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Immunology

Definition of a population of $CD4^-8^-$ T cells that express the $\alpha\beta$ T-cell receptor and respond to interleukins 2, 3, and 4

(lymphocytes/lymphokines/thymus/ontogeny)

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ABSTRACT **Whereas most T cells express surface CD4 or CD8 molecules, a minority lacks both. $CD4^-8^-$ cells usually express the $\gamma\delta$ T-cell receptor, but here we describe a population of $CD4^-8^-$ T cells from the peripheral blood that express the $\alpha\beta$ heterodimer. These cells have different surface antigens than $\gamma\delta^+$ T cells, expressing CD5 but lacking CD16, and differ in function from $\gamma\delta^+$ T cells. $CD4^-8^-$ $\alpha\beta^+$ cells lack non-major histocompatibility complex-restricted cytolytic function but can be induced to lyse their target cells after activation of their T-cell receptors. A peculiar characteristic of these cells is their responsiveness to interleukin 3. Since these cells have not altered their phenotype or function over a 12-month period in culture, they appear to be mature T cells. The results indicate that normal human peripheral blood contains two subsets of $CD4^-8^-$ T cells, expressing either $\gamma\delta$ or $\alpha\beta$ receptors, that differ in function, phenotype, and growth control.**

A small proportion of the peripheral blood T ($CD3^+$) lymphocytes do not express the CD4 or CD8 differentiation antigen and hence are called "double-negative" T cells (1). Most of these $CD4^-8^-$ cells do not use the same T-cell antigen receptor (TCR) as $CD4^+$ or $CD8^+$ T cells, whose TCR is composed of α and β polypeptides, but employ instead one composed of chains termed γ and δ. In the murine thymus, another subset of $CD4^-8^-$ lymphocytes has been detected, expressing the usual $\alpha\beta$ TCR (2–4). A similar subset has recently been identified in human thymus (5). $CD4^-8^-$ $\alpha\beta^+$ T cells have also been found in large numbers in MLR-*lpr*/*lpr* mice, which are prone to autoimmune disease. There is evidence that these cells are involved in the development of the autoimmune state (6).

Here we report the properties of a $CD4^-8^-$ T-cell line, derived from the peripheral blood of a healthy individual, that expresses the $\alpha\beta$ TCR. The properties of this cell line were found to differ markedly from those of $CD4^-8^-$ $\gamma\delta^+$ T cells. Since their characteristics did not alter over a 12-month period in culture, these cells appear to be a mature population. Unlike other T-cell subsets, $CD4^-8^-$ $\alpha\beta^+$ T cells respond to interleukin 3 (IL-3), a lymphokine not previously demonstrated to stimulate mature T cells (reviewed in ref. 7).

MATERIALS AND METHODS

Derivation of T-Cell Line. Peripheral blood mononuclear cells isolated by Ficoll/Hypaque gradient from a healthy donor were incubated at 4°C for 30 min with optimal concentrations of the phycoerythrin (PE)-conjugated anti-CD4 monoclonal antibody (anti-Leu-3a), and fluorescein isothiocyanate (FITC)-conjugated anti-CD8 (anti-Leu-2a). The cells were then washed twice in cold RPMI 1640 (GIBCO) supplemented with 5% fetal bovine serum (Flow Laboratories). Double-negative cells were sorted with a FACStar (Becton Dickinson). The purified $CD4^-8^-$ T cells were cultured and expanded by weekly stimulation with phytohemagglutinin (1:1000 PHA-P, Difco), recombinant human IL-2 (20 ng/ml, Ajinomoto, Japan), and autologous irradiated [4000 rads (40 Gy)] peripheral blood mononuclear cells; IL-2 (20 ng/ml) was added again at midweek. After 4 weeks of continuous culture the cells were stained with a combination of PE-conjugated monoclonal antibodies, G17-2 (anti-CD4) and G10-1 (anti-CD8; ref. 8), and subsequently resorted.

