PERIPHERAL HUMAN T CELLS SENSITIZED IN MIXED LEUKOCYTE CULTURE SYNTHESIZE AND EXPRESS Ia-LIKE ANTIGENS*

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It has been shown in the mouse that antigen triggers distinct subpopulations of thymus-derived lymphocytes to express specific immunoregulatory activities, and that these activities are mediated in part by intercellular signals involving products of the I-region (Ia antigens) of the major histocompatibility complex. Thus, discrete Ia antigens have been detected on soluble T-cell factors which have either helper (1) or suppressor (2) activities, and are also present on the surface membrane of suppressor T cells (3), and Con A-induced T-cell blasts (4).

Although Ia antigens have been generally thought not to be expressed by normal or leukemic T cells in man (5, 6), this view was re-examined when we found that antibody to a human Ia-like antigen, p23,30 occasionally reacted with leukemic blasts which also expressed thymus-dependent markers. p23,30 is a glycoprotein complex of 23,000 and 30,000 dalton subunits, isolated from the papain-solubilized membrane of a human lymphoblastoid B-cell line (7). A rabbit anti-p23,30 serum binds to peripheral B cells, monocytes, and a subpopulation of null cells, but is unreactive with normal human T cells or thymocytes (6, 8). In addition to conforming in molecular weight and normal tissue distribution with murine Ia antigen, the detergent-solubilized form of p23,30, p29,34, reacts with B-cell alloantisera which are specific for determinants coded by the HLA-D locus, or I-region counterpart in man (9).

We report here that determinants recognized by anti-p23,30 are expressed on the surface membrane of T cells which are transformed by alloantigen in mixed leukocyte culture (MLC), but are not detectable on T cells which are either freshly purified or maintained without stimulation in culture for 6 days. Moreover, allosensitized T cells were shown to elaborate and to incorporate into their surface membranes a 29,000 and 34,000 dalton, HLA-D-related complex.

Materials and Methods

Isolation of Human T Lymphocytes. T cells were isolated from peripheral blood mononuclear cells by nylon wool purification followed by formation and density gradient sedimentation of sheep erythrocyte-rosetting cells (10).

Sensitization Cultures. T cells were sensitized to allogeneic mitomycin-C-treated mononuclear cells (10). Of the cells recovered after a 6-day sensitization, greater than 98% were reactive with a heterologous antiserum which was specific for thymus-derived lymphocytes.

Cell-Mediated Lympholysis (CML) Assay.   CML was determined in a 6-h ^{51}Cr-release assay (10).

Antisera.   Anti-p44,12 serum was raised in rabbits to HLA-A and -B antigens and β_2-microglobulin purified from the spleen of a patient with poorly-differentiated histiocytic lymphoma (11). This antiserum has a 1:1,000 cytotoxic titer for lymphocytes and recognizes only HLA-A and -B antigens and B$_2$-microglobulin in immunoprecipitates of [^{35}S]methionine-labeled, detergent-solubilized cells. The preparation of anti-p23,30 serum has been described previously (7). Anti-p23,30 serum recognizes the native 29,000 and 34,000 dalton HLA-D complex in immunoprecipitates of detergent solubilized cells.

Labeling of Cells with [^{35}S]Methionine.   T cells were recovered from a 6-day one-way MLC, washed, and resuspended in 5 ml RPMI-1640 medium lacking methionine (Grand Island Biological Co., Grand Island, N.Y.). After incubation for 2 h with a preparation of neutralized hydrolysate of [^{35}S]labeled *Escherichia Coli* containing 1-2 mCi [^{35}S]methionine (0.065 ml) (12), the cell membranes were harvested.

Isolation of Cell Membranes.   Cells were resuspended in 0.01 M Tris-HCl buffer pH 8.1 (5 ml) and centrifuged at 2,400 g for 5 min. The pellets were re-extracted by this procedure and the supernatant solutions from each pellet were pooled and centrifuged at 110,000 g for 1 h to sediment the plasma membranes.

Double Antibody Coprecipitation of Antigens.   [^{35}S]methionine-labeled membranes were solubilized in 1% Triton X-100 detergent in normal saline. This solution was centrifuged at 100,000 g for 1 h and the supernatant solution was cleared by the addition of a normal rabbit serum (NRS) and an aliquot of formalin-treated *Staphylococcus aureas*. Specific precipitations were then performed on the cleared supernate (13). These precipitates were suspended in 1 ml Hanks' balanced salt solution, and layered over 1 ml 24% sucrose solution (1 ml), and centrifuged at 4,500 g for 15 min. The precipitates were washed, eluted from the bacteria with 6 M urea, 4% sodium dodecyl sulfate (SDS) solution, reduced with dithiothreitol, and alkylated with iodoacetic acid.

Polyacrylamide Gel Electrophoresis.   The SDS polyacrylamide gel system of King and Laemmli was employed (14).

Results and Discussion

Initially we studied the binding of anti-p23,30 to allosensitized T cells by indirect immunofluorescence using a fluorescence-activated cell sorter (FACS 1). As shown by a representative experiment in Fig. 1, neither freshly purified T cells, nor T cells incubated for 6 days with autologous cells exhibited fluorescence above a NRS control. However, approximately 50% of the T cells sensitized to alloantigen in a standard 6 day one-way MLC reacted significantly above background. Anti-p23,30 was found to bind a large but variable fraction of alloantigen-activated T cells from a number of normal donors. The magnitude of this fraction depended on T-cell transformation in culture, because appropriate gating of the FACS 1 indicated that the p23,30$^+$ population was composed almost entirely of transformed (large) cells, which in turn, were entirely p23,30$^+$.

