MORPHOLOGICAL AND HISTOCHEMICAL ANALYSES OF TWO HUMAN T-CELL SUBPOPULATIONS BEARING RECEPTORS FOR IgM OR IgG*

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Heterogeneity of distinct subpopulations of thymus-derived (T) lymphocytes in mice has been defined by assays that discriminate T-cell functions, surface alloantigens, and the presence or absence of receptors for IgG (1, 2). Recently, subpopulations of the human T lymphocytes that form rosettes with sheep erythrocytes have been defined (reviewed in 3, 4). Two functionally distinct subpopulations can be identified and isolated by their ability to bind either IgM or IgG immune complexes. So far we have shown that T cells with receptors for the Fc portion of IgM (TM) and T cells with receptors for the Fc portion of IgG (To) respond differently to phytohemagglutinin, react in a similar manner to concanavalin A, and exert opposing effects on B-cell responses to pokeweed mitogen (PWM) in vitro (5, 6). TM lymphocytes provide the required help for B-cell proliferation and differentiation into plasma cells in response to PWM, whereas To lymphocytes do not help but rather can suppress the proliferation and differentiation induced by helper TM cells (6).

Here we describe studies demonstrating that the two T-cell subpopulations, TM and To, in human peripheral blood have distinguishing morphological and histochemical features easily visualized at either the light or electron microscope levels.

Materials and Methods

Preparation of Cell Suspensions. Lymphocyte preparations from peripheral blood samples of normal human volunteers or from normal umbilical cord blood were isolated by techniques described in detail elsewhere (6); here the basic protocol is briefly outlined. Mononuclear cells were obtained by Ficoll-Hypaque (Pharmacia Fine Chemicals, Piscataway, N. J.) density-gradient separation of defibrinated whole blood. T lymphocytes were allowed to form rosettes

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Abbreviations used in this paper: ANAE, a-naphthyl acid esterase; DMSO, dimethylsulfoxide; FCS, fetal calf serum; OE-IgG, ox erythrocytes coated with rabbit IgG antibodies; OE-IgM, ox erythrocytes coated with rabbit IgM antibodies; PAS, periodic acid-Schiff; PWM, pokeweed mitogen; SEN, neuraminidase-treated sheep erythrocytes; To, T cells with receptors for the Fc portion of IgG; TM, T cells with surface receptors for the Fc portion of IgM.

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with neuraminidase-treated sheep erythrocytes (SE_{E}), and the rosetting T cells then separated from the other mononuclear cells by pelleting them through two Ficoll-Hypaque gradients. The B cell-enriched fraction of blood mononucleated cells was obtained as a by-product of T-cell depletion, performed twice by this technique. T_{0} cells were isolated from freshly prepared T cells by further density-gradient separation of the subpopulation of T cells that formed rosettes with ox erythrocytes coated with rabbit IgG antibodies (OE-IgG). After erythrocyte lysis, the T_{0} cells were routinely incubated overnight before further analysis. Blood T cells depleted of T_{0} cells were cultured overnight in TC 199 medium (Grand Island Biological Co., Grand Island, N. Y.) supplemented with 20% fetal calf serum (FCS). T_{W} cells were isolated then from the T_{0} cell-depleted population of T cells by density-gradient separation of those T cells that formed rosettes with OE coated with rabbit IgM antibodies (OE-IgM).

For comparative analysis with isolated lymphoid cell subpopulations, routine smears of fresh whole blood were prepared. 

**Cell Cultures.** Unseparated T cells and T_{W} cells were cultured in Falcon plastic tubes (Falcon 2001, Div. BioQuest, Oxnard, Calif.) for 48 h at a concentration of 10^6 cells/ml in RPMI 1640 (Grand Island Biological Co.) containing 20% heat-inactivated FCS, 2 mM glutamine (Grand Island Biological Co.), and 50 μg/ml gentamicin (Schering Corporation, Kenilworth, N. J.). In some experiments, cells were cultured in the presence of cytochalasin B (Calbiochem., San Diego, Calif.) dissolved in dimethylsulfoxide (DMSO) at a concentration of 50 μg/ml of medium. Control cells were cultured in medium containing the same amount of DMSO.