Immunophenotyping. For each analysis 2×10^5 T cells were incubated for 30 min at 4°C with the optimal concentration of the monoclonal antibodies listed below. When monoclonal antibodies not directly conjugated were used, the cells were incubated with FITC-conjugated goat anti-mouse IgG (Southern Biotechnology Associates, Birmingham, AL) at the working dilution of 1:100 for 30 min at 4°C. Cells (10^4) were analyzed with the FACStar. The following monoclonal antibodies were used: PE-anti-Leu-1 (CD5), PE-anti-Leu-2a (CD8), FITC-anti-Leu-3a (CD4), PE-anti-Leu-4 (CD3), FITC-anti-Leu-7 (CD57), PE-anti-Leu-8 (no CD defined yet), FITC-anti-Leu-11 (CD16), FITC-anti-Leu-18 (CD45R), and FITC-anti-Leu-19 (CD56) from Becton Dickinson; 9.3 (CD28; ref. 8); 9.6 (CD2; ref. 8); WT31 and BMA031 ($\alpha\beta$ TCR; refs. 9 and 10); Tiγ A (TCR $V_{\gamma 9}$ variable gene product; ref. 11); Tδ1 (TCR δ chain; ref. 12).

Cell Proliferation Assay. T cells at the end of their weekly cycle were incubated (2×10^4 cells per well) in 96-well flat-bottom microplates (GIBCO) with various lymphokines and harvested for measurement of [^{3}H]thymidine incorporation as described (13, 14). Purified recombinant human lymphokines tested in proliferative assays included IL-1β, IL-4, and IL-6 (Immunex, Seattle); IL-3 and granulocyte/macrophage colony-stimulating factor (GM-CSF) (Sandoz Pharmaceutical and Genetics Institute, Boston); tumor necrosis factor α (TNF-α) (Genentech); and IL-2 (Ajinomoto). Synthetic IL-3 (J. Schrader and I. Clark-Lewis, Biomedical Centre, Vancouver, Canada) was used in some experiments.

To study specific inhibition of the proliferative response to IL-3, a rabbit anti-human IL-3 was used at neutralizing activities of 50 units/ml and 5 units/ml (dilutions, 1:100 and 1:1000). A sheep anti-human GM-CSF at a neutralizing activity of 50 units/ml (dilution, 1:500) was used as control. These antisera were provided by S. C. Clark (Genetics Institute, Boston). Test samples containing various concentrations of IL-2, IL-3, and IL-4 were incubated for 30 min at

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Abbreviations: IL-*n*, interleukin *n*; TNF-α, tumor necrosis factor α; GM-CSF, granulocyte/macrophage colony-stimulating factor; PE, phycoerythrin; FITC, fluorescein isothiocyanate; TCR, T-cell antigen receptor; *V*, variable.

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room temperature with the appropriate dilution of the antiserum to human IL-3 or GM-CSF.

Analysis of mRNA for the α, β, and γ Chains of the TCR. RNA preparation, agarose gel electrophoresis, transfer to nitrocellulose, hybridization with ^{32}P-oligolabeled probes, and autoradiography were performed as described (15). The cDNA probes used (provided by M. J. Owen, Imperial Cancer Research Fund, London) were specific for the constant region of the TCR α (pJa2), β (pB400) or γ (pTcRγ) chain.

Immunoprecipitation. Viable (>95% by trypan blue exclusion) CD4$^-$8$^-$ lymphocytes and Jurkat T-cell lymphoma cells were radioiodinated by the lactoperoxidase technique (16) and solubilized in 1% (vol/vol) Triton X-100 before immunoprecipitation with the β-chain specific antibody βF1 (17) at 1 μg/ml. Immunobeads coated with rabbit anti-mouse IgG (Bio-Rad) were used instead of protein A as described elsewhere (16). The immunoprecipitates were analyzed by SDS/10% polyacrylamide gel electrophoresis under reducing conditions. The ^{125}I-labeled molecules were visualized as described (16).

Assay of Cell-Mediated Cytotoxicity. A ^{51}Cr-release assay was performed as described (18). Effector/target cell ratios of 30:1 to 1:1 were used, and in some experiments target cells were preincubated with UCHT1 (anti-CD3) for 30 min (18). Specific release of ^{51}Cr for each target and effector/target ratio was estimated as follows: % specific release = [(experimental release − spontaneous release)/(maximum release − spontaneous release)] × 100. For spontaneous release, target cells were incubated in medium only; for maximum release, target cells were incubated in medium containing 5% (vol/vol) Nonidet P-40.

