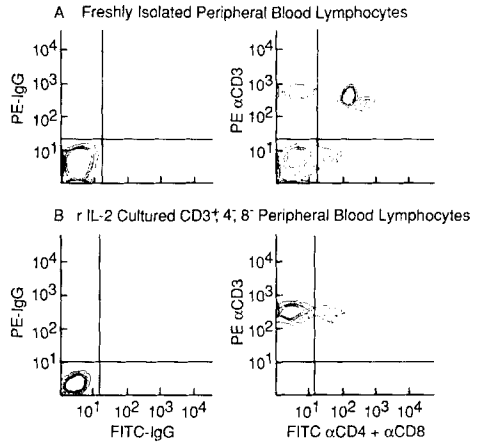

FIGURE 1. $CD3^+$ T cells expressing neither CD4 nor CD8. (*A*) Lymphocytes were stained with FITC-IgG and PE-IgG control antibodies (*left*) or PE anti-Leu-4 (CD3) and a mixture of FITC anti-Leu-3 (CD4) and FITC anti-Leu-2 (CD8) (*right*). Based on the control sample, the contour plot was divided into quadrants representing unstained cells (*lower left*), cells with only red fluorescence (*upper left*), cells with red and green fluorescence (*upper right*), and cells with only green fluorescence (*lower right*). Lymphocytes expressing CD3, but lacking both CD4 and CD8 would therefore be present in the upper left quadrant (i.e., red is positive, green is negative). In this individual, 79.8% of lymphocytes expressed the CD3 antigen, and 8.5% were $CD3^+,4^-,8^-$. In the Ig isotype–matched control sample, <0.01% of lymphocytes were present in the *upper left quadrant* and >98% were unstained (*lower left quadrant*). α, anti. (*B*) In vitro cultured $CD3^+,4^-,8^-$ lymphocytes were stained as described in (*A*). 90% of the lymphocytes were $CD3^+4^-,8^-$. Note that 10% of the lymphocytes were stained both red ($CD3^+$) and dim green (*upper left*). Immunofluorescent staining with only FITC anti-Leu-2 and PE anti-Leu-4 gave an identical profile, indicating that these cells were $CD3^+,CD8^{dim+}$ (not shown). No $CD4^+$ lymphocytes were detected in these cultures. α, anti.

Biologicals, Lexena, KS), 1 mM glutamine, 100 μg/ml gentamicin, and 1000 U/ml rIL-2 (Cetus Corp., Emeryville, CA).

Results and Discussion

Peripheral blood lymphocytes from normal donors were stained with PE anti-Leu-4 (CD3) and a mxiture of FITC anti-Leu-2 (CD8) and FITC anti-Leu-3 (CD4) and were analyzed by flow cytometry. A small subset of cells expressed CD3 antigen (a component of the T cell antigen receptor), but lacked both CD4 and CD8 (Fig. 1). Based on analysis of >20 normal blood donors, ~3% of blood lymphocytes comprised $CD3^+,4^-,8^-$ T lymphocytes (range, <0.5–23%) (~78% of lymphocytes were $CD3^+$). The specificity of the staining was confirmed by showing that an identical percentage of lymphocytes was observed using a reciprocal set of reagents (i.e., FITC anti-Leu-4 and a mixture of PE anti-Leu-2 and PE anti-Leu-3), and by direct three-color immunofluorescence analysis using FITC anti-Leu-2, PE anti-Leu-3, and APC anti-Leu-4 (not shown).[1] By three-color immunofluorescence,[2] we also determined that >90% of the $CD3^+,4^-,8^-$

[1] Identical results have also been obtained using the T3, T4 and T8 mAbs (Coulter Immunology, Hialeah, FL).