**Light Microscope and Histochemical Analysis.** Cell suspensions were sedimented onto glass slides using a cytocentrifuge (Shandon Southern Instruments Inc., Sewickley, Pa.). For morphological studies, cells were fixed in methanol for 15 min and stained with May-Grunwald-Giemsa.

For cytochemical demonstration of enzyme activities, cells were fixed after or before cytocentrifugation. The latter procedure proved to be advantageous for evaluation of α-naphthyl acid esterase (ANAE) activity. This allowed a shorter incubation period and improved the detail of histochemical localization.

**For ANAE activity** cell suspensions were fixed in cold Baker's formalin calcium (pH 6.7) for 10 min and cytocentrifuged. After they were washed in distilled water, the slides were incubated for 60 min at 37°C with hexazotized pararosaniline and α-naphthyl acetate at pH 5.8 (7). The positive control for this reaction was a preparation of adherent cells separated from human peripheral blood. The adherent cells were detached by lidocaine treatment before fixation and cytocentrifugation (8).

**Peroxidase activity** was detected after fixation for 15 min at 4°C with 2% glutaraldehyde in 0.1 M phosphate buffer at pH 7.4. Slides were incubated for 30 min at room temperature in 3,3'-diaminobenzidine tetrahydrochloride according to the method of Graham and Karnovsky (9). Positive controls for this histochemical reaction were white blood cells adherent to glass slides, and granulocytes prepared by dextran sedimentation; they were treated in parallel with the lymphocyte suspensions.

**For histochemical detection of acid phosphatase,** cytocentrifuged preparations were fixed for 30 min in cold formalin-sucrose and incubated in sodium-α-glycerophosphate for 30 min at 37°C following the Gomori technique as modified by Holt and Hicks (10). Cryostat sections of rat liver and kidney fixed overnight in cold formalin-sucrose were the positive controls for this reaction.

Neutral and acidic glucosaminoglycans were localized via the periodic acid-Schiff (PAS) reaction in cells fixed in Carnoy's fluid and counterstained with Alcian blue at pH 2.5.

**Assay for C2 Synthesis.** Supernates for 48-h cultures of purified T_{W} and T_{W} cells were assayed for C2 activity by Dr. Harvey R. Colten, Children's Hospital, Boston, Mass., by a previously described method (11).

**Immunofluorescence Staining.** Viable suspensions of cells were stained with the globulin fraction of a rabbit anti-monkey thymocyte antiserum conjugated with fluorescein isothiocyanate (molar F/P ratios, 3-6) (12) or with purified goat antibodies to human F(ab')_{2} fragments conjugated with tetramethylrhodamine isothiocyanate (P/R ratio, 1.5). After thorough washing in RPMI 1640, containing 5% FCS and 0.1% sodium azide, cells were examined with a Leitz Orthoplan microscope (E. Leitz, Inc., Rockleigh, N. J.) equipped with incidence light illumination and phase contrast optics (13).

**Electron Microscope Analysis.** The purified T-cell population and separated subpopulations were fixed for 30 min at 4°C in 1.25% glutaraldehyde in 0.1 phosphate buffer, pH 7.4, washed in
the buffer, and postfixed for 1 h at 4°C in 1% osmium tetroxide. After fixation the cells were dehydrated and embedded in Epon 812 (Epoxy Resins, Shell Chemical Co., Houston, Texas, 14). Thin sections cut with an ultramicrotome were stained with uranyl acetate and lead citrate and examined under a Siemens Elmiskop 101 transmission electron microscope (Siemens Corp., Iselin, N. J.).

Results

Use of Discriminating Markers to Establish the T-Cell Nature of the Isolated T\textsubscript{M} and T\textsubscript{G} Subpopulations of Cells. The T\textsubscript{M} and T\textsubscript{G} subpopulations were isolated from a total T-cell fraction that formed SE\textsubscript{N} rosettes. However, the possibility that a minor population of B cells or macrophages contaminating the SE\textsubscript{N}-rosetted cells might be selected with the T\textsubscript{G} fraction through binding of OE-IgG via an Fc receptor was considered.

