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IDENTIFICATION OF Ia ON A SUBPOPULATION OF HUMAN T LYMPHOCYTES THAT STIMULATE IN A MIXED LYMPHOCYTE REACTION[1]

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The delineation of discrete subpopulations of human T lymphocytes has permitted preliminary analyses of the complex cellular network regulating the immune response in man. We previously showed that a subset of T lymphocytes, designated as theophylline-sensitive because of their inability to bind sheep red blood cells in the presence of the drug, are responsible for antigen-specific suppression or regulation in an *in vitro* plaque-forming cell assay. We now show that 25 to 45% of these theophylline-sensitive T cells were Ia-positive by immunofluorescence with a rabbit antiserum raised against purified B lymphoblast surface antigenic material. These data suggested that 4 to 7% of peripheral blood T cells carry Ia determinants. The presence of Ia determinants on this T cell subset was confirmed by gel analysis of radioiodinated surface material. Furthermore, in mixed lymphocyte culture, the theophylline-sensitive cells demonstrated HLA-D determinants and were 10-fold more potent stimulators than equal numbers of B lymphocytes. The presence of Ia determinants on these T cells indicates the expression of major histocompatibility complex-related regulatory gene products on a specific human T lymphocyte subpopulation.

By using a variety of allo and heteroantisera, DR or Ia-like determinants have been demonstrated on human B lymphocytes and monocytes (1–3). The heteroantisera recognize a basic structure, common to all allotypes, which is composed of two components, 27,000 daltons and 35,000 daltons; in this respect it is similar to murine Ia (4). The main lymphocyte-activating determinant in the mixed lymphocyte reaction (MLR), encoded by the HLA-D region, appears to be identical or closely linked with Ia (5). In the mouse, Ia is known to play a major role in the regulation of the immune response, and in this species a number of groups have described Ia determinants, expressed predominantly on a T cell subset, responsible for suppression of the immune response (6–8).

We have isolated a subpopulation of human T cells that contain antigen-specific suppressor cells when assayed in a direct plaque-forming cell (PFC) assay (9). These cells, identified by their inability to bind sheep red blood cells (SRBC) in the presence of theophylline, have been labeled theophylline-sensitive T lymphocytes (T-sens)[5] (10). In this report we demonstrate that 25 to 45% of T-sens are Ia-positive and express HLA-D determinants.

MATERIALS AND METHODS

Cell surface markers. The E-rosette assays in which were used untreated or aminoethylisothiouronium bromide- (AET) treated SRBC and incubation with theophylline have been described previously (11). After theophylline treatment, all E-rosette assays were carried out by using AET-treated SRBC (E_{AET}). Surface immunoglobulin- (sIg) positive cells were identified by using direct immunofluorescence, and monocytes were enumerated after α-naphthyl esterase (NSE) staining and latex particle ingestion. C3 receptor-bearing cells were assessed by using a rosette technique with intermediates generated by incubating ox red blood cells (E_{ox}) with rabbit IgM anti-ox antiserum and normal mouse serum as the source of complement (12).

Isolation of lymphocyte subpopulations. The isolation technique is illustrated in Figure 1. Heparinized or defibrinated peripheral blood was subjected to Isopaque-Ficoll gradient centrifugation (9). The mononuclear cells (PBL) were depleted of adherent cells by incubation in plastic Petri dishes for 30 min at 37°C in the presence of 20% FCS. The nonadherent cells (NAC) were incubated with 3 mM theophylline for 45 min at 37°C. SRBC were then added in the presence of 30% FCS, and the mixture was spun for 5 min at 200 × G, maintained at 4°C for 30 min, gently resuspended, and placed on gradients of Isopaque-Ficoll. After 30 min centrifugation at 400 × G, the interface containing T-sens and non-T cells were recovered and treated separately from the pellet containing theophylline-resistant T cells (T-res). The interface cells were incubated with $E_{ox}AC3$ (1:40 ratio) for 5 min at 37°C, kept at room temperature for 30 min, gently resuspended, and layered onto an Isopaque-Ficoll gradient. After centrifugation at 200 × G for 30 min, the

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[5] Abbreviations used in this paper: AET, aminoethylisothiouronium bromide; MHC, major histocompatibility complex; NAC, nonadherent cells; NSE, α-naphthyl esterase (nonspecific esterase); PBL, peripheral blood mononuclear cells; T-res, theophylline-resistant T-lymphocytes; T-sens, theophylline-sensitive T-lymphocytes; E_{AET}, AET-treated SRBC; sIg, surface immunoglobulin; E_{ox}, ox red blood cells; SDS, sodium dodecylsulfate.

