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FUNCTIONAL PROPERTIES OF A UNIQUE SUBSET OF CYTOTOXIC CD3+ T LYMPHOCYTES THAT EXPRESS Fc RECEPTORS FOR IgG (CD16/LEU-11 ANTIGEN)

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Human T lymphocytes were initially identified (1) and distinguished from B lymphocytes based on the ability of T cells to form spontaneous rosettes with sheep erythrocytes (E).[1] Within the E^+ subset, Moretta et al. (2) defined a discrete subpopulation of cells that bound IgG-coated erythrocytes (EA) via a specific cell surface receptor for the Fc region of IgG (FcR). This population was designated the Tγ subset. These cells have been shown to mediate suppressor cell function (2), antibody-dependent cellular cytotoxicity (ADCC) (3), and natural killer (NK) cell–mediated cytotoxicity (4, 5).

Using monoclonal antibodies (mAb) against human T cell differentiation antigens, two antigenically and functionally distinct subsets of E^+ cells have been identified. The majority of E^+ lymphocytes express the pan–T cell–associated antigens, CD3 and CD5; whereas a minor subset of E^+ cells lack CD3 (5–7). However, most IgG-FcR+ cells are contained within this small $CD3^-$ fraction (5–7). NK cell–mediated cytotoxicity is confined to E^+ lymphocytes lacking the CD3 antigen (5). Based on these findings, it was unclear whether functions attributed to the Tγ subset were mediated by the $CD3^-$ (non-T) NK cell fraction or the very small proportion of $CD3^+$ T cells that express FcR for IgG.

Recently, several mAb (anti-Leu-11, VEP 13, B73.1, and 3G8) have been produced (8–12) that specifically react with the IgG FcR expressed on NK cells and neutrophils. These antibodies do not react with the IgG FcR expressed on monocytes or B lymphocytes (8–10, 12). Essentially all NK and ADCC function is contained within the small fraction of lymphocytes that express this IgG FcR (i.e. CD16/Leu-11 antigen) (8–10, 12, 13).

We (8, 12) and other investigators (9–11) reported that these anti-FcR antibodies did not react with a significant proportion of $CD3^+$ T lymphocytes isolated

from peripheral blood, concanavalin A–activated cultures, or mixed lymphocyte response cultures. However, several patients with T cell lymphoproliferative disorders have been shown (14–17) to coexpress both CD3 antigen and IgG FcR. This finding prompted us to investigate whether or not any CD3$^+$ T lymphocytes obtained from normal individuals express the IgG FcR detected by the anti-Leu-11 mAb. In this report, we show that a functionally unique subset of cytotoxic T lymphocytes in peripheral blood can be identified by coexpression of the CD3 and CD16 (Leu-11) antigens.

Materials and Methods

Preparation of Human Peripheral Blood Leukocytes (PBL).   Human peripheral blood from random, normal donors was obtained from volunteer donors at our institutes, the American Red Cross (San Jose, CA) or the Stanford Blood Center (Palo Alto, CA). Mononuclear cells were isolated using Ficoll/Hypaque. In sorting experiments, monocytes and B cells were depleted by plastic adherence and nylon wool columns, as described (18).

Antibodies.   All Leu series mAb were prepared at the Becton Dickinson Monoclonal Center. Anti-Tac (interleukin 2 receptor [IL-2-R]) antibody was provided by T. Waldmann, National Institutes of Health, Bethesda, MD (19). Anti-Leu-1, anti-Leu-2a, anti-Leu-3a, anti-Leu-4, and anti-Leu-5b (E rosette receptor) recognize the CD5, CD8, CD4, CD3, and CD2 T cell differentiation antigens, respectively. Anti-Leu-11a (IgG1 antibody), anti-Leu-11b (IgM antibody) and anti-Leu-11c (IgG1 antibody that binds a different epitope than anti-Leu-11a and -b) recognize the IgG FcR (CD16) present on essentially all NK cells and neutrophils (12). Anti-Leu-15 detects an epitope on the complement receptor type 3, CR$_3$ (CD11) (20). Anti-Leu-M3 recognizes an antigen expressed exclusively on monocytes. Anti-Leu-7 reacts with an antigen present on a subset of NK cells and T lymphocytes (8, 21). Anti-Leu-12 detects the CD19 antigen, expressed on essentially all B lymphocytes.

Isotype switch-variant antibodies of ME1 or MA2.1 were used to detect ADCC activity (13). The ME1 antibody reacts with HLA-B7, Bw22, Bw42, and B27, whereas the MA2.1 antibody reacts with HLA-A2.

FcR were detected using covalently linked dimeric rabbit IgG and fluorescein isothiocyanate (FITC)-conjugated F(ab')$_2$ goat anti–rabbit IgG, provided by J. Titus (NIH) (22, 23).

F(ab')$_2$ fragments of anti-Leu-11a and anti-Leu-4 antibodies (both IgG1 isotype) were prepared by pepsin digestion (Worthington Biochemicals, Freehold, NJ) (25 μg pepsin per milligram IgG, pH 4.0, 5 h, 37°C) as described by Parham (24). The F(ab')$_2$ fragments were purified by size-exclusion high-pressure liquid chromatography. Fragments were analyzed for purity by sodium dodecyl sulfate–polyacrylamide gel electrophoresis using Coomassie Brilliant Blue R-250 dye for visualization of proteins (25). Antibodies were conjugated with FITC (Molecular Probes, Beaverton, OR) or N-hydroxysuccinimidobiotin (Pierce Chemical Co., Rockford, IL) as described by Goding (26). All antibodies were titrated for specific binding activity by direct or indirect immunofluorescence. The titers of the F(ab')$_2$ antibodies were equivalent to the intact molecules.

Immunofluorescence and Flow Cytometry.   Methods of immunofluorescence, flow cytometry, and data analysis have been presented elsewhere (8, 27, 28). Fluorochrome-conjugated, isotype-matched mAb that do not react with human cells were used to control for nonspecific binding in the immunofluorescence assays. For quantitation of fluorescence, the variable logarithmic amplifiers were calibrated to span four log decades. On the fluorescence histograms, the abscissa was arbitrarily divided into 256 channels, with 64 channels representing a 10-fold increase in fluorescence. By determining the mean channel of a population and converting this number from a logarithmic to a linear value, it was possible to compare the relative amounts of fluorescence of different populations.

Tumor Cells.   All tumor cell lines were obtained from the American Type Culture

Collection, Rockville, MD, and were tested monthly to insure against mycoplasma infection.

Cytotoxicity Assays. For NK cytotoxicity assays, ^{51}Cr-labeled K562 cells were used as targets in a 4-h radioisotope-release assay, as described (8, 29). ADCC assays were performed using the relatively NK-insensitive B lymphoblastoid cell line JY, as described (13). JY expresses HLA antigens A2 and B7.