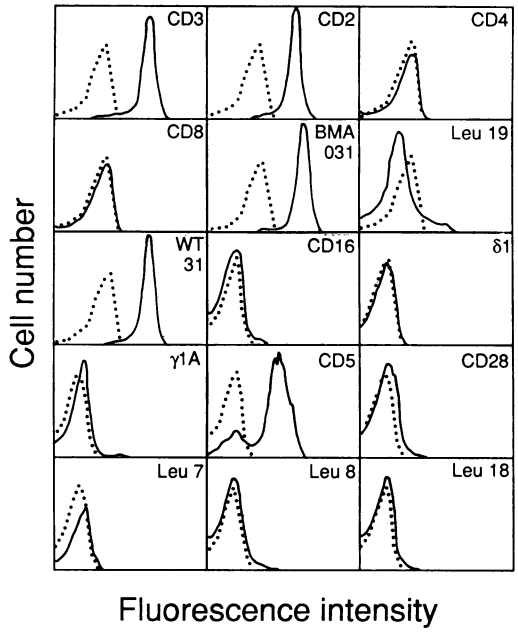

FIG. 1. Flow cytofluorometric analysis of surface antigens expressed by the double-negative population after the second sorting. Before these experiments were performed the cells were cultured for 4 weeks in the presence of phytohemagglutinin (1:1000), IL-2 (20 ng/ml) and autologous irradiated (4000 rads) peripheral blood mononuclear cells as feeders. Vertical axis, frequency of cells; horizontal axis, logarithm of FITC or PE fluorescence intensity. The cells were passed through Ficoll/Hypaque before analysis and were positive for CD3, CD2, CD5, WT31, and BMA031 but negative for all the other differentation molecules tested. The dotted line represents the negative control of each of two experiments. The first seven graphs are from the first experiment. Antibody specificities are indicated (see *Materials and Methods*).

RESULTS

Phenotypic Analysis of the Double-Negative αβ$^+$ T Cells. Over a 12-month period, multiple phenotypic analyses were performed, with consistent results. Results from two experiments are shown. The cells were stained by monoclonal antibodies directed to CD2 and CD3, but not by monoclonal antibodies specific for CD4 and CD8 differentiation antigens (Fig. 1). The cells were not stained by anti-Leu 7 (19) anti-Leu-11 (anti-CD16; ref. 20), or anti-Leu-19 (21), which are markers of "natural killer" cells. As previously noted for CD4$^-$8$^-$ WT31$^-$ (αβ$^-$) γδ$^+$ cells (22), they were not stained by anti-CD28 (8), suggesting that both αβ$^+$ and γδ$^+$ double-negative cells lack this pathway of activation (Fig. 1). They also did not react with anti-Leu-8 and anti-Leu-18, monoclonal antibodies that define regulatory (inhibitory) subpopulations of T cells (23) in both the CD4$^+$ and CD8$^+$ populations (Fig. 1). Anti-Leu-1 (anti-CD5; ref. 24) stained brightly up to 85% of the cell line (Fig. 1). The cells in culture were also stained by monoclonal antibody (anti-Tac; T. Waldmann, National Institutes of Health) to the IL-2 receptor (data not shown). No staining was detected with Tiγ A, directed against the $V_{\gamma 9}$ gene product (11), or Tδ1, specific for the δ chain of the TCR (12). The cells reacted with the monoclonal antibodies WT31 and BMA031 (Fig. 1), both of which are considered to be directed against either the αβ heterodimer of the TCR or a CD3 epitope expressed only on αβ$^+$ T cells (9, 10).

