

ANTIBODIES TO MEMBRANE STRUCTURES THAT  
DISTINGUISH SUPPRESSOR/CYTOTOXIC AND HELPER  
T LYMPHOCYTE SUBPOPULATIONS BLOCK THE  
MIXED LEUKOCYTE REACTION IN MAN\*

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Since the description of Lyt subclasses of T lymphocytes in the mouse (1-3), two subpopulations of T cells in man have been defined by antibodies to heterologous membrane determinants (4, 5). Those determinants restricted to suppressor/cytotoxic T cells (herein termed Leu-2) belong to a membrane antigen that is similar in distribution, structure, and function to the mouse Lyt-2,3 molecule (6-8), whereas those restricted to helper T cells (Leu-3) belong to a single-chain membrane component of 55,000 mol wt (6, 8). Recent evidence that suggests that the Leu-2 molecule may act as an antigen receptor on alloantigen-activated cytotoxic T cells (7), prompted us to examine the possibility that both Leu-2 and Leu-3 antigens are involved in the cognitive functions of their respective subsets. Experiments detailed below indicate that high concentrations of antibodies to Leu-2 and Leu-3 sites block the proliferative responses of Leu-2 and Leu-3 T cells in the mixed leukocyte reaction (MLR). The implications of these findings with regard to the possible roles of Leu-2 and Leu-3 antigens in alloantigen recognition are discussed.

**Materials and Methods**

*Monoclonal Antibodies.* Mouse monoclonal anti-Leu-1, anti-Leu-2a, anti-Leu-3a, and anti-HLA-A2 antibodies were produced by the method of somatic cell hybridization and were purified from BALB/c ascites fluid by ammonium sulfate precipitation and ion-exchange chromatography (anti-Leu antibodies) or size-exclusion chromatography (anti-HLA-A2). All of these antibodies bind with similar affinity to human T cells and fail to lyse their target cells in the absence of complement. Anti-Leu-1 (clone L17F12) is an IgG2a antibody that binds to all peripheral T cells (9). The other three antibodies are of the IgG1 isotype. Anti-HLA-A2 antibody (designated PA2.1) was generously provided by Dr. Peter Parham of Stanford University, Stanford, Calif. (10).

*Isolation of E-Rosetting and Nonrosetting Cells.* Peripheral blood mononuclear leukocytes were obtained from normal volunteers by Ficoll-Hypaque density-gradient centrifugation of fresh defibrinated blood. Populations enriched for T and non-T cells were prepared by a sheep erythrocyte rosetting technique in which  $5 \times 10^6$  cells/ml were suspended in 40% fetal calf serum in phosphate-buffered saline (FCS-PBS) and mixed with an equal volume of 3% sheep

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erythrocytes in 40% FCS-PBS. The mixture was maintained at 4°C for 1 h. It was then sedimented over a second Ficoll-Hypaque gradient to separate the rosetted T cells (E-RFC) from the nonrosetted (non-T) cells. Cells recovered from the rosetted pellet by treatment with hypotonic NH<sub>4</sub>Cl solution contained <3% surface Ig<sup>+</sup> cells on the basis of direct immunofluorescence. In addition, <3% of the T cell-enriched fractions were monocytes, based on staining with  $\alpha$ -naphthyl acetate (11). The nonrosetting fraction was again treated with sheep erythrocytes as above, and the resultant population consisted of 48–55% surface Ig<sup>+</sup> cells and 24–46% monocytes.

*Isolation of T Cell Subsets.* To obtain purified subsets of T cells, a panning technique as modified by Wysocki and Sato (12) was used to fractionate the E-RFC into Leu-2<sup>+</sup>/Leu-2<sup>-</sup> or Leu-3<sup>+</sup>/Leu-3<sup>-</sup> subpopulations. In this technique  $2 \times 10^7$ – $3 \times 10^7$  unfractionated E-RFC were incubated with ~20  $\mu$ g of monoclonal antibody for 20 min. The cells were then washed twice in 5% FCS-PBS and poured into 15- $\times$ 100-mm plastic petri dishes (Lab-Tek Products, Naperville, IL) previously coated with affinity-purified goat anti-mouse IgG (Tago, Inc., Burlingame, Calif.) at 10  $\mu$ g/ml in 0.05 Tris buffer (pH 9.5). The dishes were then incubated at 4°C for 2 h, after which, nonadherent cells were collected, and the dishes were gently washed five times with 1% FCS-PBS before recovery of bound cells by vigorous pipetting. When anti-Leu-2a was used as the first-stage incubating antibody, <5% of the unbound cells were Leu-2<sup>+</sup> and >98% of the bound cells were Leu-2<sup>+</sup> on the basis of analysis in a fluorescence-activated cell sorter. Similar subset purity was achieved when anti-Leu-3a antibody was used as the first-stage incubating antibody.