Proliferation Assays. Lymphocytes were cocultured with mitomycin C–treated tumor cell lines as described (30). Proliferation was measured by incorporation of [^{3}H]thymidine (30). Lectin-free, purified IL-2 was purchased from Electronucleonics, Inc. (Silver Spring, MD). For short term growth, cells were cultured in RPMI-1640 (Irvine Scientific, Santa Ana, CA) supplemented with 10% heat-inactivated fetal calf serum (KC Biologicals, Lexena, KS), 1 mM glutamine (Gibco, Grand Island, NY), antibiotics, and 10% purified IL-2.

Morphology. Giemsa-stained specimens were prepared as described (29).

Results

Coexpression of CD3 and IgG-FcR (CD16/Leu-11 Antigen) on PBL. In a routine phenotypic analysis of the PBL obtained from normal, healthy donors at our institute, we observed two individuals (D. H. and L. G.) who possessed a significant proportion of lymphocytes coexpressing CD3 and CD16 (Leu-11) antigens. As shown in Fig. 1, 23.6% of D. H.'s lymphocytes and 9.4% of L. G.'s lymphocytes were CD3$^+$,CD16$^+$; whereas 46.6% (D. H.) and 61.0% (L. G.) of lymphocytes were typical T lymphocytes with the phenotype CD3$^+$,CD16$^-$. The proportion of CD3$^-$,CD16$^+$ NK cells was within the normal range for both individuals (18.0%, D. H.; 14.0%, L. G.). Peripheral blood from both L. G. and D. H. have been sequentially monitored over the past 6 and 18 mo, respectively, with no significant change in the proportion of CD3$^+$,CD16$^+$ lymphocytes or absolute lymphocyte number.

The specificity of antibody binding was established by the findings that: (*a*) An equal percentage of CD3$^+$ cells were costained with both IgM (anti-Leu-11b) and IgG1 (anti-Leu-11a) mAb against a common epitope on the IgG-FcR. Neither control IgM nor IgG1 mAb reacted with a subset of T lymphocytes (Fig. 1). (*b*) Coexpression of CD16 and CD3 antigens was also demonstrated using the anti-Leu-11c/B73.1 (Fig. 4) and OKT3 antibodies (not shown). Anti-Leu-11c/B73.1 reacts with a different epitope on the Fc-IgG receptor that does not inhibit the binding of the anti-Leu-11a and -b antibodies (12). (*c*) Two-color immunofluorescence using F(ab')$_2$ fragments of anti-Leu-4 and anti-Leu-11a confirmed that a subset of CD3$^+$ T lymphocytes coexpressed the CD16 antigen (Fig. 2).

FcR-bearing CD3$^+$ T cells could be distinguished by the levels of FcR and CD3 surface antigen expression compared to other cell types. The apparent density of CD16 antigen on the CD3$^+$ T lymphocytes was significantly less than on the CD3$^-$ subset. Moreover, the apparent amount of CD3 antigen was significantly higher on the CD3$^+$,CD16$^+$ compared with the CD3$^+$,CD16$^-$ cells (Figs. 1 and 2). In an independent experiment, the amount of fluorescence was quantitated by determining the mean fluorescence value for each population (Fig. 3). The upper panels display the fluorescence histograms for FITC–anti-Leu-11a and phycoerythrin (PE) anti-Leu-4 binding to L. G.'s lymphocytes. Note that the anti-Leu-11–stained cells show three distinct populations: an unstained population, cells with low density (dim) CD16 antigen (11.6% of lymphocytes),
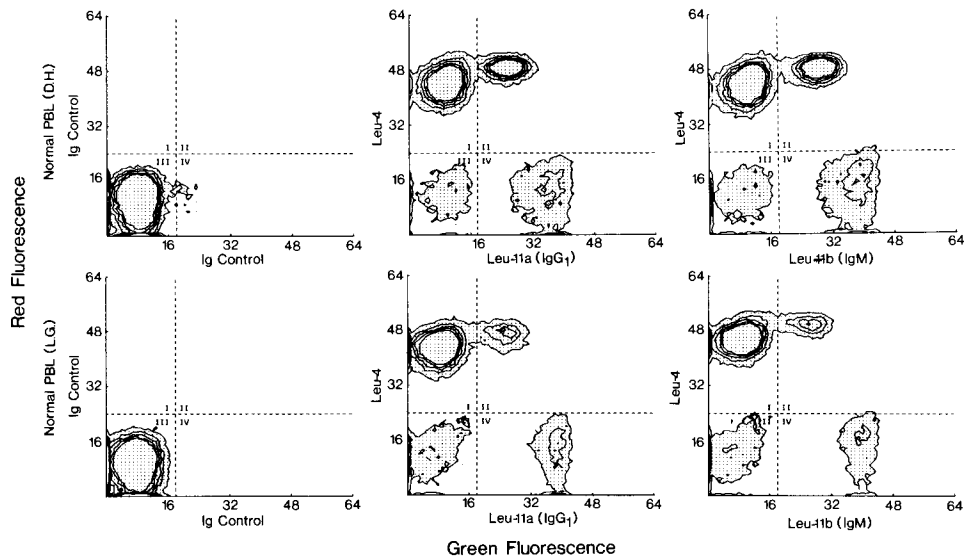
FIGURE 1.   Coexpression of CD3 and CD16 on normal PBL. Peripheral blood mononuclear cells from two individuals (D. H. [upper panels] and L. G. [lower panels]) were stained with fluorochrome-conjugated control antibodies (*left*); FITC–anti-Leu-11a (IgG1 antibody) and PE–anti-Leu-4 (*middle*); and FITC–anti-Leu-11b (IgM antibody) and PE–anti-Leu-4 (*right*). Lymphocytes were distinguished from monocytes on the basis of their characteristic 90° and forward angle light scatter profiles. Correlated green (abscissa, four-decade log scale) and red (ordinate, four-decade log scale) fluorescence of the lymphocytes are displayed as contour plots. Based on the Ig control sample, the contour plots were divided into quadrants: I, cells with red fluorescence only; II, cells with red and green fluorescence; III, unstained cells; and IV, cells with green fluorescence only. The number of cells within each quadrant was determined and the percentage of the total number of lymphocytes calculated. Analysis of D. H.'s lymphocytes indicated: 46.6% CD3+,CD16−; 23.6% CD3+,CD16+; and 18.1% CD3− ,CD16+. For L. G.'s lymphocytes, 61.0% CD3+,CD16−; 9.4% CD3+,CD16+; and 14.0% CD3−,CD16+. In the control contour plots, >98% of lymphocytes were in quadrant III (unstained).
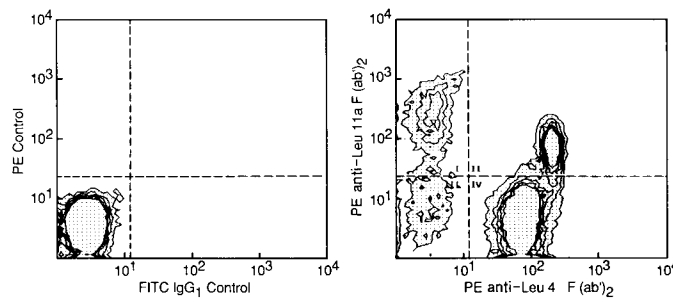