Definition of the TCR by Northern Blot and Immunoprecipitation Analysis. To confirm that αβ TCR molecules were expressed on these cells, total RNA was extracted from the cell line and cDNA probes specific for the α, β, or γ constant regions were used to establish which TCR mRNAs were transcribed. Northern blotting analysis demonstrated that

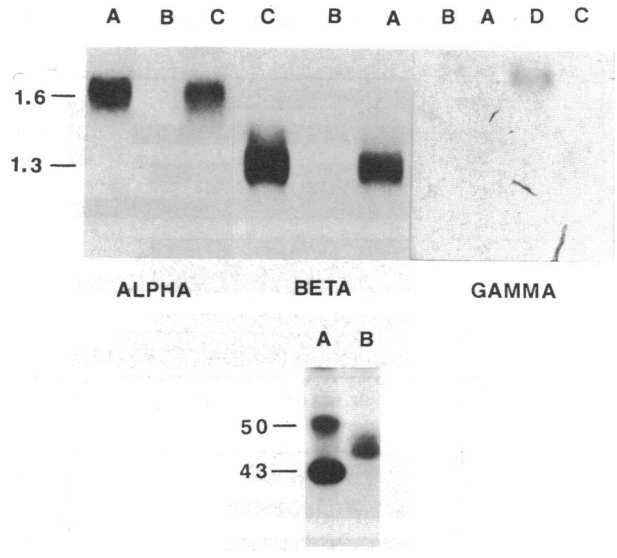

FIG. 2. (*Upper*) Northern blot analysis of total RNA with cDNA probes specific for the constant region of the TCR α, β, or γ chain. Lanes A, peripheral blood from the individual used to raise the CD4$^-$8$^-$ αβ$^+$ cells. Lanes B, hepatoma cell line Hep G2, used as a negative control. Lanes C, CD4$^-$8$^-$ αβ$^+$ cell line. Lane D, γδ$^+$ cell line raised from the same individual. The double-negative αβ$^+$ cell line showed full-length message for the α and β chain (1.6 and 1.3 kilobases) but no message for the γ chain. All the blots were subsequently probed with 7B6 (25) to show presence of mRNA in each track (data not shown). (*Lower*) Immunoprecipitation of lysates of surface-radioiodinated cells with monoclonal antibody βF1, directed against the β chain of the TCR (17). Lane A, immunoprecipitate from the Jurkat cell line. Lane B, immunoprecipitate from the CD4$^-$8$^-$ αβ$^+$ cell line. SDS/polyacrylamide gel electrophoresis was done under reducing conditions.

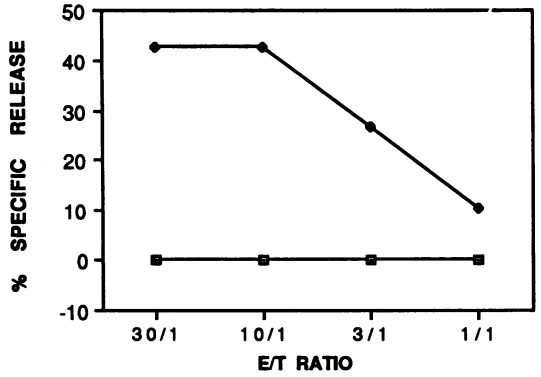

FIG. 3. Cytotoxicity assay using K562 target cells. The $CD4^-8^-$ $\alpha\beta^+$ effector cells did not kill uncoated K562 target cells (□) but did kill K562 cells that were coated with anti-CD3 (UCHT1; ♦). E/T, effector/target.

