Phenotypically and Functionally Distinct Subpopulations of Human Lymphocytes With T Cell Markers Also Exhibit Different Cytochemical Patterns of Staining for Lysosomal Enzymes

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Human peripheral blood lymphocytes that express T cell markers, when reacted for the cytochemical localization of lysosomal acid hydrolases, display two major patterns of staining, i.e., dot-like and scattered granular. Previous attempts to fractionate T cells according to surface markers have yielded populations of cells with heterogeneous patterns of cytochemical staining. In this study, peripheral blood cells forming rosettes with sheep erythrocytes have been fractionated by sequential staining with two monoclonal antibodies, D12 and 2D2, followed by fluorescence-activated cell sorting. These reagents have been shown previously to recognize a subpopulation of cells capable of suppressing T cell proliferation. All of the cells positive for D12 and 2D2 stained for acid hydrolases with the scattered granular pattern, whereas the large majority of the cells negative for both markers stained with the dot-like pattern. It is concluded that suppressor cells within the E⁺ cell fraction have the cytochemical characteristics of large granular lymphocytes.

In this study, E⁺ cells, isolated by rosetting human peripheral blood mononuclear cells with sheep erythrocytes, have been further fractionated by fluorescence-activated cell sorting, according to the presence or absence of surface antigens detected by two new monoclonal antibodies, D12 and 2D2. These antibodies have been shown previously to define a subset of OKT8/Leu-2⁺ cells that suppresses T cell proliferative responses. In this report, we show that all of these suppressor cells expressing both the D12 and 2D2 markers stain for AP and ANAE with the scattered granular pattern, whereas the vast majority of cells lacking both markers exhibit the dot-like type of cytochemical staining.

MATERIALS AND METHODS

Preparation of T Cells

Mononuclear cell suspensions separated from heparinized blood samples of adult healthy volunteers by Ficoll-Hypaque density gradient centrifugation were depleted of adherent cells by incubation on plastic petri dishes for 1 hr at 37°C. E⁺ cells were isolated from the nonadherent cell fraction by E rosette formation with sheep erythrocytes treated with 2-aminoethylisothiouronium bromide (AET) and density gradient centrifugation. Lymphocytes prepared with the above procedure were >95% pure as determined by

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immunofluorescent staining with an anti-pan-T cell monoclonal antibody (OKT3, Ortho Pharmaceuticals, Raritan, NJ).

Isolation of T Cell Subsets

For the further fractionation of the E rosetting cells into subsets, two monoclonal antibodies, D12 and 2D2, were used in positive selection experiments, employing a fluorescence activated cell sorter (FACS IV, Becton Dickinson, Mountain View, CA). The D12 reagent is a $\gamma_2a,k$ that recognizes an antigen expressed by cells of the granulocytic-monocytic lineage as well as a proportion of E rosetting cells ranging from 8% to 36%. This pattern of reactivity closely resembles that of the OKM1 monoclonal antibody, although cross-blocking studies indicate that the D12 and OKM1 do not recognize the same antigenic determinant.

The E' cell subpopulations recognized by the D12 and 2D2 monoclonal antibodies were isolated sequentially with the FACS. E rosetting cells stained with D12 and affinity-puriﬁed, FITC-conjugated, goat anti-mouse $\gamma_2a$ antibodies were fractionated into D12' and D12' populations with the cell sorter. The D12' fraction was then stained with 2D2 and FITC-conjugated goat anti-mouse $\gamma_1$ antibodies, and separated into D12' -2D2' and D12' -2D2' fractions. The fractionation of the D12' cells with the 2D2 antibody was then accomplished in similar fashion. The ability to separate subpopulations of these ﬂuorochrome-labeled D12' cells was possible because the ﬂuorescent staining intensities of 2D2 and D12 are dramatically different. Hence, D12' cells (which are dimly stained) were then labeled with a known excess of 2D2 and FITC-conjugated goat anti-mouse $\gamma_1$ antibodies. The additive ﬂuorescent staining intensity achieved by the brightly staining 2D2 antibody allowed clear deﬁnition of two subpopulations of D12' cells. These D12'-2D2' (bright) and D12'-2D2' (dim) subpopulations were then puriﬁed by cell sorting. Hence, four phenotypically distinct E' cell subpopulations were isolated by these techniques for subsequent study.