*MLR.* Responder cells were obtained from individuals known to be HLA-A2<sup>+</sup> both on the basis of binding to monoclonal anti-HLA-A2 antibody and on the basis of typing by standard lymphocytotoxicity with alloantisera. Stimulator cells were obtained from HLA-A2<sup>-</sup> donors.

MLR were performed in round-bottomed microtiter wells containing RPMI-1640 medium supplemented with 25 mM Hepes buffer, 2 mM glutamine, 100 U/ml of penicillin, 100  $\mu$ g/ml streptomycin, and 10% de complemented pooled human serum. Triplicate cultures were incubated at 37°C in a 10% CO<sub>2</sub> humidified atmosphere. Cultures consisted of  $5 \times 10^4$  responder T cells,  $5 \times 10^3$ – $2.5 \times 10^4$  allogeneic stimulator non-T cells irradiated to 3,000 rad (<sup>136</sup>Cs Irradiator; J. L. Shepherd & Associates, Glendale, Calif.), and the indicated antibody in a total vol of 0.20 ml. Proliferation was measured by addition of [<sup>3</sup>H]thymidine (1  $\mu$ Ci/well) 18 h before harvesting with a MASH II apparatus (Microbiological Associates, Walkersville, Md.).

## Results

Table I summarizes the effects of various monoclonal antibodies on the response of unseparated T lymphocytes in MLR. As shown, 10  $\mu$ g (final concentration, 50  $\mu$ g/ml) of either anti-Leu-2a or anti-Leu-3a significantly inhibited proliferation by T cells responding to  $5 \times 10^3$  allogeneic non-T cells. Little or no inhibition of MLR was produced by the same concentration of anti-Leu-1 or anti-HLA-A2 antibodies. The effect of anti-Leu-3a was somewhat greater than the effect of anti-Leu-2a, an observation reproduced in five of six separate experiments. However, inhibition was never complete and was diminished by either reducing the concentration of antibody or increasing the number of stimulator cells (Table I).

Because proliferation was measured on the 7th d of culture, the effects of these antibodies might have been a result of alteration of the proliferative kinetics. This was excluded by harvesting replicate cultures after varying periods of incubation. As shown in Fig. 1A, suppression of the T cell response by anti-Leu-2a or anti-Leu-3a was greatest on days 5–7. The same figure also shows that the inhibitory effects of these antibodies were additive. Thus, when both antibodies were present in high concentration, the response was almost abolished.

To assess the effects of anti-Leu-2a and anti-Leu-3a antibodies on the response of isolated T cell subsets, E-RFC were fractionated into Leu-2 and Leu-3 subpopulations

TABLE I  
*Inhibitory Effects of Anti-Leu-2a and Anti-Leu-3a Antibodies on T Cell Proliferation in MLR\**

| Antibody    | µg/culture | Background | Proliferation induced by |                       |                          |                       |
|-------------|------------|------------|--------------------------|-----------------------|--------------------------|-----------------------|
|             |            |            | Stimulator 1, No. cells: |                       | Stimulator 2, No. cells: |                       |
|             |            |            | 5 × 10 <sup>3</sup>      | 2.5 × 10 <sup>4</sup> | 5 × 10 <sup>3</sup>      | 2.5 × 10 <sup>4</sup> |
| None        |            | 137        | 18,867                   | 37,233                | 23,870                   | 52,840                |
| Anti-Leu-1  | 10         | 201        | 20,166                   | 35,519                | 24,955                   | 50,373                |
|             | 1          | 175        | 19,880                   | 36,812                | 25,044                   | 53,831                |
| Anti-Leu-2a | 10         | 105        | 9,863‡                   | 33,104                | 12,419‡                  | 44,076                |
|             | 1          | 116        | 14,040                   | 39,619                | 26,812                   | 53,301                |
| Anti-Leu-3a | 10         | 161        | 5,099§                   | 29,586                | 8,514§                   | 28,361‡               |
|             | 1          | 123        | 10,369‡                  | 33,911                | 12,352‡                  | 39,435                |
| Anti-HLA-A2 | 10         | 98         | 19,865                   | 32,301                | 22,517                   | 44,131                |
|             | 1          | 110        | 16,194                   | 34,455                | 20,532                   | 42,718                |