FIGURE 2.   Two-color immunofluorescence using $F(ab')_2$ fragments of anti-Leu-11 and anti-Leu-4. Nonadherent PBL from D. H. were stained with FITC-conjugated $F(ab')_2$ anti-Leu-4 and biotin-conjugated $F(ab')_2$ anti-Leu-11a mAb (*B*) or appropriate control IgG antibodies (*A*). PE-conjugated streptavidin was used to detect binding of biotin-conjugated antibodies. Data are presented as described in Fig. 1.

and cells with high density (bright) CD16 antigen (13.2%). As shown previously (8, 12, 31), this dim CD16+ subset is absent from most normal individuals. Gates were set on these three distinct subsets, and the PE–anti-Leu-4 staining was
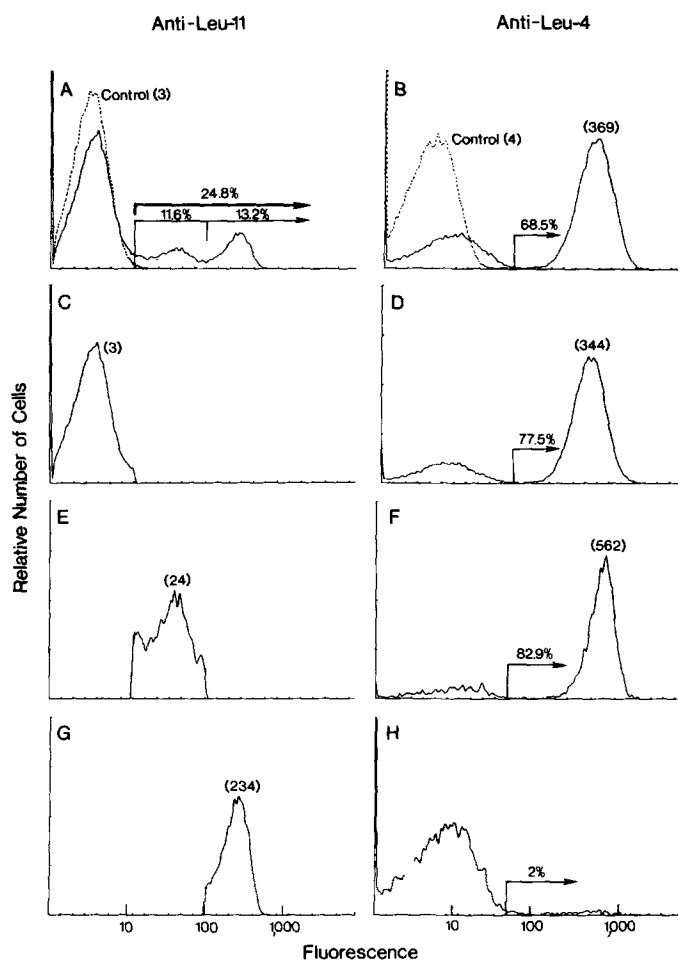
Anti-Leu-11   Anti-Leu-4

FIGURE 3.   Quantitation of CD3 and CD16 antigen expression. Peripheral blood mononu-
clear cells (L. G.) were stained with: FITC–IgG1 and PE–IgG1 control antibodies, or FITC–
anti-Leu-11a and PE–anti-Leu-4 antibodies. Data were collected in list-mode files and displayed
as histograms (abscissa, 256 channels, four-decade log scale; ordinate, relative number of cells).
In A and B, the fluorescence from the stained sample (solid line) is superimposed over the
fluorescence of the control sample (broken line). The percentage of stained cells and relative
amount of fluorescence (indicated in the parenthesis as arbitary linear fluorescence units) are
shown. Note that in A there are three distinct populations of cells in the anti-Leu-11a–stained
sample: unstained, dim CD16$^+$ (11.6%), and bright CD16$^+$ (13.2%) cells. In C, E, and G, a
gate was placed on each of these subpopulations and the anti-Leu-4 staining profile was
displayed for the selected population. The ordinate was rescaled in each panel to permit
optimal visualization of the results.

displayed for each population. Within the dim CD16$^+$ cells, 82.9% expressed the
CD3 antigen. In contrast, only 2% of the bright CD16$^+$ cells expressed CD3.
Quantitative comparison of the populations indicated that the dim CD16$^+$ cells
expressed ~10% of the CD16 antigen compared to the bright CD16$^+$ cells. The
dim CD16$^+$ cells were also unique in that they showed about twice as much CD3
antigen as the typical CD3$^+$,CD16$^-$ T lymphocytes (Fig. 3). Quantitative studies

with D. H.'s lymphocytes were essentially identical to those presented for L. G.'s lymphocytes (not shown).