[2] This was determined by three-color immunofluorescence by staining lymphocytes with APC streptavidin/biotin anti-Leu-4 (CD3), a mixture of PE anti-Leu-2 and PE anti-Leu-3, and a third FITC-conjugated antibody (e.g., FITC anti-Leu-1 [CD5], FITC anti-Leu-5 [CD2], etc.). By gating on $CD3^+,4^-,8^-$ (APC^+,PE^--stained) lymphocytes, it was possible to determine the proportion of $CD3^+,4^-,8^-$ cells that expressed the antigen detected by the FITC-conjugated antibody.

cells coexpressed the CD2 and CD5 pan T cell antigens, but did not express antigens associated with B cells (CD19), NK cells (CD16), thymocytes (CD1), or monocytes (Leu-M3).

$CD3^+,4^-,8^-$ lymphocytes were isolated to >90% purity using a FACS. 10^5 cells were placed into culture in medium containing rIL-2. After 2 wk, $\sim 5 \times 10^6$ cells were recovered and reanalyzed for expression of CD4 and CD8. >99% of the rIL-2-cultured lymphocytes expressed CD3, and 90% were $CD3^+,4^-,8^-$ (Fig. 1*B*). We failed to detect any cells (<0.5%) that expressed either CD4 or CD16. These results clearly show that $CD3^+,4^-,8^-$ T lymphocytes can proliferate in response to rIL-2 without deliberate antigenic stimulation and that the $CD3^+,4^-,8^-$ phenotype is stably maintained. It should be noted however that 10% of the rIL-2-cultured $CD3^+$ T cells did express CD8 antigen, but in approximately one-tenth of the amount present on typical blood $CD3^+,8^+$ T lymphocytes. Lymphocytes expressing "dim" CD8 have been described; however, in these prior reports most $CD8^{dim+}$ cells lacked CD3 and were $CD3^-$, $CD16^+$ NK cells (7). Whether $CD3^+,CD4^-,CD8^{dim+}$ lymphocytes were generated from the $CD3^+,4^-,8^-$ population or were a contaminant in the isolation procedure is unknown.

Morphological examination revealed that >80% of $CD3^+,4^-,8^-$ cells cultured only in rIL-2 were large lymphoblasts with abundant cytoplasm containing predominant azurophilic granules (Fig. 2*A*). In contrast, >90% of freshly isolated $CD3^+,4^-,8^-$ cells were agranular lymphocytes, although a small proportion of granular cells was observed in some donors (Fig. 2*B*). Since both NK cells and some CTL possess this characteristic granular morphology, these cells were tested for cytotoxic activity. $CD3^+,4^-,8^-$ T cells cultured only in rIL-2 lysed NK-sensitive K562 and NK-insensitive JY targets (Fig. 3*A*). Since K562 lacks cell surface expression of both class I and II MHC antigens, these results show that rIL-2-cultured $CD3^+,4^-,8^-$ T lymphocytes recognize and lyse tumor cell targets without MHC restriction. Freshly isolated $CD3^+,4^-,8^-$ T cells did not lyse K562 (Fig. 3*B*). The rIL-2-activated $CD3^+,4^-,8^-$ cells could be distinguished from freshly isolated $CD3^+,4^-,8^-$ cells based on expression of Leu-19 antigen. >70% of rIL-2-stimulated $CD3^+,4^-,8^-$ cells expressed Leu-19, whereas generally <10% of freshly isolated $CD3^+,4^-,8^-$ cells expressed Leu-19. Recently, we have shown that the Leu-19 antigen is expressed on essentially all peripheral blood $CD3^-$ NK cells, but is also present on a small subset of freshly isolated peripheral blood $CD3^+$ T lymphocytes (most of which express CD8) that mediate non-MHC-restricted cytotoxicity (8).

The cytotoxicity mediated by rIL-2-cultured $CD3^+,4^-,8^-$ lymphocytes against K562 was significantly augmented by the presence of soluble anti-Leu-4 antibody in the cytotoxicity assay (Fig. 4). Although anti-CD3 antibodies generally inhibit the cytotoxic function of antigen-specific CTL activity against the specific target antigen, it has been observed that anti-CD3 antibodies actually induce non-specific lyse of inappropriate targets (10). The finding that anti-CD3 *induces*, rather than inhibits, the cytotoxicity of the rIL-2-activated $CD3^+,4^-,8^-$ cells suggests that this population may be polyclonal, containing cytotoxic cells of several specificities which are triggered in the presence of anti-CD3.