\* 5 × 10<sup>4</sup> responder T cells from an HLA-A2<sup>+</sup> donor were cultured for 7 d with 5 × 10<sup>3</sup> or 2.5 × 10<sup>4</sup> irradiated (3,000 rad) allogeneic non-T cells from two HLA-A2<sup>-</sup> donors. Results represent the mean of triplicate cultures in counts per minute.  
 ‡ Inhibition significant at P < 0.05 by two-tailed t test.  
 § Inhibition significant at P < 0.01 by two-tailed t test.

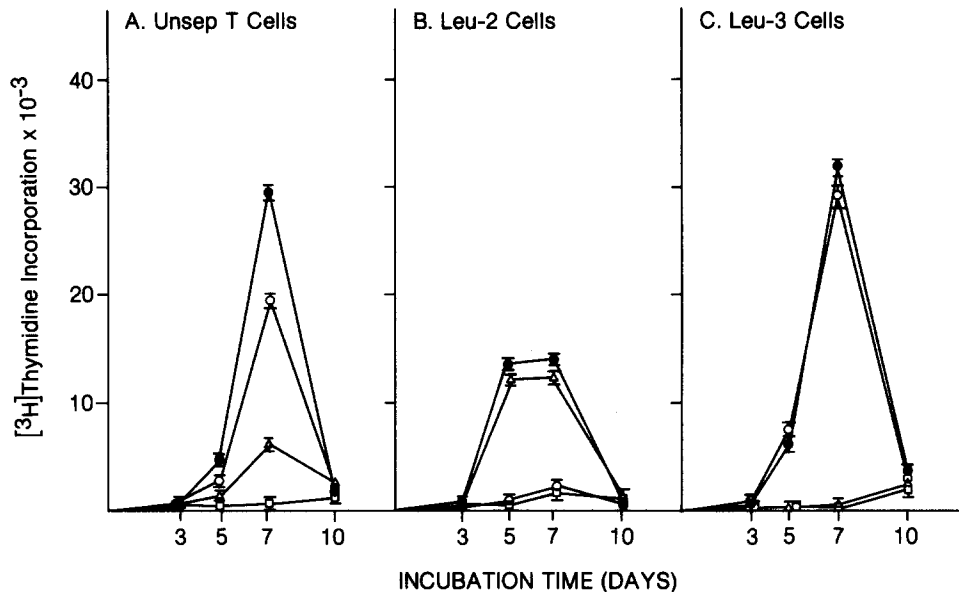


FIG. 1. Kinetics of MLR suppression by anti-Leu-2a and anti-Leu-3a antibodies. 5 × 10<sup>4</sup> unseparated T cells (A), Leu-2<sup>+</sup> cells (B), or Leu-3<sup>+</sup> cells (C) were cultured with 5 × 10<sup>3</sup> irradiated (3,000 rad) allogeneic non-T cells in the absence of added antibody (●) or in the presence of 10 µg anti-Leu-2a (○), 10 µg anti-Leu-3a (△), or 10 µg of each antibody (□). Cultures were begun on day 0 and harvested on days 3, 5, 7, and 10. Responses represent the mean of triplicate cultures ± 1 SD.

and incubated separately with allogeneic stimulator cells in the presence of specific antibody. Fig. 1B and C demonstrates that inhibition by these antibodies is limited to antibody-reactive cells. Near-complete inhibition of the response of isolated subsets was obtained with 10 µg of appropriate subset-specific antibody and was not a result

of retarded or accelerated kinetics. Although the results shown were obtained with positively selected subsets (i.e., Leu-2<sup>+</sup> and Leu-3<sup>+</sup>), similar results were obtained by using negatively selected subsets (i.e., Leu-2<sup>-</sup> and Leu-3<sup>-</sup> populations). As in the response by unseparated T cells, inhibition of the subset responses was markedly diminished when lower concentrations of antibody or higher numbers of non-T stimulator cells were used (data not shown). Anti-Leu-1 and anti-HLA-A2 antibodies had no effect on the subset responses (data not shown).