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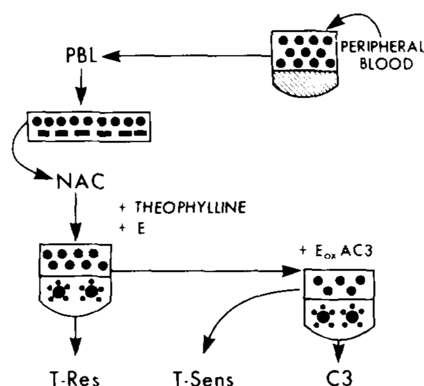

Figure 1. Isolation of theophylline-sensitive peripheral blood T-cells. E, sheep red blood cells; $E_{ox}AC3$, Ox erythrocyte-IgM-C3 intermediates; C3, C3-positive cell fraction.

interface cells and pellet were recovered. The SRBC and E_{ox} in the cell pellets were lysed in Tris-ammonium chloride (13).

Preparation of the anti-Ia antiserum. Membrane vesicles were prepared from cultured B lymphoblasts by solubilization in Tween 40 (14). The vesicles were solubilized in 0.5% Triton X-100, and the material was applied to a column of Sephadex G-200 equilibrated in 0.01 M Tris-HCl (pH 8.0) and 1% deoxycholate. The position of B cell-specific antigen originally characterized as a molecule of 30 to 35,000 daltons was recognized by precipitation of labeled material with an absorbed anti-B lymphoblast serum (15, 16). Subsequently, material with a m.w. of 20 to 50,000 was recovered, and rabbits were immunized with 50 μg of this material in complete Freund's adjuvant. A second immunization with 10 μg in incomplete adjuvant was carried out, and the animals were bled 7 days later. The serum obtained was absorbed three to five times with cultured T lymphoblasts until the absorbed antiserum no longer precipitated surface labeled material from these lines. This absorbed antiserum was capable of precipitating the B cell antigen, which was resolved into two components of 27,000 and 35,000 daltons from cultured B lymphoblasts, chronic lymphatic leukemia cells, E-rosette-negative acute lymphoblastic leukemia (ALL) cells and acute myelocytic leukemia cells. Precipitation studies with E-positive lymphoblastic leukemia, chronic myelocytic leukemia cells, thymocytes, four different T cell lines, or the K562 line were negative.

Identification of Ia on T lymphocytes

Indirect immunofluorescence. The first incubation was carried out with a 1:60 dilution of the anti-Ia antiserum followed by labeling with a 1:10 dilution of fluoresceinated goat anti-rabbit IgG (Meloy Laboratories). Because of the weak staining of the T cells (compared with B cells or monocytes) and for convenience, all cell preparations were incubated overnight with the anti-Ia reagent. This did not significantly alter the number of positive cells in the different cell preparations but did improve the intensity of staining in T-sens. Control incubations were with a preimmunization serum from the same rabbit.

Lactoperoxidase-catalyzed radioiodination. T-sens were labeled by the lactoperoxidase method, and the labeled antigens were solubilized in 0.5% Triton X-100. The lysates were passed over a DEAE column and precleared with normal rabbit serum and *Staphylococcus aureus* Cowan strain I. Specific immunoprecipitations were carried out with Ig, Ia, and β2-microglobulin antisera and a control normal rabbit serum, followed by incubation with *S. aureus*. The precipitated materials were eluted in 10% sodium dodecylsulfate (SDS), reduced (2 mM 2-mercaptoethanol), and boiled for 2 min. The eluates were analyzed on 10% SDS-acrylamide gels (17).