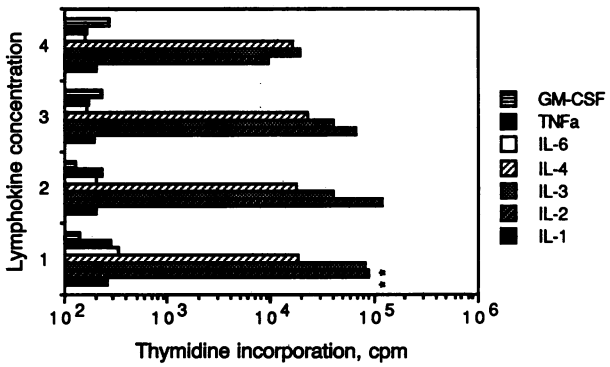

FIG. 4. Proliferative response of $CD4^-8^-$ $\alpha\beta^+$ cells to various lymphokines. Twenty thousand cells were plated in triplicate in a 96-well flat-bottom microplate at the end of their weekly feeding cycle. Lymphokine concentrations were as follows, for bar sets 1–4, respectively: 5000, 500, 50, and 5 units/ml for IL-1β, IL-6, and TNF-α; 1000, 500, 50, and 5 units/ml for IL-3; 500, 50, 5, and 0.5 units/ml for GM-CSF; 500, 50, 5, and 0.5 ng/ml for IL-2 and IL-4. Stars (at $\approx 10^5$ cpm) indicate proliferative response in the presence of UCHT1 (anti-CD3) coated on plastic. All the experiments were performed in triplicate, and SE was within 5% of the mean of the proliferative response.

these cells expressed full-length mRNA for the α (1.6 kilobases) and β (1.3 kilobases) chains but not for the γ chain (Fig. 2 *Upper*). As positive controls for the α and β cDNA probes we used total RNA extracted from peripheral blood lymphocytes. As a control for the γ cDNA probe, we used total RNA obtained from a $CD4^-8^-$ $\gamma\delta^+$ cell line derived from the same individual.

To demonstrate the presence of TCR β chain on the cell surface, cells were surface-labeled with ^{125}I (lactoperoxidase method) and solubilized with Triton X-100, and immunoprecipitation was performed using monoclonal antibody βF1, directed against the β chain of the TCR (17). Electrophoresis of the immunoprecipitate under reducing conditions showed two closely running bands at $\approx$47 kDa from the lysate of ^{125}I-labeled $CD4^-8^-$ cells (Fig. 2 *Lower*, lane B). In contrast, βF1 immunoprecipitated two distinct proteins, of 43 and 50 kDa from the T-cell line Jurkat (lane A). Under nonreducing conditions a single band at 80 kDa was detected from $CD4^-8^-$ $\alpha\beta^+$ cells (data not shown).

Modulation of the CD3 Complex Induces Cytotoxicity. No "natural killer"-like activity was detected against target cells such as K562 (human erythroleukemia), P815 (murine mastocytoma), and Raji (Burkitt lymphoma), even when the effector cells were pretreated with IL-2 (data not shown). In contrast, modulation of the CD3 complex by coating the target cells with anti-CD3 induced lysis of the K562 cells (Fig. 3) as well as Raji and P815 cells (data not shown).