Purified T\textsubscript{G} cells, T\textsubscript{M} cells, and unfractionated T cells were incubated overnight and mixed again with SE\textsubscript{N} under the standard conditions for rosette formation. Approximately 85% of the cells in all three populations formed SE\textsubscript{N} rosettes.

Additional evidence supported the T-cell nature of T\textsubscript{M} and T\textsubscript{G} lymphocytes. All of the cells in both populations were stained by the fluorescein-conjugated globulin fraction of an anti-T-cell heteroantiserum. They lacked surface immunoglobulin and complement (C3b and C3d) receptors characteristically found on B lymphocytes (6). These observations indicated that the T\textsubscript{G} cells, as well as T\textsubscript{M} cells and unfractionated T cells, possess a receptor for SE\textsubscript{N} and that this receptor becomes undetectable on a small proportion of T cells after culture.

Cytoplasmic markers for cells of the granulocytic and the monocytic-macrophage series, endogenous peroxidase and acid phosphatase, were not detected in purified T\textsubscript{M}- or T\textsubscript{G}-cell preparations. These enzyme activities were present in granulocyte and macrophage preparations as well as in rat liver and kidney sections used as controls. Finally, C2 activity was not found in the supernates of cultured T\textsubscript{M} and T\textsubscript{G} cells although macrophage synthesis of C2 is easily detectable by the assay used.

Morphology of T\textsubscript{M} and T\textsubscript{G} Subpopulations at the Light Microscope Level. Human T lymphocytes, when isolated from the peripheral blood by rosetting twice with SE\textsubscript{N} and stained with the May-Grünwald-Giemsa dye mixture, were morphologically heterogeneous. The majority of the T cells, 80-90% in adult blood and 60-70% in cord blood, were small (9.3 ± 1.3 μm in diameter when measured in cytocentrifuged cell samples) and exhibited typical lymphoid morphology. These cells had a large nucleus, densely stained in patches, and a thin rim of basophilic cytoplasm (Fig. 1). The remaining cells were larger (14.2 ± 1.7 μm in diameter) and displayed a homogeneously stained nucleus surrounded by abundant cytoplasm that was either neutrophilic or slightly basophilic. The latter cells were readily identifiable by the presence in the cytoplasm of azurophilic granules which varied in size, number, and distribution from cell to cell (Fig. 2). The granules were not stained by the PAS-reaction or by Alcian blue (pH 2.5), and therefore lacked constituents with the histochemical characteristics of glucosaminoglycans. Among similarly stained cells in fresh whole blood smears, cells with features identical to those described for isolated T\textsubscript{G} cells could be identified in appropriate frequencies. This observation makes it
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Fig. 1. TM-lymphocyte preparation stained with May-Grünwald-Giemsa. The morphological homogeneity of this cell type is shown in two microscope fields. A large nucleus with patchy chromatin is surrounded by a thin rim of lightly basophilic cytoplasm that does not contain visible granules.

Fig. 2. T\textsubscript{n}-lymphocyte preparation stained with May-Grünwald-Giemsa. Compared with TM cells, T\textsubscript{n} lymphocytes are larger, with lower nuclear to cytoplasmic ratios and a more homogeneous distribution of the nuclear chromatin. In most T\textsubscript{n} cells, the cytoplasm contains azurophilic granules that vary in number, size, and distribution.
unlikely that the characteristic features of $T_o$ cells described here resulted from artifactual changes during the isolation procedures.

Morphological analysis of $T$-cell subpopulations isolated by the presence of receptors either for IgM or IgG, revealed that all of the cells in the $T_M$-cell fraction had the characteristics of the type of small lymphocytes described above, whereas the $T_o$ cells were identified as the large cells with cytoplasmic granules. In five experiments in which 400–500 cells were counted in each fraction, lymphocytes with the morphological characteristics of the $T_o$ subpopulation were not observed among the $T_M$ cells and vice versa.