Mixed lymphocyte reactions (MLR). One-way MLR were performed in microtiter plates by using a constant number of responder cells obtained by Isopaque-Ficoll gradient centrifugation of peripheral blood from normal donors (5×10^4/well) (18). Varying numbers of irradiated stimulator cells in the mixtures were incubated for 7 days. The stimulatory capacity of individual cell suspensions was expressed as a percent of the maximal stimulation in any one experiment. At a concentration of 3×10^5 cells, both T-sens and C3-positive cells were able to stimulate responder cells maximally (range: 47,000 to 158,000 cpm), and these responses were taken as maximal stimulation (100%) in any given experiment.

RESULTS

Table I summarizes the results of four experiments after the cell separation protocol illustrated in Figure 1. The population designated T-sens contained 81 ± 9% E_{AET}, and of these more than 90% were identified as theophylline-sensitive. Less than 5% were positive for C3, sIg, NSE, or latex particle ingestion. Of these cells, 25 to 45% were positively stained with the Ia antiserum, whereas T-res contained <5% Ia-positive cells. The C3-positive fraction contained >90% Ia-positive cells. Absorption of the anti-Ia antiserum with B lymphoblasts removed the reactivity against both the T-sens and the C3-positive fractions. In agreement with previous results for cell recoveries (19), T-sens represented approximately 20% of PBL. These data indicated that Ia-positive T cells could be identified in T-sens, and it was calculated that 4 to 7% of peripheral blood T-cells were Ia-positive.

The presence of Ia on T-sens was confirmed in two additional ways. After incubation of T-sens with anti-Ia, E_{AET} were added, and rosettes formed. Often T lymphocytes were covered with a large "morula" of SRBC and did not permit visualization of Ia on their surface. However, the addition of 1% formaldehyde during the incubation with SRBC permitted the detection of a receptor for SRBC on most of the cells identified as Ia-positive. In the mixed NAC suspension, we were able to detect the SRBC receptor on 10 to 15% of the Ia-positive cells but not in the C3-positive cell preparation.

The presence of Ia determinants on T-sens was demonstrated after lactoperoxidase-catalyzed iodination of T-sens. Figure 2 illustrates the pattern of one such immunoprecipitation with the anti-Ia antiserum and a control serum. The presence of the 27,000- and 35,000-dalton components confirms the presence of the Ia-like determinants on T-sens. Parallel immunoprecipitations with anti-immunoglobulin reagents detected the presence of sIgD/sIgM on only unseparated, NAC, and C3-positive preparations.

It has generally been accepted that B lymphocytes and

TABLE I

Characterization of the T-sens

% Positive Cells		
E_{AET}		Ia
81 ± 9[a]		34 ± 10
C3	sIg	NSE[b]
6 ± 4	1 ± 1	3 ± 2

[a] Results are expressed as the mean ± 1 S.D. of four experiments.
[b] NSE: α-naphthyl esterase stain for monocytes.

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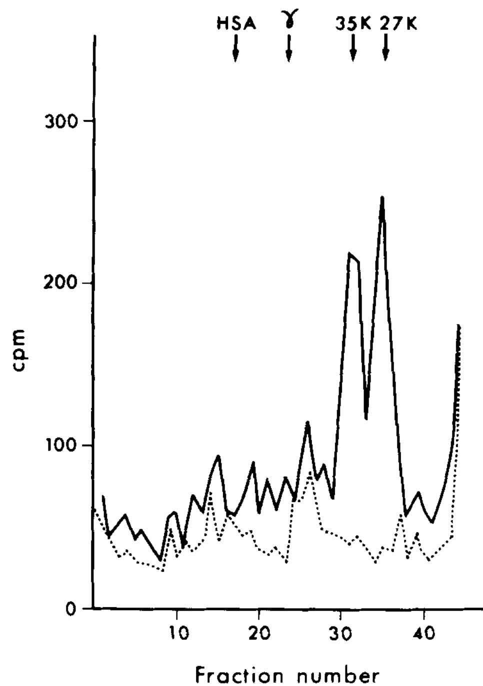

Figure 2. SDS-acrylamide gel analysis of radiolabeled surface material from T-sens. *Solid line,* immunoprecipitate obtained with the rabbit anti-Ia antiserum. Dotted line, immunoprecipitate obtained with the control rabbit serum. *Arrows* indicate the 27,000 and the 35,000 bimolecular complex of Ia, and the markers represent human serum albumin (HSA) and human IgG heavy chain (γ).