Cytochemical Staining for AP and ANAE

In five different experiments, unfractionated E rosetting cells, as well as the four E' subpopulations obtained as detailed above, were ﬁxed in suspension with 1.25% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.6, for 30 min at room temperature and washed with phosphate-buffered saline. For cytochemical staining, ﬁxed cells were cytocentrifuged onto slides and incubated with the substrates for 1 hr at 37°C in the dark. The substrate for the localization of ANAE contained alpha-naphthyl acetate (Sigma Chemical Co., St. Louis, MO) and hexazonitized pararosanilin (Sigma) in phosphate buffer, pH 5.8. The substrate for AP staining was naphthol-AS-BI phosphoric acid (Sigma) and Fast Garnet GBC salt (Sigma) in acetate buffer, pH 5.2. Following incubation, slides were washed in deionized water and mounted with Fluoromount G (Southern Biotechnology Associates, Birmingham, AL).

At least 200 cells were counted in each preparation and recorded as being positive with the dot-like pattern, positive with the scattered granular pattern, or negative (data are reported as mean percentages ± SD).

RESULTS

In Figs. 1 and 2, the proportion of cytochemically identifiable cell types and the patterns of staining for all of the E' cell preparations examined are summarized.

Virtually all cells forming SE rosettes stained for acid phosphatase. The majority of the cells displayed a dot-like pattern of staining (73.6% ± 1.5%), while in the remaining cells, the reaction product was found in scattered granules (Fig. 2A). When stained for ANAE, the percentage of cells with the dot-like pattern closely corresponded with that determined for AP staining (78.7% ± 4.9%). However, less than half of the residual cells (9.0% ± 2.0% of the total) stained with the scattered granular pattern, with the majority of these cells (12.3% ± 6.6%) being ANAE-negative (Fig. 2D).

As shown in Fig. 1, virtually all of the cells staining with the dot-like pattern were found in the D12 fractions. However, when D12' cells were fractionated further, according to the expression of the 2D2 marker, differences were observed in the two subsets so obtained. Thus, D12' -2D2' fractions were highly enriched for cells staining with both AP and ANAE with the dot-like pattern (86.2% ± 8.0% and 90.4% ± 9.8%, respectively) (Fig. 2, B and E), whereas D12' -2D2' cells showed a wide range of variation, with a predominance of either the dot-like or the scattered granular cytochemical pattern in the different donors. Furthermore, the large majority of the cells staining with the scattered granular pattern was positive for AP but negative for ANAE.

As expected from the above results, cells staining with the scattered granular pattern were greatly
Fig. 2. Cytochemical staining for acid phosphatase (A, B, C) or alpha naphthyl acetate esterase (D, E, F). (A and D) Cells forming E rosettes show different patterns of staining. The majority of the cells stain with the dot-like pattern; cells displaying the scattered granular pattern (---) or those that are negative for esterase (----) are also present. (B and E) Cells lacking the D12 and the 2D2 markers show predominant dot-like cytochemical staining. (C and F) Cells expressing both D12 and 2D2 markers are stained with the scattered granular pattern (mag 500×).

enriched in the D12+ fractions. Again, marked differences were observed between the two D12+ subfractions sorted according to the expression of the 2D2 marker. Thus, the large majority of the cells in the D12+2D2+ fraction stained with the scattered granular pattern for both AP and ANAE (98.5% ± 1.3% and 81.0% ± 8.5%, respectively) (Fig. 2, C and F). The majority of the cells in the D12+2D2+ fraction also exhibited a scattered granular pattern. However, whereas virtually all of these cells (93.2% ± 3.6%) contained AP, only 20.0% ± 5%, stained for ANAE.

DISCUSSION

Previous attempts to fractionate T cells according to a given surface marker and to relate the subsets so obtained with cytochemical patterns of staining for lysosomal acid hydrolases have achieved enrichment to some extent for cells exhibiting either the dot-like or the scattered granular cytochemical patterns.4,7 However, subpopulations of E+ cells homogeneous for their cytochemical staining pattern have not been previously obtained.

In this study, we have used two monoclonal antibodies, D12 and 2D2, and the FACS to isolate four different E+ cell subpopulations. The subset lacking both markers (D12-2D2-) contained 90% or more cells with the dot-like staining for both AP and ANAE. It is of note that >98% of the cells in this fraction express the OKT3 and T4-Leu-3 markers. The D12+2D2- subset contained a variable proportion of cells displaying the dot-like pattern of staining (the range was 30%–95% in 5 different experiments). However, the large majority of the cells staining with the scattered granular pattern expressed AP but was negative for ANAE. The heterogeneity found in this subset may reflect in part the variability of the granular versus the dot-like cytochemical pattern that has been described in the OKT8+ subset.7