PBL obtained from L. G. and D. H. were analyzed by two-color immunofluorescence using PE-conjugated anti-Leu-11c/B73.1 and a panel of FITC-conjugated antibodies against other T cell– and NK cell–associated differentiation antigens. The results using L. G.'s lymphocytes are presented in Fig. 4. As shown in Figs. 1–3, two distinct populations of CD16$^+$ (Leu-11c$^+$) lymphocytes could be identified based on the amount of CD16 antigen present on the cell surface. The majority of dim CD16$^+$ cells coexpressed the pan–T cell–associated antigens CD3 (Leu-4) and CD5 (Leu-1). In contrast, the bright CD16$^+$ cells lacked these pan–T cell antigens. However, as shown in the lower left panel, CD8 (Leu-2) antigen was apparently expressed in low amounts on a small proportion of both the dim and bright CD16$^+$ lymphocytes. Expression of low amounts of CD8 antigen on a minor proportion of CD16$^+$ lymphocytes has been reported previously (8). CD4 (Leu-3) was not coexpressed on CD16$^+$ lymphocytes (Fig. 4), indicating that most CD16$^+$ lymphocytes (both dim and bright subsets) lack expression of either the CD4 or CD8 antigen. Furthermore, HLA-DR, CD19 (Leu-12), IL-2-R, and Leu-M3 also were not coexpressed on CD16$^+$ lymphocytes (not shown). A subset of CD16$^+$ cells coexpressed the Leu-7 antigen (Fig. 4), and a majority of CD16$^+$ lymphocytes (both dim and bright CD16$^+$) were found to coexpress the CR$_3$ (Leu-15) and CD2 (Leu-5) antigens (not shown). Qualitatively,
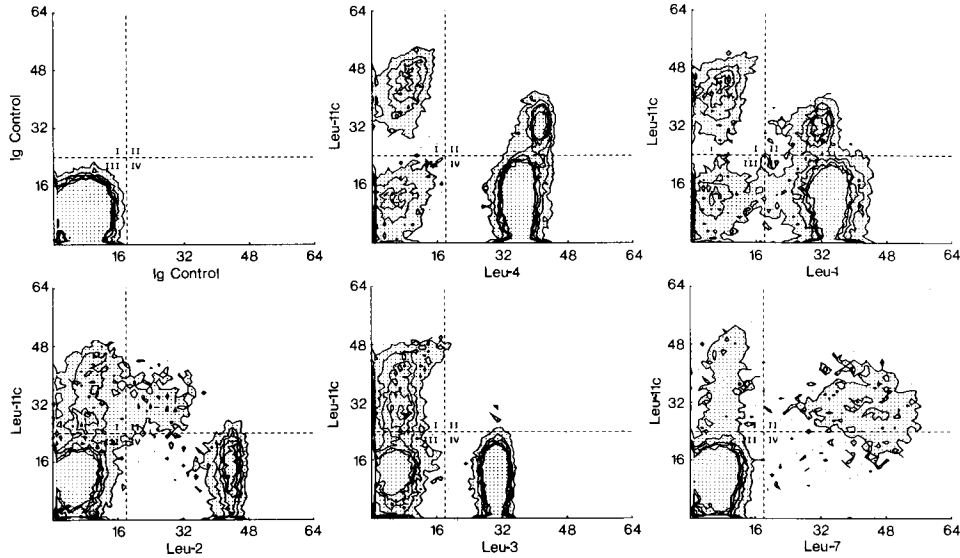

FIGURE 4.   Coexpression of T cell–associated antigens on CD16$^+$ lymphocytes. Peripheral blood mononuclear cells (L. G.) were stained with: FITC–IgG and PE–IgG control antibodies; FITC–anti-Leu-4 and PE–anti-Leu-11c/B73.1; FITC–anti-Leu-1 and PE–anti-Leu-11c/B73.1; FITC–anti-Leu-2a and PE–anti-Leu-11c/B73.1; FITC–anti-Leu-3a and PE–anti-Leu-11c/B73.1; and FITC–anti-Leu-7 and PE–anti-Leu-11c/B73.1. Data are presented as described in Fig. 1. Quantitation of the data revealed: 10.6% CD3$^+$ (Leu-4$^+$), CD16$^+$; 11.1% CD5$^+$ (Leu-1$^+$), CD16$^+$; 6.3% CD8$^+$ (Leu-2$^+$), CD16$^+$; <1% CD4$^+$ (Leu-3$^+$), CD16$^+$; and 13.3% Leu-7$^+$,CD16$^+$ lymphocytes. >98% of lymphocytes were in quadrant III (unstained) in the control sample.

essentially identical profiles were observed when D. H.'s lymphocytes were examined using this panel of reagents (not shown).

PBL from 46 normal, random blood donors were examined for coexpression of the CD3 and CD16 surface antigens. Most individuals examined (40/46) had low (<2%) to undetectable levels of circulating $CD3^+,CD16^+$ cells. Six individuals expressed >2% $CD3^+,CD16^+$ lymphocytes. In three of these six individuals, the $CD3^+,CD16^+$ cells comprised 2–5% of lymphocytes. Only lymphocytes from three individuals, including D. H. and L. G., contained >5% $CD3^+,CD16^+$ cells. In all cases observed, the amount of CD16 antigen expressed on the $CD3^+$ T cell subset was significantly less than on the $CD3^-$ NK cell population.

L. G.'s parents were analyzed and found to have <1% $CD3^+,CD16^+$ lymphocytes. The families of two other individuals (caucasians of european ancestry) who demonstrated significantly higher proportions of these cells were also examined (Table I). Most family members had <1% $CD3^+,CD16^+$ lymphocytes (pedigree charts given in Fig. 5).

Cytolytic Activity of $CD3^+,CD16^+$ and $CD3^-,CD16^+$ Lymphocytes. Lymphocytes from D. H. were stained with FITC–anti-Leu-11a and PE–anti-Leu-4 (CD3). The $CD3^-,CD16^+$ and $CD3^+,CD16^+$ cells were isolated by two-color cell sorting. Reanalysis of the purified subpopulations indicated >95% purity. As shown in Fig. 6, $CD3^+,CD16^+$ cells were mononuclear lymphocytes with distinct azurophilic granules. The cells were tested for cytotoxic activity against the NK-sensitive tumor cell line K562 immediately after isolation and after 1 wk of culture in IL-2 (Fig. 7). The $CD3^-,CD16^+$ NK cells mediated cytolysis of K562, which was augmented by IL-2 treatment. Additionally, IL-2 activation enabled

TABLE I

Coexpression of CD3 and CD16: Normal Donors and Family Studies

Individuals tested	Phenotype (percent of lymphocytes)		
	$CD3^-,CD16^+$	$CD3^+,CD16^+$	$CD3^+,CD16^-$
Family 1			
1	8	7	77
2	7	<1	84
3	5	<1	86
4	3	3	86
5	5	<1	86
Family 2			
1	7	3	77
2	6	1	81
3	2	4	82
4	4	3	86
5	4	1	83
6	5	<1	83
7	3	1	82
8	7	1	81

Peripheral blood mononuclear cells were stained with FITC anti-Leu-11a and Texas Red–avidin/biotin anti-Leu-4, or appropriate fluorochrome-conjugated Ig controls. Based on the control sample, the percentage of lymphocytes with the indicated antigenic phenotype was determined.
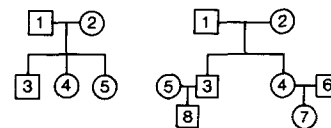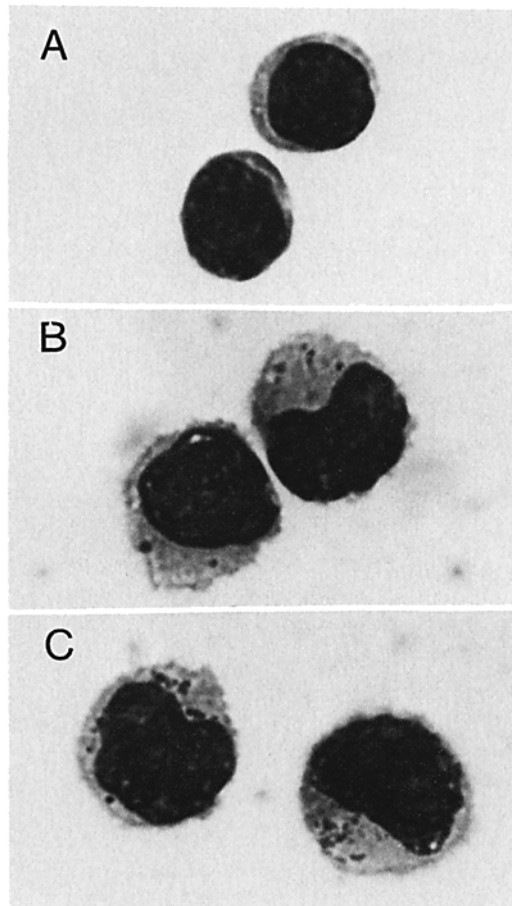
FIGURE 5. Pedigree charts of families studied in Table I.