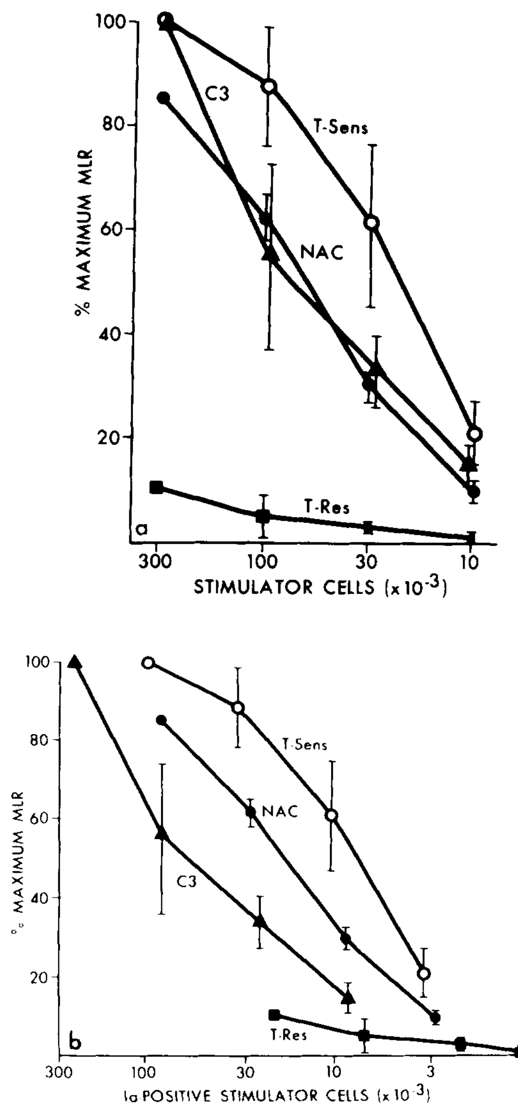

Figure 3. Limiting dilution analysis of MLR stimulating capacity of isolated lymphocyte subpopulations. *a,* Values expressed as a percent of the maximum MLR induced in responder cells by any of the cell populations. Mean (±1 S.D.) of four experiments is shown. *b,* As above except that the MLR stimulatory capacity was expressed on the basis of the number of Ia-positive cells added as stimulator cells.

monocytes are the most potent stimulator cells in MLR, and T cells have been reported to only stimulate weakly, if at all (20–25). When the isolated subpopulations were used as stimulator cells in the MLR, it was obvious on analysis of cell dilution protocols that not only did T-sens (but not T-res) stimulate in MLR, they appeared to be more potent stimulators than the B cell fraction on a per cell basis (Fig. 3*a*). Further, the number of Ia-positive cells in the B cell fraction was 2- to 3-fold greater than in the T-sens fraction, and when this capacity to stimulate in MLR was expressed as a function of the number of Ia-positive cells, it appeared that T-sens were about 10-fold more potent than the Ia-C3-positive B cells (Fig. 3*b*).

DISCUSSION

Certain products of the major histocompatibility complex (MHC) in man such as HLA-A, B, and C antigens, can be readily identified by serologic techniques and appear to be expressed equally on all lymphocytes. In contrast, another series of antigenic determinants, designated DR (or Ia-like), appear to be expressed preferentially on B lymphocytes and monocytes (5). Although the presence and functional role of Ia antigens has been demonstrated on certain murine T lymphocyte populations (reviewed in 26), the presence of similar antigens on human T cells is the subject of much discussion and, with one exception (27), appears restricted to activated or proliferating T cells (28).