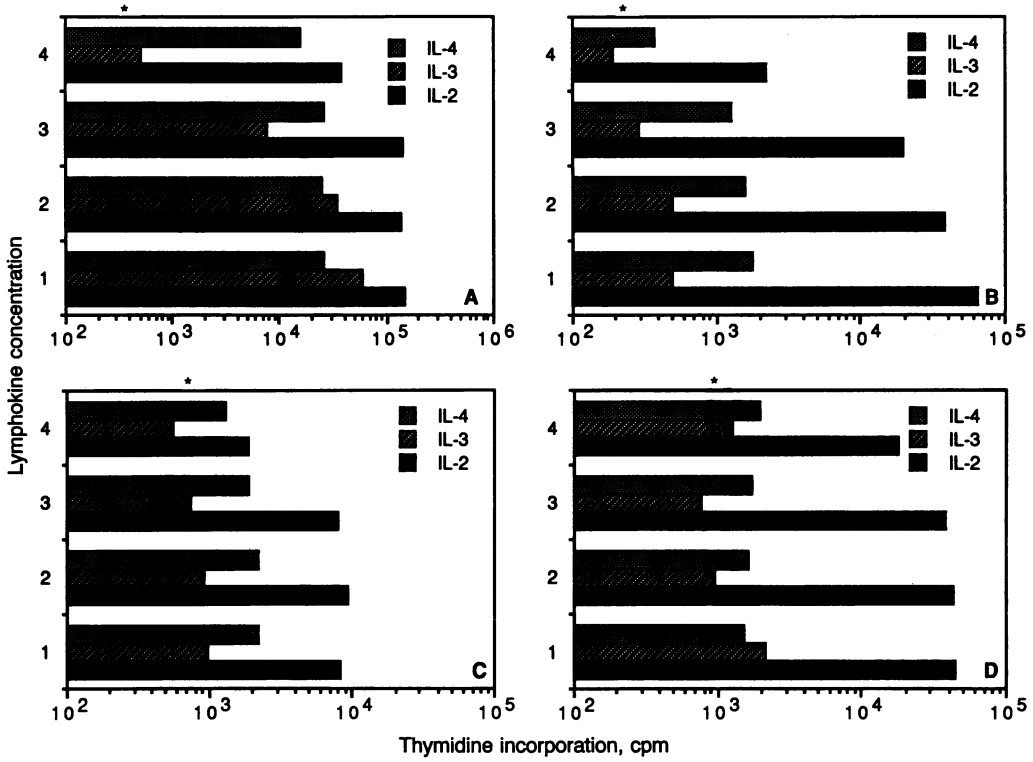

FIG. 5. Comparison of the proliferative response to IL-2, IL-3, and IL-4 of four different T-cell clones or lines raised from the same individual. IL-2 and IL-4 concentrations were 500, 50, 5, and 0.5 ng/ml (bar sets 1–4, respectively); IL-3 was used at 1000, 500, 50, and 5 units/ml. (*A*) $CD4^-8^-$ $\alpha\beta^+$ T-cell line. (*B*) $CD4^+$ $\alpha\beta^+$ T-cell clone. (*C*) $CD8^+$ $\alpha\beta^+$ T-cell clone. (*D*) $CD4^-8^-$ $\gamma\delta^+$ T-cell line. Stars indicate background proliferation. All the experiments were performed in triplicate; SE was within 5% of the mean proliferative response.

Responsiveness to Growth Factors. We studied the effect of a wide range of purified recombinant lymphokines previously reported to induce proliferation of T cells or thymocytes (e.g., IL-1, IL-6, and TNF-α) or that act on hemopoietic precursors (GM-CSF and IL-3). No response was observed to IL-1, IL-6, TNF-α, or GM-CSF over a wide range of concentrations (Fig. 4). As expected, because the cells were maintained with IL-2 in culture, IL-2 induced a strong proliferative response. IL-4, as reported for other T cells (26), also induced a strong proliferative response. However, the most striking result was the proliferative response of these cells to recombinant human IL-3 (Fig. 4).

Comparative experiments were performed using four different phenotypes of T cells, $CD4^-8^-$ $\gamma\delta^+$, $CD4^-8^-$ $\alpha\beta^+$, $CD4^+$ $\alpha\beta^+$, and $CD8^+$ $\alpha\beta^+$, expanded under identical experimental conditions and used at the end of their weekly cycle of culture after Ficoll separations to remove feeder cells. Only the $CD4^-8^-$ $\alpha\beta^+$ cells responded significantly to IL-3 (Fig. 5). Antisera that specifically neutralize human IL-3 and GM-CSF (at neutralizing activities of 50 units/ml) were used to block the proliferative response of $CD4^-8^-$ $\alpha\beta^+$ T cells stimulated with IL-2, IL-3, or IL-4. Rabbit anti-human IL-3 antiserum completely blocked the response to IL-3, whereas the response to IL-2 or IL-4 was not affected. The antiserum against GM-CSF did not affect the proliferative response to any of the cytokines (Fig. 6).

DISCUSSION

Recently, there has been considerable interest in $CD4^-8^-$ T cells, which were found to use a $\gamma\delta$ TCR instead of the $\alpha\beta$ TCR used by $CD4^+$ or $CD8^+$ T cells (e.g., ref. 27). During investigations of $CD4^-8^-$ T cells we generated from the blood of a healthy individual a line of $CD4^-8^-$ T cells expressing the more usual $\alpha\beta$ TCR. The properties of this line, described here, establish that $CD4^-8^-$ T cells in the periphery are heterogeneous, with two subsets, $\alpha\beta^+$ and $\gamma\delta^+$. Both the subsets were present in the peripheral blood of this donor.