These results are compatible with the hypothesis that the peripheral blood $CD3^+,4^-,8^-$ population is polyclonal, and that a $Leu\text{-}19^+$ cytotoxic subset within

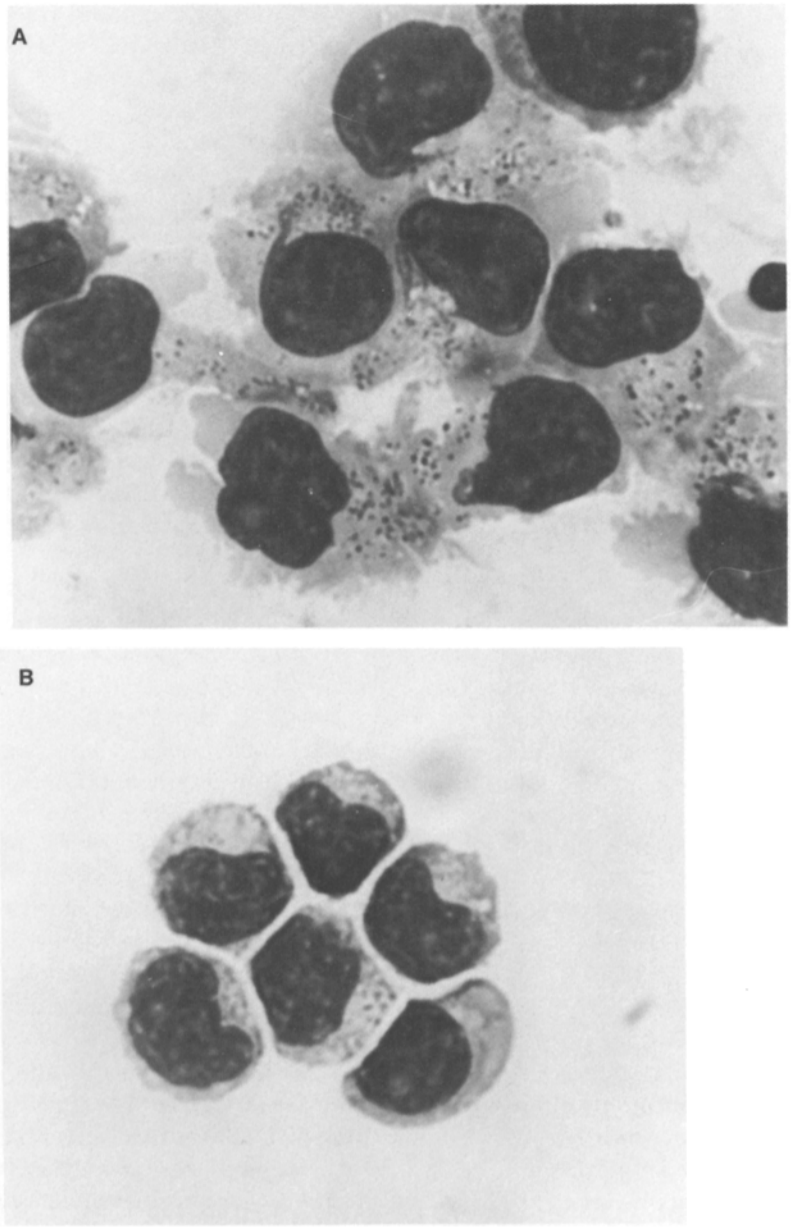

FIGURE 2. Morphology of $CD3^+,4^-,8^-$ T lymphocytes. Cells were cytocentrifuged onto glass slides and stained with Giemsa. (*A*) rIL-2-cultured $CD3^+,4^-,8^-$ lymphocytes; (*B*) freshly isolated peripheral blood $CD3^+,4^-,8^-$ lymphocytes. Magnification, × 6,000.

the $CD3^+,4^-,8^-$ population can proliferate directly in response to rIL-2, without deliberate antigenic stimulation. Further studies on the precursors of the cytotoxic cells within the $CD3^+,4^-,8^-$ subset will be necessary to identify the frequency and phenotype of these cells, and investigate their relationship to the lymphokine-activated killer phenomenon.