We demonstrated Ia-like determinants on a subpopulation of human T lymphocytes by immunofluorescence with a rabbit anti-Ia antiserum. This antiserum was raised against purified B lymphoblast surface antigenic material and was rendered specific for the 27,000/35,000-dalton bimolecular complex of Ia by suitable absorption. After a three-step depletion procedure, Ia-positive T cells were identified in the theophylline-sensitive T cell subset, previously shown to mediate antigen-specific suppressor cell activity in an antigen-dependent PFC assay (9, 29). Approximately 20% of all peripheral blood T cells were isolated as T-sens cells. Since 25 to 45% of these cells were shown to be Ia-positive, we estimate that 4 to 7% of peripheral blood T cells carry Ia-like determinants. This figure is very similar to that proposed by Fu *et al.* (27) by using a different antiserum and different separation techniques.

The presence of Ia-like antigens on a significant proportion of theophylline-sensitive T cells was confirmed by surface labeling and immunoprecipitation studies. It is unlikely that the cells responsible for the binding were not T cells (but a B cell, monocyte, or null cell), since a) up to 90% of T-sens cells had E-receptors; b) less than 5% of these cells were non-T cells carrying sIg, C3, stained with the esterase reagent, or were found to be phagocytic; c) the intensity of staining on T cells was less than that observed on B cells; and d) in a double-marker assay, both Ia and SRBC were found on the same cells.

The many contradictory reports on the identity of the cell

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responsible for the major allogeneic stimulation in MLR (20–25) prompted us to test the various subpopulations obtained during the depletion protocol. Genes localized in the I region of the H-2 complex of the mouse control several basic immunologic phenomena, including the stimulation of allogeneic cells (26). Stimulation in the human MLR also appears to be controlled by genes of the MHC localized in the D region of chromosome 6. Our studies showed that a minor population of T lymphocytes, the theophylline-sensitive cells, were potent stimulators in the MLR, whereas the majority of the T cells, namely theophylline-resistant lymphocytes, were inactive. The stimulation in MLR by theophylline-sensitive cells could not be explained by a small degree of contamination by B cells or monocytes. In addition, the postulated "back stimulation" of responder cells by mitogenic factors released by irradiated T cells is unlikely, since the theophylline-resistant T cells were incapable of stimulation; both T cell subsets did respond in MLR.

In cell dilution experiments, theophylline-sensitive T cells appeared to be more potent stimulators than either the mixed nonadherent or purified C3-positive cell preparations. The stimulatory capacity of the theophylline-sensitive cells did not reflect (in a quantitative fashion) the expression of surface Ia molecules, since the B cell fraction contained more than twice the number of Ia-positive cells. Thus, when differences in stimulatory capacity were expressed as a function of the number of Ia-positive stimulator cells added, at a level of 50% maximum MLR, the T-sens fraction was about 10-fold more potent than the B cell fraction. Although the results presented are confined to peripheral blood T lymphocytes, similar results were obtained by using tonsillar lymphocytes and an alternative depletion protocol (9).

The strong association between specific HLA-D and Ia-like (DR) antigens makes it difficult to differentiate between the possibility that the genes that code for them are situated on the same locus or are on closely linked loci (30, 31). The MLR findings would suggest that either the Ia/D determinant is more exposed on T lymphocytes or that Ia and D are not identical but that the theophylline-sensitive fraction contains more D-bearing cells. It is also possible that all T-sens are Ia-positive but some cells are below the threshold for detection in our system.

In summary, we have demonstrated the presence of Ia-like determinants on a small subpopulation of human peripheral blood T cells known to contain specific T-suppressor cells and their precursors. This same subpopulation of cells has also been shown to carry MLR-stimulating determinants. Although additional studies are required to document that it is the Ia-positive cells that mediate these activities, we speculate that these "I-region" determinants expressed on theophylline-sensitive cells may be related to MHC-linked regulatory gene products equivalent to murine I-J determinants.

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