**Characteristic Localization of Esterase Activity in $T_M$ Cells.** When unseparated mononuclear blood cells, taken from the interface of a Ficoll-Hypaque density gradient, were stained for esterase activity, macrophages were intensely stained (Fig. 3A), and a large proportion of the lymphocytes contained one or two large cytoplasmic accumulations of esterase activity. 70–80% of $T$ cells separated as $S_{en}$ rosettes were esterase-positive (Fig. 3A); a single large spot or sometimes two such vesicles were the only sites of activity detectable within the cytoplasm. Separation of $T$-cell subsets according to the presence of receptors for IgM or IgG showed that up to 95% of the $T_M$ cells were esterase-positive (Fig. 3B). 90% of the $T_o$ cells were completely esterase-negative (Fig. 3C). Less than 1% contained one or two of the large "$T_M$-type" accumulations of esterase activity and ~10% contained dispersed small granules with esterase activity presumed to represent lysosomes. In the B-enriched population, ~40% of the cells exhibited the latter pattern of esterase activity (Fig. 3D).

Using whole blood smears, other investigators have observed lymphocytes with cytoplasmic ANAE activity in the same large "spot" pattern that we show here to be characteristic of $T_M$ cells (15, 16). In a direct comparison of this ANAE activity pattern among lymphocytes on whole blood smears and lymphocytes isolated from the interface of a Ficoll-Hypaque density gradient, we found the same frequencies of lymphocytes displaying the ANAE activity pattern of $T_M$ cells in both preparations. This indicates that artifactual changes in ANAE activity during lymphocyte isolation procedures are negligible, and suggests that the $T_M$ cells can be accurately enumerated by histochemical evaluation of ANAE activity in lymphocytes on routine blood smears.

**Phagocytosis of OE-IgG by $T_o$ Cells.** A variable percentage of $T_o$ cells in adult blood (5–10%) as well as in umbilical cord blood was observed to have phagocytosed OE-IgG within 30 min of incubation. (Phagocytosis of OE-IgM by $T_M$ cells or of unsensitized OE by $T_o$ cells was not observed.) Although adult $T_o$ cells contained only one to two phagocytosed erythrocytes, some newborn $T_o$ cells contained as many as five red cells. We noted no characteristic redistribution or loss of the azurophilic granules in $T_o$ cells that had engulfed erythrocytes. Further studies of this phenomenon under conditions optimal for phagocytosis were not done.

**Morphology of $T_M$ and $T_o$ Subpopulations at the Electron Microscope Level.** Examination of the fine structure by transmission electron microscopy confirmed light microscope observations showing that $T_M$ and $T_o$ cells have different structural characteristics and that very little morphological variation exists within each population. The ultrastructural appearance of $T_M$ cells
FIG. 3. Demonstration of ANAE activity in lymphocyte subpopulations. (A) Most of the SE<sub>E</sub>-rosetted T cells show one or two large cytoplasmic spots of ANAE positivity, occasional cells have small dispersed dots of esterase activity (arrow), and some cells appear to be ANAE-negative; the inset shows the intense ANAE-reaction of a macrophage. (B) The great majority of T<sub>M</sub> cells separated as rosettes with OE-IgM show striking focal accumulations of cytoplasmic ANAE activity. (C) T<sub>o</sub> cells separated as rosettes with OE-IgG are mostly ANAE-negative; in a few of them (~10%), the histochemical reaction stains small granules dispersed within the cytoplasm. (D) B cell-enriched preparations (peripheral blood mononuclear cells twice depleted of SE<sub>E</sub>-rosetting T lymphocytes) are either ANAE-negative or show the fine positive granular pattern (arrow) occasionally seen among T<sub>o</sub> cells. The cells shown in (C) and (D) were photographed using phase contrast illumination, whereas those in (A) and (B) were not. × 1,250.
correlated well with the general morphological features of typical small- or medium-sized lymphocytes. These cells showed a high nuclear to cytoplasmic ratio with margined heterochromatin together with a patchy chromatin distribution in the nucleus. Other distinguishing features included a small Golgi apparatus, rare mitochondria, occasional inclusion bodies and lipid droplets, isolated profiles of rough endoplasmic reticulum, and monoribosomes uniformly dispersed throughout the hyaloplasm. An ultrastructural correlate of the large cytoplasmic accumulation of ANAE activity was not noted in the thin sections of T_M cells examined. The cell surface of T_M cells appeared smooth with only occasional short microvillous projections visible in the plasma membrane of sectioned cells (Fig. 4).