### Discussion

These results demonstrate that monoclonal anti-Leu-2a and anti-Leu-3a antibodies block proliferation in MLR of Leu-2 and Leu-3 T cells, respectively. The failure of either antibody to completely inhibit the response of unfractionated T cells is explained by the observation (Fig. 1 B and C) that both subsets proliferate independently in response to alloantigen. Blocking is a result of the direct effect of antibody on responder T cells, which bind the antibodies, rather than the non-T stimulator cells. Time-course experiments ruled out the possibility that inhibition was a result of alteration of MLR kinetics. Although it is possible that the inhibitory effects of anti-Leu-2a and anti-Leu-3a could result from destruction of the responding cells by antibody-dependent cell-mediated cytotoxicity (13), a monoclonal anti-HLA-A2 of the same subclass (IgG1) had little or no effect on the response of HLA-A2<sup>+</sup> individuals. IgG2a monoclonal antibody to Leu-1, an antigen distributed on all peripheral T cells, also had no inhibitory effect on the MLR. Alternatively, our results might be explained by steric hindrance of structures adjacent to the Leu-2a and/or Leu-3a sites which have functions critical to T cell activation. However, this would seem unlikely to occur on cells of both subsets.

Because blocking by both Leu antibodies was augmented by increasing their concentration and decreasing the concentration of stimulator cells, it would seem that anti-Leu-2a and anti-Leu-3a compete with stimulator cell antigens for T cell membrane sites which are involved either in antigen recognition by T cells or in other events which lead to T cell activation. At least in the case of the Leu-2 molecule, this possibility is strengthened by the observation that anti-Leu-2 antibodies inhibit killing by cytotoxic T cells in cell-mediated lympholysis (7). Moreover, experiments described elsewhere suggest that different histocompatibility antigens are involved in the activation of Leu-2 and Leu-3 T cells.<sup>1</sup> In light of those data, the present findings might be interpreted as indicating that the Leu-2 and Leu-3 molecules function as receptors for the target alloantigens of their respective subsets.

Previous studies in mice have shown that antibodies to Lyt-2,3 sites block cytotoxicity by alloactivated T cells (14-18) and block proliferation in the MLR (16, 17). The tight linkage of genes coding for Lyt-2,3 determinants and genes coding for immunoglobulin  $\kappa$  chains, has therefore raised the possibility that part of the Lyt-2,3 molecule is a clonally determined antigen receptor (19-21). Although this hypothesis may be relevant to the functional nature of the human Leu-2 antigen, which is homologous to the Lyt-2,3 molecule (6-8), no mouse counterpart to Leu-3 has been identified.

<sup>1</sup> Engleman, E. G., C. J. Benike, F. C. Grumet, and R. L. Evans. Activation of human T lymphocyte subsets: helper and suppressor/cytotoxic T cells recognize and respond to distinct histocompatibility antigens. Manuscript submitted for publication.

### Summary

Two major subsets of human T lymphocytes that are functionally analogous to the mouse Lyt-2<sup>+</sup> and Lyt-2<sup>-</sup> subsets have been defined by their expression of two thymus-dependent membrane antigens, Leu-2 and Leu-3. Leu-2<sup>+</sup>,3<sup>-</sup> cells have suppressor/cytotoxic functions and Leu-2<sup>-</sup>,3<sup>+</sup> cells have helper functions. These studies were designed to determine the effects of adding IgG1 monoclonal anti-Leu-2 and anti-Leu-3 antibodies to the mixed leukocyte reaction (MLR). At high concentrations, each antibody partially inhibited the proliferative response of unseparated T cells and abolished the response of the isolated subset having the appropriate phenotype. An IgG1 monoclonal antibody to HLA-A2 and an IgG2a antibody to Leu-1, a pan-T antigen, failed to inhibit the MLR. These results suggest that the Leu-2 and Leu-3 antigens may have a direct role in the mechanism whereby T cells recognize and respond to alloantigen.

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