FIGURE 6.   Morphology. $CD3^+,CD16^-$ T cells (A); $CD3^+,CD16^+$ T cells (B); and $CD3^-,CD16^+$ NK cells (C), isolated as described in Fig. 7, were cytocentrifuged onto glass slides and stained with Giemsa. Magnification, × 5,500.

the $CD3^-,CD16^+$ cells to lyse the relatively NK-insensitive CCRF-SB cell line (Fig. 7). The $CD3^+,CD16^+$ cells showed little cytolytic activity, either before or after IL-2 treatment (Fig. 7). Comparison of the cytotoxicity of unstained cells and cells stained with anti-Leu-4 or anti-Leu-11 indicated that these antibodies did not inhibit activity. K562 lysis was slightly increased in the anti-Leu-4–stained cells compared to unstained cells (not shown).

We were unable to directly access the ADCC activity of the freshly isolated $CD3^+,CD16^+$ and $CD3^-,CD16^+$ cells, since the anti-Leu-11 antibody efficiently inhibits ADCC function (12, 13). However, to determine whether ADCC activity was present in D. H.'s T cell population, nonadherent peripheral blood mononuclear cells were stained with FITC–anti-Leu-1 (CD5) antibody, and the cells were separated into $CD5^-$ and $CD5^+$ subpopulations using a fluorescence-activated cell sorter (FACS). As shown in Fig. 8, significant ADCC activity was detected in both the $CD5^+$ and $CD5^-$ populations. Furthermore, addition of anti-Leu-11 antibody to the assay completely inhibited the ADCC mediated by
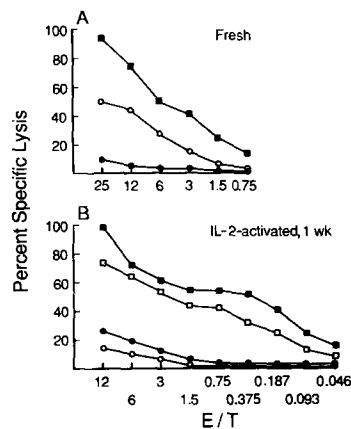
FIGURE 7. NK cell activity of CD3⁺,CD16⁺ and CD3⁻,CD16⁺ lymphocytes. Nonadherent PBL (D. H.), depleted of B cells using a nylon wool column, were stained with FITC–anti-Leu-11a and PE–anti-Leu-4. The CD3⁺,CD16⁺ and CD3⁻,CD16⁺ cells were separated using the FACS. Reanalysis of the separated subpopulations indicated >95% purity. Morphological examination revealed that >99% of the sorted cells were lymphocytes. The sorted subpopulations and stained, unsorted lymphocytes were tested for cytotoxic activity against ⁵¹Cr-labelled K562 tumor cells in a 4-h radioisotope-release assay (A). The CD3⁺,CD16⁺ and CD3⁻,CD16⁺ cells were placed in culture medium containing purified IL-2, and again tested for cytotoxic activity against K562 and CCRF-SB tumor cells after 1 wk (B). Analysis of the antigenic phenotypes after 1 wk in culture indicated that the CD3⁻,CD16⁺ and CD3⁺,CD16⁺ populations contained ~98% CD3⁻,CD16⁺ and CD3⁺,CD16⁺ cells, respectively. 2% of the cells in the CD3⁺,CD16⁺ culture were CD3⁻,CD16⁺ cells. By extrapolation of the effector/target (E/T) curves, this small contamination of activated CD3⁻,CD16⁺ NK cells may account for the low levels of cytotoxicity observed against K562 in the CD3⁺,CD16⁺ population.

both the CD5⁻ and CD5⁺ subsets. In contrast, antibody against CR₃ (Leu-15), an antigen present on both the CD3⁺,CD16⁻ and CD3⁺,CD16⁺ cells, failed to affect ADCC activity. By direct two-color immunofluorescence analysis, ~21.4% of the CD5⁺ T lymphocytes coexpressed CD16, whereas 63.4% of the CD5⁻ lymphocytes were CD16⁺ NK cells. These data suggest that a CD16-associated FcR was involved in the ADCC mediated by both populations.

Proliferation of CD3⁺,CD16⁺ Lymphocytes Cocultured with Allogeneic Tumor Cell Lines. The CD3⁺,CD16⁺ and CD3⁻,CD16⁺ cells were cocultured with irradiated NK-sensitive K562 tumor cells, irradiated NK-insensitive CCRF-SB B lymphoblastoid cells, or purified IL-2 for 6 d. As shown in Table II, CD3⁻,CD16⁺ cells proliferated vigorously in response to K562 and IL-2, but less well in response to CCRF-SB. Consistent with our previous findings (32), the response of the CD3⁻,CD16⁺ NK cells was only partially inhibited (~20%) by the presence of anti-IL-2-R (TAC) antibody in the culture. In contrast, the CD3⁺,CD16⁺ population was strongly stimulated by both K562 and CCRF-SB. Furthermore, anti-IL-2R antibodies inhibited the response against CCRF-SB by 75%, compared to only 25% against K562. The proliferative response by the CD3⁺,CD16⁺ cells cultured in IL-2 similarly was greatly inhibited (89%) by the presence of anti-IL-2-R antibody.