Characterization of the $CD4^-8^-$ $\alpha\beta^+$ T-cell line with monoclonal antibodies revealed that it has a different surface phenotype than $CD4^-8^-$ $\gamma\delta^+$ cells: it expresses CD5 but lacks CD16 (low-affinity Fc receptor) (Fig. 1). Functionally, the $CD4^-8^-$ $\alpha\beta$ T cells lack non-major histocompatibility complex-restricted lytic activity, reported for $\gamma\delta^+$ T cells (26), even when preactivated with high doses of IL-2 (data not shown). However, the $CD4^-8^-$ $\alpha\beta^+$ T cells are cytolytic after their TCR complex is activated, as demonstrated experimentally by using anti-CD3 (Fig. 3). These results and the induction of proliferation by anti-CD3 (Fig. 4) demonstrate that these cells are mature T cells, with a functional CD3 complex. $CD4^-8^-$ $\alpha\beta^+$ T cells have been reported previously, in the murine thymus (2–4) and more recently in human thymus (5). This report demonstrates that $CD4^-8^-$ $\alpha\beta^+$ T cells are also present in the periphery, but the relationship of those in the thymus to those cells in the periphery is not clear. The $CD4^-8^-$ $\alpha\beta^+$ T-cell line described here is a fully differentiated population, as no change of phenotype was observed under different experimental conditions such as culture with different cytokines (M. L., unpublished data).

An unexpected characteristic of these cells was noted during an investigation of their growth properties. The cell line responds to IL-2, like all T cells, and to IL-4, like many T cells (26), but surprisingly it responds also to IL-3, a growth factor previously shown to affect hemopoietic cells, chiefly progenitor cells and mast cells (7). There have been no previous conclusive reports of its action in T cells (28), but a recent report described its synergy with IL-2 in inducing T-cell proliferation (29). The response of this cell line emphasizes the complexity of T-cell growth control.

We investigated the effects of IL-3 in detail. To verify that it was indeed IL-3 acting as a growth factor, purified recombinant human IL-3 from two independent sources (Genetics Institute and Sandoz) was used, as well as IL-3 prepared by peptide synthesis (donated by I. Clark-Lewis and J. Schrader). The proliferative effect of IL-3 on the cell line (without feeder cells) was shown to be blocked by neutralizing antiserum to IL-3 (Fig. 6).

Having demonstrated that IL-3 was a growth factor for the $CD4^-8^-$ $\alpha\beta^+$ T cells, we investigated the specificity of the IL-3 effect with other T-cell populations: $CD4^-8^-$ $\gamma\delta^+$, $CD4^+$ $\alpha\beta^+$, and $CD8^+$ $\alpha\beta^+$. None of these responded to IL-3 (Fig. 5).

The function of these $CD4^-8^-$ $\alpha\beta^+$ cells in the periphery, like that of $CD4^-8^-$ $\gamma\delta^+$ cells, is not known. However, MRL-*lpr/lpr* mice, which develop a variety of autoimmune