As compared with T_M cells, T_G cells showed a lower nuclear to cytoplasm ratio...
and greater margination of nuclear heterochromatin (Fig. 5A). The Golgi apparatus was typically more extensive (Fig. 5C) as was the rough endoplasmic reticulum; mitochondria were more numerous, often occurring in clusters. Similar density and distribution of monoribosomes were seen in T<sub>G</sub> and in T<sub>M</sub> cells. The T<sub>G</sub>-cell surface appeared relatively rough as a result of the presence of numerous microvilli of variable lengths (Fig. 5A).

The cytoplasmic granules, which were a distinguishing feature of T<sub>G</sub> cells viewed under the light microscope, had no apparent consistent relationship with the cell organelles mentioned above, except for their frequent proximity to the Golgi apparatus. Firm morphological evidence for Golgian origin of the granules, however, was not established. Each granule was surrounded by a membrane unit and contained a matrix which was either homogeneously electron dense or contained a single darker inclusion; an electron transparent space often separated the matrix from the limiting membrane (Fig. 5B).

Phagocytosis of OE-IgG by T<sub>G</sub> cells was also documented at the electron microscope level (Fig. 6) after a 12-h incubation interval before cell fixation. Fusion of the cytoplasmic granules with the phagosome was never observed.

Effect of Cytochalasin B on Release of Granules from Cultured T<sub>G</sub> Lymphocytes. The percentages of lymphocytes with T<sub>G</sub> characteristics in the unseparated T-cell preparations, determined by counting cells with granules on slides stained with May-Grünwald-Giemsa, closely correlated with the number of cells forming OE-IgG rosettes in both adult blood (12 compared with 13.5%) and cord blood (28 vs. 33.5%). However, in five separate experiments, only 55–85% of the cells in the purified T<sub>G</sub>-cell preparations showed detectable granules although all of these cells exhibited other morphologic features characteristic of T<sub>G</sub> cells. This suggested the possibility of some release of granules from T<sub>G</sub> lymphocytes during the cell separation procedures and overnight incubation before examination.

To test this hypothesis, two T<sub>G</sub> fractions containing 55 and 81% cells with granules were cultured for 48 h. In the absence of cytochalasin B only 8 and 16% of the cells, respectively, carried detectable granules at the end of the culture period, whereas in the presence of cytochalasin B (50 μg/ml) the percentages in both cases were close to the preincubation values (51 and 72%, respectively). Cytochalasin B was therefore able to prevent loss of granules from T<sub>G</sub> cells.

Discussion

We have previously described two functionally distinct subpopulations of human T cells based on their ability to bind the Fc portion of IgM (T<sub>M</sub>) or IgG.
Fig. 6. Phagocytosis of OE-IgG by T\(\alpha\) lymphocytes. The cell contains a red cell within its phagosome. T\(\alpha\) granules (arrows) do not show any relationship with the phagosome. × 14,000.

(T\(\alpha\)) (3, 5, 6). In this study we have shown that not only do these subpopulations differ functionally but they are also easily distinguished on the basis of their morphological and histochemical characteristics.

Efficient purification of the newly described subpopulations of T lymphocytes was considered a primary requirement for these studies. It was deemed especially important to exclude that a contaminant cell belonging to another mononuclear cell line had been enriched for by the separation techniques employed. For this reason, we have examined purified T\(\alpha\) and T\(\beta\) subpopulations extensively for markers identifying B cells, and monocytes as well as T cells.
The results reported here and in a previous communication (6) indicate that both subpopulations are T lymphocytes and are not significantly contaminated with other mononuclear cell types. The apparent morphological homogeneity of the purified fractions as visualized at both light and electron microscope levels substantiates the latter conclusion.