Culture of CD3⁺,CD16⁺ Lymphocytes. CD3⁺,CD16⁺ and CD3⁻,CD16⁺ lymphocytes were placed into culture and maintained as IL-2-dependent cell lines. The CD3⁺,CD16⁺ cells had a 24–48-h generation time, whereas the CD3⁻,CD16⁺
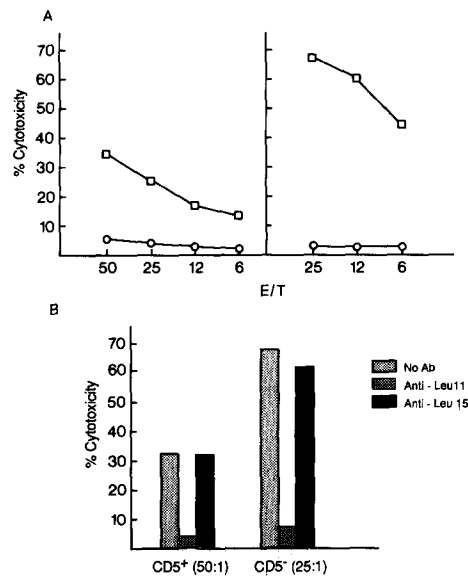
FIGURE 8.   ADCC activity of CD5⁻ and CD5⁺ lymphocytes and inhibition by anti-Leu-11 antibody. Nonadherent peripheral blood mononuclear cells from D. H. were stained with FITC–anti-Leu-1 (CD5) and separated into CD5⁺ and CD5⁻ subsets using a FACS. Reanalysis indicated 98% purity. A: CD5⁻ and CD5⁺ lymphocytes were tested for ADCC activity against ⁵¹Cr-labelled JY tumor cells preincubated with an IgG2a anti-HLA mAb, ME1. No cytotoxicity was observed against JY cells not preincubated with antibody. B: Anti-Leu-11 or anti-Leu-15 mAb were added to the ADCC assay at 5 μg/ml. JY target cells do not express the CD16 (Leu-11) or CR₃ (Leu-15) antigens.

cells grew more slowly. The growth of these cells was dependent on the presence of IL-2. After 3 wk in culture, these cells were assayed for ADCC activity and NK cell–mediated cytotoxicity against the JY lymphoblastoid B cell line. IL-2 cultured CD3⁻,CD16⁺ NK cells demonstrated high levels of cytotoxicity against JY in the absence of antibody (Fig. 9). This is consistent with prior observations (32) that CD16⁺ NK cells can lyse both NK-sensitive and NK-insensitive targets after activation with IL-2. The high background of NK activity against JY in the absence of antibody (78% specific lysis at an effector/target ratio of 4:1) obscured any ADCC activity. In contrast, CD3⁺,CD16⁺ cells mediated only low levels of JY cytotoxicity in the absence of antibody. Using an IgG2a switch-variant of the anti-HLA mAb MA2.1, we observed significant ADCC activity. The parent IgG1 MA2.1 antibody, however, failed to cause any increase in JY cytolysis. The IgG2b switch-variant of MA2.1 directed only a modest increase in cytotoxicity.

Phenotypic analysis of the CD3⁺,CD16⁺ cells after 3 wk in culture indicated that ~95% retained the CD3 and CD16 antigens. The amount of CD16 and CD3 antigen on these cells after short-term culture in IL-2 was approximately the same as present on the freshly isolated lymphocytes. Furthermore, the cells expressed IL-2-R (40%), HLA-DR antigens (95%), CD2 (83%), and CD5 (95%). As with the freshly isolated cells (Fig. 3), the majority of the cultured cells (~80%) expressed neither the CD4 nor CD8 antigens. That CD3⁺,CD16⁺ cells expressed

TABLE II

Alloantigen and IL-2 Stimulation of CD3⁺,CD16⁺ Lymphocytes

Responder	Stimulator	Antibody	[³H]Thymidine incorporation (cpm ± SD)	SI	Percent inhibition with antibody
Control PBL	None	None	6,790 ± 230	—	—
	IL-2	Control	81,640 ± 310	14.1	—
	IL-2	Anti–IL-2-R	35,898 ± 620	6.2	56
	K562	Control	136,644 ± 740	23.6	—
	K563	Anti–IL-2-R	105,378 ± 1,120	18.2	22
	CCRF-SB	Control	160,962 ± 1,260	27.8	—
	CCRF-SB	Anti–IL-2-R	55,005 ± 891	9.5	65
D. H. PBL	None	None	7,600 ± 820	—	—
	IL-2	Control	89,680 ± 360	11.5	—
	IL-2	Anti–IL-2-R	45,600 ± 420	6.0	48
	K562	Control	123,880 ± 1,160	16.3	—
	K562	Anti–IL-2-R	75,520 ± 1,000	10.2	37
	CCRF-SB	Control	209,000 ± 1,421	27.5	—
	CCRF-SB	Anti–IL-2-R	99,560 ± 680	13.1	52
CD3⁻,CD16⁺ (D. H.)	None	None	230 ± 160	—	—
	IL-2	Control	8,809 ± 165	38.3	—
	IL-2	Anti–IL-2-R	6,785 ± 220	29.5	22
	K562	Control	21,919 ± 631	95.3	—
	K562	Anti–IL-2-R	17,296 ± 412	75.2	21
	CCRF-SB	Control	1,886 ± 206	8.2	—
	CCRF-SB	Anti–IL-2-R	1,081 ± 151	4.7	42
CD3⁺,CD16⁺ (D. H.)	None	None	294 ± 110	—	—
	IL-2	Control	7,908 ± 375	26.9	—
	IL-2	Anti–IL-2-R	823 ± 120	2.8	89
	K562	Control	28,224 ± 410	96.0	—
	K562	Anti–IL-2-R	21,109 ± 593	71.8	25
	CCRF-SB	Control	11,789 ± 295	40.1	—
	CCRF-SB	Anti–IL-2-R	2,881 ± 173	9.8	75

CD3⁺,CD16⁺ and CD3⁻,CD16⁺ lymphocytes from D. H. were isolated as described in Fig. 7. CD3⁺,CD16⁺; CD3⁻,CD16⁺; and unseparated PBL from D. H. and a random, normal donor were cocultured with purified IL-2 (10% vol/vol) or with mitomycin C–treated K562 or CCRF-SB cells at a responder/stimulator ratio of 1:1. Anti–IL-2-R or an isotype-matched control Ig were added to the indicated cultures at a final concentration of 100 μg/ml. After 5 d, wells were pulsed with [³H]-thymidine and harvested. The mean cpm ± SD of triplicate wells are presented. The stimulation index (SI) was calculated as: (mean cpm stimulated cultures)/(mean cpm unstimulated culture).

functional FcR was confirmed by their ability to specifically bind dimeric rabbit IgG. This binding was completely inhibited by anti-Leu-11 mAb.