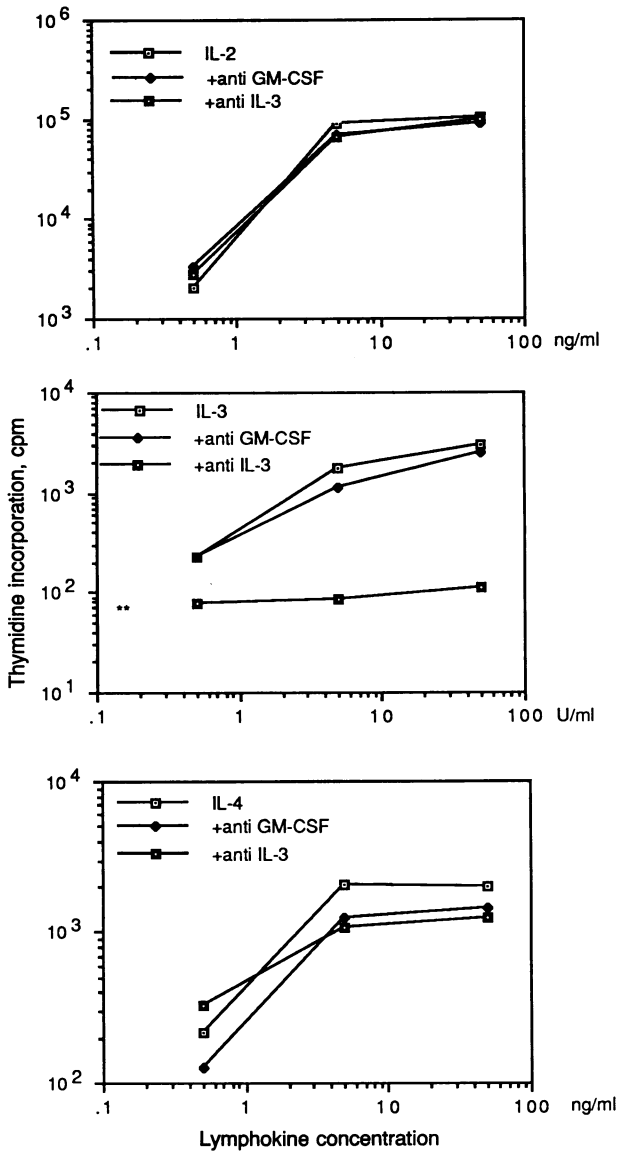

FIG. 6. Specific inhibition of the proliferative response by antiserum to IL-3. Test samples containing various concentrations of IL-2 (0.5–50 ng/ml), IL-3 [0.5–50 units (U)/ml], or IL-4 (0.5–50 ng/ml) were incubated for 30 min at room temperature with rabbit antiserum to human IL-3 (dilution, 1:100; neutralizing activity, 50 units/ml) or with sheep antiserum to human GM-CSF (dilution, 1:500; neutralizing activity, 50 units/ml) provided by S. C. Clark (Genetics Institute). Control samples (stars) were incubated with normal medium. SE in all experiments was within 5% of the mean proliferative response.

manifestations resembling systemic lupus erythematosus, such as arthritis and vasculitis, possess a large population of $CD4^-8^-$ T cells. These cells express the $\alpha\beta$ receptor (6) and are involved in the generation of autoantibodies and in the autoimmune manifestations (30). Recently increases in $CD4^-8^-$ $\gamma\delta^+$ T cells were detected in joints and blood of patients with rheumatoid arthritis and Sjögren syndrome (31, 32).

Although we have described the phenotypic and functional characteristics of $CD4^-8^-$ $\alpha\beta^+$ T cells, their antigen specificity and their repertoire are not yet known. $CD4^-8^-$ $\alpha\beta^+$ T cells, in contrast to $\gamma\delta^+$ T cells, which have a small V_γ and V_δ repertoire (e.g., ref. 33), may employ the same range of V_α, V_β, and J (joining) regions as $CD4^+$ and $CD8^+$ $\alpha\beta^+$ cells. If so, they will have a much wider repertoire than $CD4^-8^-$ $\gamma\delta^+$ cells. It seems that the cell line had limited heterogeneity, as judged by the relative sharpness and unusual molecular size of the immunoprecipitated TCR bands (Fig. 2 *Lower*). Moreover, a predominant pattern of rearrangement has been observed for the TCR β-chain gene (B. Flanagan and M. J. Owen, personal communication). This may have been due to "self-cloning" as the cell line aged in culture.

We sought to determine whether $CD4^-8^-$ $\alpha\beta^+$ T cells were present in other individuals. T cells obtained from peripheral blood of five individuals by erythrocyte rosetting were treated with anti-CD4, anti-CD8, and complement and then stained with FITC-conjugated anti-CD4 and anti-CD8 and with PE-conjugated BMA031, to detect $\alpha\beta^+$ cells, or Tδ1 to detect $\gamma\delta^+$ cells. In all the preparations double-negative $\alpha\beta^+$ T cells were detected, in a minority ranging from 2–12% of the double-negative T cells. After this study was completed, a related study of $CD4^-8^-$ T-cell clones was reported; included were a few $CD4^-8^-$ $\alpha\beta^+$ clones (34).

We have also established, from the spleen of another individual, T-cell lines of the same phenotype ($CD3^+4^-8^-$ $WT31^+$) by using a similar protocol. These cells also respond to IL-3.

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