The existence of $CD3^+,4^-,8^-$ T lymphocytes shows that a minor subset of

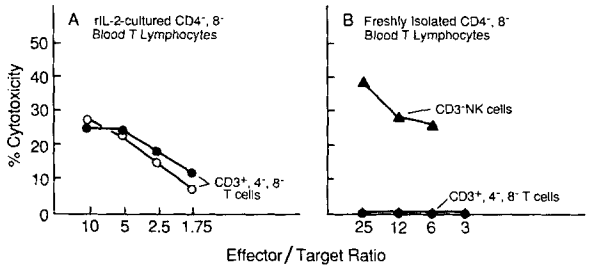

FIGURE 3. Cytotoxicity mediated by $CD4^-,8^-$ T lymphocytes (*A*) An rIL-2-cultured $CD3^+,4^-,8^-$ cell line shown in Fig. 1B, was assayed for cytotoxicity against K562 (*closed symbols*) and JY (*open symbols*). (*B*) Lymphocytes were stained with PE anti-Leu-1 and a mixture of FITC anti-Leu-2, FITC anti-Leu-3, and FITC anti-Leu-12. Lymphocytes that showed exclusively red immunofluorescence (i.e., $CD5^+,4^-,8^-,12^-$) were isolated with a FACS to >95% purity. These freshly isolated $CD5^+,4^-,8^-$ T lymphocytes (●) were assayed for cytotoxicity against K562. In these experiments, T cells were identified with an antibody against CD5 rather than CD3, since antibodies against CD3 have been shown to either induce or inhibit cytotoxic function (10). >95% of the $CD5^+,4^-,8^-,12^-$ lymphocytes coexpressed CD3, as determined by three-color immunofluorescence analysis. FITC anti-Leu-12 (CD19, pan B cell antigen) was used to exclude any $CD5^+$ B lymphocytes from the T lymphocyte population. As a positive control, NK cells (▲), i.e., the $CD5^-,4^-,8^-,19^-$ population, were isolated from the same individual's blood and assayed for cytotoxicity.

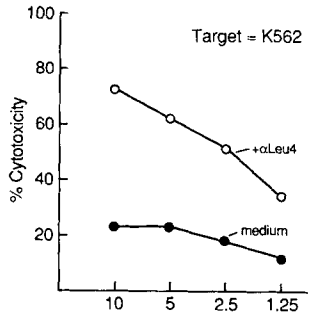

FIGURE 4. Effect of anti-CD3 on cytotoxicity. rIL-2-cultured $CD3^+,4^-,8^-$ cells were assayed for cytotoxicity against K562 in the presence (○) or absence (●) of soluble anti-Leu-4 antibody (final concentration 2 μg/ml) in the 4 h radioisotope release assay. α, anti; abscissa, E/T ratio.

functionally competent peripheral T cells apparently does not require either CD4 or CD8 expression. Furthermore, these studies raise questions regarding the origin of these unique T cells. Recently, De La Hera et al. (11) have reported that in vitro culture of $CD3^+,4^-,8^-$ thymocytes in IL-2 results in the generation of non-MHC-restricted cytotoxic cells. It seems likely that these thymocytes are the immediate precursor for the $CD3^+,4^-,8^-$ T lymphocytes that we have detected in peripheral blood.

Summary

$CD3^+$ T lymphocytes expressing neither CD4 nor CD8 antigens exist in normal human peripheral blood in low frequency (~3% of lymphocytes). The $CD3^+,4^-,8^-$ phenotype was stably maintained after in vitro culture in IL-2. Culture of $CD3^+,4^-,8^-$ cells in only rIL-2 generated cytotoxic T cells that lysed NK-sensitive and NK-insensitive tumor cell targets without MHC restriction. These experiments clearly show that phenotypically and functionally competent T cells expressing neither CD4 nor CD8 are present in normal peripheral blood.

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