Anti-CD3 Directs Non–antigen-specific Cytotoxicity by CD3⁺,CD16⁺ Cells. Recently (33, 34) it has been reported that antigen-specific, major histocompatibility complex (MHC)-restricted cytotoxic CD3⁺ T cell clones may kill inappropriate target cells in the presence of anti-CD3 antibodies. As shown in Fig. 10, cultured CD3⁺,CD16⁺ cells kill the relatively NK-insensitive JY and LCL 207 B lymphoblastoid cell lines in the presence of anti-Leu-4 (CD3). Because the targets
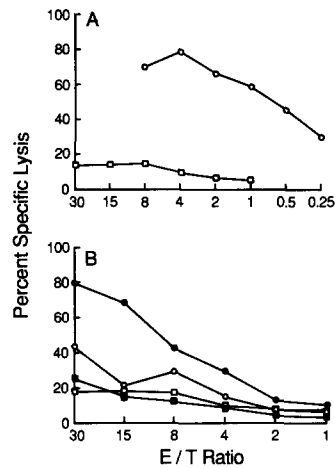
FIGURE 9.   ADCC activity of $CD3^+$,$CD16^+$ lymphocytes. After 3 wk culture in IL-2-containing medium, $CD3^-$,$CD16^+$ and $CD3^+$,$CD16^+$ lymphocytes were tested for NK cell–mediated cytotoxicity against the JY B lymphoblastoid cell line in a 4-h radioisotope-release assay (A). In B, the ADCC activity of the $CD3^+$,$CD16^+$ cells was tested in a 4-h radioisotope-release assay using ^{51}Cr-labelled JY cells preincubated with isotype switch-variants of the MA2.1 (anti-HLA-A2–specific) mAb.

do not express CD3, this cytotoxicity was induced by the interaction of anti-CD3 with the effector cells. As demonstrated elsewhere[2] (35), the cytolytic activity of $CD3^-$,$CD16^+$ NK cells was unaffected by anti-CD3 mAb.

The role of class I MHC molecules in the anti-CD3-induced cytotoxicity was examined using Daudi as a target cell line. Although Daudi does not express class I MHC molecules, these B lymphoblastoid cells were lysed efficiently by $CD3^+$,$CD16^+$ cells, but only in the presence of anti-Leu-4 (CD3) antibody (Fig. 10). Unlike anti-Leu-4, the addition of anti-Leu-11 antibody to the cultures failed to induce cytotoxicity against Daudi (Fig. 10).

Discussion

In most individuals, cells with the phenotype $CD3^+$,$CD16^+$ comprise <2% of total PBL (8–10, 12, 30, 31). However, we have recently identified several healthy individuals who possess significant levels of these cells in their peripheral blood. In this study, we have examined the antigenic, morphological, and functional properties of this unique subset of T lymphocytes.

FcR for IgG are expressed on two distinct populations of human lymphocytes. The majority of these FcR^+ lymphocytes lack the CD3 antigen, and mediated NK cell–mediated cytotoxicity (5–7). Expression of IgG FcR on a very minor subpopulation of $CD3^+$ T lymphocytes has been previously identified (5–7, 23) using either EA rosette procedures or dimeric rabbit IgG complexes. However, due to the low levels of these cells in most individuals, and the technical difficulties involved in efficiently separating the $CD3^+$,FcR^+ and $CD3^-$,FcR^+ subsets, there

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[2] Phillips, J. H., and L. L. Lanier. 1985. Lectin-dependent and anti-CD3-induced cytotoxicity are mediated by peripheral blood cytotoxic T lymphocytes expressing the Leu-7 antigen. Manuscript submitted for publication.
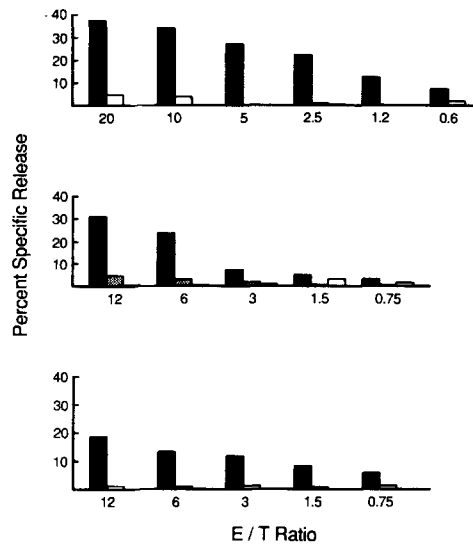
FIGURE 10. Anti-CD3-induced cytotoxicity mediated by $CD3^+,CD16^+$ lymphocytes. After 3 wk in culture in IL-2-containing medium, $CD3^+,CD16^+$ lymphocytes were tested for cytotoxic activity against the JY, Daudi, and LCL 207 B cell lines in the presence or absence of anti-Leu-4 mAb (4 μg/ml) or anti-Leu-11 mAb (4 μg/ml; Daudi experiment).

have been few functional studies comparing these populations. Furthermore, it has been unclear whether or not FcR present on $CD3^+$ T lymphocytes would be recognized by anti-Leu-11 and similar antibodies that react with the FcR present on $CD3^-$ NK cells and granulocytes. In our experiments, we have shown that, in certain individuals, a subset of T cells can express the FcR recognized by the anti-Leu-11 antibody, and that these $CD3^+,CD16^+$ T cells can mediate ADCC via this FcR. Consistent with these cells being within the T cell lineage, we have shown that essentially all of the $CD3^+,CD16^+$ lymphocytes coexpress CD2 (E rosette receptor) and CD5 (Leu-1) antigens. Furthermore, anti-Leu-4 antibody immunoprecipitates a 22-29 kD protein complex from the cell surface of these $CD3^+,CD16^+$ lymphocytes, similar to the CD3 antigen isolated from $CD3^+,CD16^-$ T lymphocytes (our unpublished observation). It is unresolved whether the $CD3^+,CD16^+$ cells represent a monoclonal or polyclonal population of T lymphocytes. Since it is possible to culture these cells in IL-2, we may be able to examine this question in future studies.

These $CD3^+,CD16^+$ lymphocytes are different than typical T cells in several aspects. Unlike resting T lymphocytes, $CD3^+,CD16^+$ have numerous azurophilic granules, similar to NK cells and in vitro–activated cytotoxic T lymphocytes. Furthermore, unlike most T lymphocytes $CD3^+,CD16^+$ lymphocytes lack detectable surface expression of either CD4 or CD8. Since these antigens are implicated (36-38) in cell-cell interactions involving class II and class I antigens, respectively, failure to express CD4 and CD8 suggests that $CD3^+,CD16^+$ cell may not require MHC gene products for target cell recognition. $CD3^+,CD16^+$ cells were also distinct in that they expressed approximately twice the level of CD3 as $CD16^-$ T lymphocytes, and expressed lower levels of CD5 than most other T cells.

Furthermore, this subset expressed approximately one-tenth the level of CD16 found on the CD3$^-$,CD16$^+$ NK cells. This is corroborated by the low level of binding observed when these cells were stained with FITC-labeled dimeric rabbit IgG. Due to the low levels of FcR expression, the frequency of this cell population may have been underestimated in many studies. Unless immunofluorescent reagents and immunofluorescence detection systems are optimized, this population may be undetectable.