A number of general structural features distinguish TM and TG cells, including differences in cell size, nuclear to cytoplasmic ratio, and the extent of development of cellular organelles. On the basis of our observations, we cannot exclude the possibility that some of these morphological differences (e.g., the difference in the villous projections from the surfaces of TM and TG cells) could be due to changes induced by cell activation during the isolation procedures. Certain characteristic features of TM and TG cells, however, were clearly seen in unseparated whole blood preparations, thus indicating that these differences were not a result of cell modification induced during their separation. One striking characteristic of TM cells was their distinct cytoplasmic accumulations of ANAE activity; the large spots of ANAE staining, not found in TG and B cells, were also very different from the ANAE reaction pattern of macrophages, and thus may serve as a practical marker for rapid enumeration of cells of this subpopulation.

TG cells, on the other hand, contained characteristic granules that could be visualized under both light and electron microscopy. These granules did not contain the enzyme markers of lysosomes in granulocytes and macrophages, nor were they ever observed in confluence with phagosomes containing OE-IgG. Within individual TG cells, we noted considerable heterogeneity in the morphological appearance of the single membrane-lined granules. They differed in size, electron density of the matrix, and presence or absence of a central dense core. This type of heterogeneity is suggestive of a maturational pattern as defined in other cell types, e.g., the protein-secreting serous cell (17, 18). The release of the granular contents from TG cells with suppressor activity (6) after in vitro incubation at 37°C and the inhibition of this release by cytochalasin B, a drug known to affect microfilaments, raise the possibility that the granules contain suppressor substances. Support for this hypothesis must await further studies on the mechanism of suppression by TG cells and on the nature and function of the material enclosed within the granules. For the present, these characteristic granules may serve as a convenient additional marker for TG cells in certain lymphoproliferative and immunodeficiency diseases.

Endocytosis of OE-IgG by a significant proportion of the lymphocytes in purified TG-cell preparations was an unexpected finding that seems especially noteworthy. The possibility that such cells represented a contaminating population of non-T phagocytic cells would appear to be excluded by the fact that they lacked the markers characteristic of cells of the monocyte-macrophage and granulocytic series. Moreover, these erythropagocytic cells were indistinguishable from other TG cells on the basis of their morphological, histochemical, surface antigen, and sheep erythrocyte-binding characteristics. In view of the possession of receptors for the Fc portion of IgG by TG cells, perhaps this ability to engulf erythrocytes coated with IgG is not surprising. Nevertheless, comparison of the mechanisms by which TG cells and classic phagocytic cells accomplish
this feat, and elucidation of the fate of the endocytosed cells, could shed new light on the biologic functions of cells within the $T_C$ subpopulation.

**Summary**

Two subpopulations of circulating human T cells forming rosettes with neuraminidase-treated sheep erythrocytes were purified on the basis of the presence of receptors for IgG ($T_C$ cells) or for IgM ($T_M$ cells), and were shown to have distinguishing morphological and histochemical characteristics. $T_M$ cells had the general features of typical small- or medium-sized lymphocytes; most were easily identifiable by distinctive cytoplasmic accumulations, usually one and sometimes two large spots, of nonspecific acid esterase activity. The larger $T_C$ cells had a more complex system of cytoplasmic organelles, numerous surface villous projections, and distinctive vesicles in their cytoplasm. These vesicles were lined by a unit membrane enclosing granular material of varied electron density and intravesicular distribution. The release of the vesicular contents on short-term culture of $T_C$ cells was inhibited by cytochalasin B. Definition of these distinguishing characteristics of $T_M$ and $T_C$ cells provides a basis for practical enumeration of these functionally distinct subpopulations of human T cells. Some of the $T_C$ cells were capable of endocytosis of IgG antibody-coated erythrocytes.

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