Interestingly, virtually all of the cells expressing both markers (D12+2D2+) were of the scattered granular type and expressed both AP and ANAE, whereas the D12+2D2+ fraction (which also consisted of scattered granular cells) contained a large proportion of cells positive for AP but negative for ANAE. Therefore, the two monoclonal antibodies used in this study appear capable not only of providing homogeneous populations of cells staining with the dot-like or with the scattered granular pattern, but also of distinguishing between granular cells expressing both AP and ANAE and granular cells expressing only AP.
The relationship of the 2D2 and D12 monoclonal antibodies to other reagents has been previously investigated. Several lines of evidence indicate that the 2D2 monoclonal antibody recognizes an antigenic determinant closely related, if not identical, to that of the OKM1 antibody. First, the Leu-2 and 2D2 T cell subpopulations are mutually inclusive, i.e., identical. Pretreatment of T cells with 2D2 abrogates the binding of anti-Leu-2 antibodies, and the molecular characteristics of the antigen precipitated by 2D2 are virtually identical to those reported for the Leu-2 antigen. Furthermore, studies using anti-Leu-2 antibodies rather than 2D2 indicate that the cytochemical characteristics of Leu-2 subsets are identical to those presented here for 2D2 (data not shown). Hence, 2D2 and anti-Leu-2 antibodies can be regarded as homologous reagents for phenotypic analyses. The relationship of the D12 antibody to previously described antibodies is less certain. The pattern of cellular reactivity of D12 is virtually identical to that of the OKM1 antibody. However, D12 does not block the binding of OKM1 antibodies. Although the antigenic determinants recognized by these antibodies therefore appear to be different, the subsets of cells defined by the D12 and OKM1 antibodies are virtually identical.

Studies of the functional capabilities of the four E+ cell subpopulations identified by these antibodies are in progress. As previously noted, E+ cells that suppress T cell proliferation are confined to the AP+, ANAE+ subset of granular cells expressing the D12-2D2 phenotype. In addition, E+ cells with NK cell function appear to be present only within the D12-2D2 granular subset, whereas alloreactive cytotoxic T-lymphocyte precursor and effector cells appear to have the D12-2D2 phenotype (L. T. Clement, manuscript in preparation). These data therefore suggest that there is a significant correlation among surface antigen phenotype, expression of lysosomal enzymes, and functional properties of these E+ cell subpopulations.

The relationship of the E+ granular cell subsets to one another or to granular lymphocytes purified by other methods or expressing other surface markers remains uncertain. Cells with azurophilic granules, an extended cytoplasm, and a scattered granular staining pattern for acid hydrolases have been collectively termed large granular lymphocytes (LGL). LGL are commonly related to natural killing (NK) cells, although this function may also be exerted by other cell types. Cells with a scattered granular pattern of staining are also found among E+ non-T, non-B, cells and constitute the bulk (up to 80%) of the so-called third population cell (TPC) fraction. The latter cells are indistinguishable cytochemically from those found in the E+ cell fraction in the present study. In like fashion, the lack of ANAE activity in the D12-2D2 E+ cells suggests that they might be related to immature LGL. Cytochemical studies on immature LGL from various sources (normal bone marrow LGL, a subset of LGL from the normal peripheral blood and cells from patients with abnormally expanded LGL populations), have shown that, as in other lymphocyte lineages (e.g., T or B cells), acid hydrolases are expressed sequentially during LGL maturation, with AP always preceding the appearance of ANAE activity. Cells with D12-2D2 E+ phenotype express both AP and ANAE and, thus, might represent more mature LGL. However, the existence of distinct subsets of granular cells differing for their surface phenotype and for the expression of lysosomal enzymes cannot be excluded.

The relationship between D12-2D2, scattered-granular suppressor cells and other populations of LGL with suppressor capabilities is also unclear at present. LGL (separated as Leu-7+ cells) have been shown to exert suppressor functions. Approximately 80% of the D12-2D2 E+ cells (and 50%-60% of the D12-2D2 E+ subset) are recognized by the Leu-7 antibody (L. T. Clement, unpublished observations). However, as previously shown for Tc, yet contrary to the D12-2D2 cells found in the E+ cell fraction, Leu-7+ LGL must be activated by preliminary exposure to immune complexes in order to acquire the suppressor capability. Several lines of evidence suggest that certain LGL might be myeloid cells or, alternatively, of a bone marrow-derived lineage that is distinct from both T and myeloid cells. Analysis of the fine structure and ontogeny of the D12-2D2 suppressor cells should provide more information on this issue.

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