This CD3$^+$,CD16$^+$ population lacked NK activity. However, these cells could be rendered cytotoxic by two mechanisms. The CD3$^+$,CD16$^+$ cells efficiently mediated ADCC activity against antibody-coated targets. These cells may mediate a similar function in vivo, thereby providing a cytotoxic T lymphocyte with the capacity to recognize and lyse an antibody-coated target through a structure distinct from the T cell antigen receptor. Although there have been reports (15, 16) that T cells isolated from patients with certain lymphoproliferative disorders can mediate ADCC, but not NK activity, the isolation of this T cell subset from the peripheral blood of a normal, healthy individual has not been reported. As previously demonstrated for CD3$^-$,CD16$^+$ NK cells (13), the CD3$^+$,CD16$^+$ T cells preferentially mediate ADCC with IgG2a antibodies. IgG2b antibodies were less efficient, whereas IgG1 antibodies demonstrated no significant cytotoxicity. These data suggest that the FcR on these CD3$^+$ T cells is functionally and antigenically quite similar to the FcR present on CD3$^-$ NK cells. However, further comparative studies on the biochemical structure of these receptors will be required to address this issue.

CD3$^+$,CD16$^+$ lymphocytes were also rendered cytotoxic against several NK-resistant B lymphoblastoid cell lines when anti-Leu-4 (CD3) antibody was present in the assay. Recently, several investigators (33, 34) have demonstrated that antigen specific cytotoxic T cells can nonspecifically lyse tumor cell targets in the presence of anti-CD3 mAb. Since the CD3 antigen is structurally associated with the T cell antigen receptor heterodimer (39–43), anti-CD3 antibodies may induce cytotoxic activity in cytotoxic T lymphocytes by antigenic mimicry. Although the antigenic specificity of the CD3$^+$,CD16$^+$ population is unknown, we show here that anti-CD3 can induce significant cytotoxic activity. The anti-CD3-induced cytotoxicity does not require recognition of class I MHC antigens on the target, since the CD3$^+$,CD16$^+$ cells lysed Daudi efficiently.

Since CD3$^+$,CD16$^+$ can mediate both ADCC and nonspecific cytotoxicity in the presence of anti-CD3 antibodies, it is possible that the two mechanisms may be related. Binding to the FcR may perturb the CD3–T cell antigen receptor complex, thus stimulating cytotoxic activity. Contrary to this hypothesis, however, is our observation that soluble anti-Leu-11 antibody did not induce cytotoxicity in the CD3$^+$,CD16$^+$ population. Whether anti-Leu-11 complexed to an insoluble matrix may activate these cells remains to be determined.

The proliferative response of the CD3$^+$,CD16$^+$ population shares certain features with both typical T lymphocytes and NK cells. We have shown that NK cells (CD3$^-$,CD16$^+$) proliferate vigorously when cocultured with NK-sensitive K562 tumor cells, and that they proliferate poorly when cultured with a B lymphoblastoid cell line, CCRF-SB (30). In contrast, T lymphocytes (CD3$^+$,CD16$^-$) are stimulated preferentially by CCRF-SB. Furthermore, the two

cell types were functionally distinguished in that the T cell response, but not the NK cell response, could be significantly inhibited by anti-IL-2-R antibody (30). In this study, the $CD3^+,CD16^+$ population was stimulated by coculture with either K562 or CCRF-SB. The response against CCRF-SB was much more sensitive to inhibition by anti-IL-2-R antibody than the response against K562. It is unlikely that the strong response against K562 was due to contamination by NK cells, since reanalysis of the isolated population showed <1% $CD3^-,CD16^+$ cells. It is possible that the $CD3^+,CD16^+$ population is heterogeneous, with some cells responding to K562 and others to CCRF-SB. Alternatively, each cell may possess two distinct mechanisms for activation, one of which is resistant to inhibition by anti-IL-2-R antibody.

The origin and relationship of $CD3^+,CD16^+$ T cells to FcR^- T cells are not known. In previous studies we did not observe induction of CD16 antigen expression on T lymphocytes upon activation with mitogens or alloantigens. Furthermore, after 3 wk of culture in IL-2, $CD3^+,CD16^+$ cells retained expression of both the CD3 and CD16 antigens. Stable surface expression of CD16 on these $CD3^+,CD16^+$ cytotoxic T cells in both D. H. and L. G.'s peripheral blood was found over an observation period of 18 and 6 mo, respectively. This indicates that these cells are not present as a consequence of acute infection. Furthermore, both L. G. (female) and D. H. (male) are healthy individuals (25–35 y old) with no known illness. It may be noteworthy that these individuals are both oriental, since Prince et al. (44) reported the level of $CD16^+$ lymphocytes to be significantly elevated in certain Chinese individuals. However, a limited study of other Chinese individuals did not reveal many to have high levels of circulating $CD3^+,CD16^+$ lymphocytes (L. Lanier and H. Prince, unpublished observation). Family studies described here demonstrate relatively high proportions of circulating $CD3^+,CD16^+$ cells in persons of other ethnic backgrounds. The distribution of $CD3^+,CD16^+$ cells within these families suggests genetic mechanisms may operate to influence the proportion of these cells in peripheral blood. Further studies are required to test this hypothesis.

Summary

A subset of peripheral blood T lymphocytes coexpressing CD3 and IgG Fc receptors (FcR) (CD16/Leu-11 antigen) have been identified, isolated, and functionally characterized. The $CD3^+,CD16^+$ cells were established in short-term culture using growth medium containing interleukin 2 (IL-2). Both the freshly isolated cells and the cultured cell line stably expressed the $CD3^+,CD16^+$ phenotype. Furthermore, a majority of these T cells lacked either CD4 or CD8 expression. Like in vitro–activated cytotoxic T lymphocytes and natural killer (NK) cells, the $CD3^+,CD16^+$ cells showed numerous azurophilic granules. Although these cells failed to mediate significant levels of NK cell–mediated cytotoxicity even after stimulation with IL-2, they efficiently functioned as effectors of antibody-dependent cellular cytotoxicity (ADCC). The Ig isotype specificity of the ADCC was analyzed using an isotype switch-variant family of a murine anti-HLA monoclonal antibody (mAb). Similar to the $CD3^-,CD16^+$ NK cell population, the $CD3^+,CD16^+$ T cells preferentially used the IgG2a antibody to mediate ADCC. The $CD3^+,CD16^+$ cells demonstrated a proliferative response

when cocultured with either a NK-sensitive tumor cell line, K562, or a NK-insensitive B lymphoblastoid cell line, CCRF-SB. The response against CCRF-SB was significantly inhibited by anti–IL-2 receptor antibody, whereas the response against K562 was only partially diminished. Cytotoxicity was also induced in the $CD3^+, CD16^+$ population by the presence of anti-CD3 mAb, indicating that cytotoxicity can be triggered by stimulation via the CD3–T cell antigen receptor complex. By isolating these $CD3^+, CD16^+$ cells from the peripheral blood of a normal, healthy individual, it has been possible to extensively study the morphology, antigenic phenotype, and functional behavior of this unique subset of T lymphocytes expressing IgG FcR.

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