To provide conclusive evidence for the modulation of HLA-D antigens on the surface membrane of alloreactive T cells, we studied the effect of anti-p23,30 and complement on CML by T cells treated before and after sensitization in MLC. Initially, we determined the effect of this treatment on the capacity of T cells to generate killer activity in CML. Freshly purified T cells were incubated with either media, a NRS, or anti-p23,30 serum (1/20 dilution), washed, and treated with rabbit serum as a source of complement. The cells were then reconstituted to equal numbers of viable cells and sensitized to alloantigen in a 6-day one-way MLC. As shown in Fig. 2 A, anti-p23,30 and complement had no effect on the generation of killer cells, which conformed to the results of prior studies indicating that HLA-D antigens are not expressed by resting T cells. The effect of anti-p23,30 and complement on the
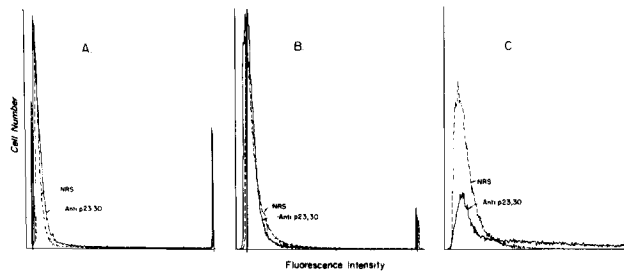
Fig. 1.   The reactivity with anti-p23,30 serum by human T cells before and after activation in MLC as shown by indirect immunofluorescence analysis on a FACS 1. Histograms show analyses of T cells either freshly-purified (A), or incubated for 6 days in a standard one-way MLC with either autologous lymphocytes (B), or allogeneic lymphocytes (C). 1 × 10^6 cells were labeled at 4°C with either a rabbit anti-p23,30 serum (——), or a normal rabbit serum (NRS) (- - -) at a 1/50 dilution, washed and stained with a goat anti-rabbit Fc antibody conjugated to fluorescein isothiocyanate. The percent T cells binding to anti-p23,30 was calculated by dividing the number fluoresceing above the NRS background by the total number analyzed (40,000).
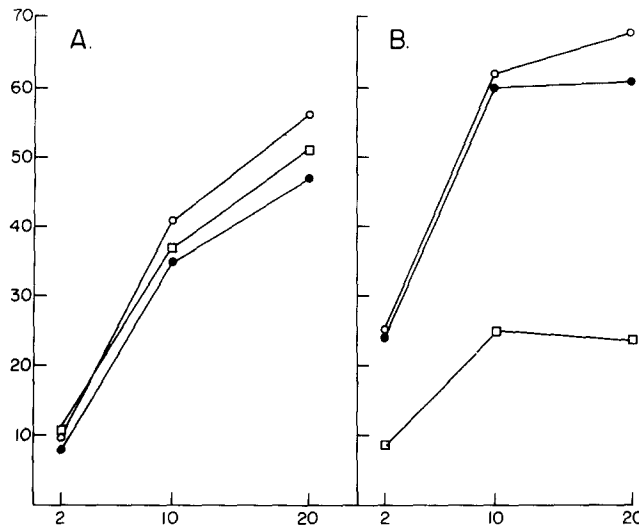

Fig. 2.   The effect of anti-p23,30 and complement on CML by T cells treated before or after sensitization to alloantigen in vitro. T cells were incubated before (A) or after (B), priming in a 6 day, one-way MLC with either media (○), a NRS (●), or anti-p23,30 (□), washed and treated with rabbit serum as a source of complement.

lytic activity of allosensitized T cells was then determined by treating 10 × 10^6 cytotoxic T cells with either media, a NRS, or anti-p23,30 serum. The cells were then washed, treated with complement, reconstituted to equal numbers of viable cells, and assayed for killer activity against the appropriate allogeneic targets. As shown in Fig. 2 B, treatment with anti-p23,30 markedly depressed killing, while the NRS had no effect, indicating that MLC-primed T cells responsible for CML bear HLA-D antigens, whereas their unactivated progenitors do not.

While these experiments clearly demonstrated that Ia-like antigens appeared on the surface membrane of T cells as the result of activation in MLC, they did not prove these antigens to be T-cell products. Because in the mouse I-region products from stimulator cells have been shown to be bound to the surface membrane of
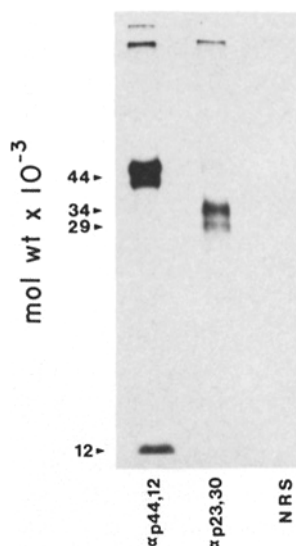
Fig. 3.   Synthesis of HLA-A, -B, and -D antigens and B₂-microglobulin by allosensitized T cells. SDS polyacrylamide gel electrophoresis of radioactive p44,12 (HLA-A and -B antigens and β_2-microglobulin) and p29,34 (HLA-D antigen) precipitated from detergent-solubilized membranes of allosensitized T cells labeled with [35S]methionine. NRS precipitated no proteins. Molecular weights are indicated on the ordinate.

responder T-cell blasts (15), it was important to establish that these antigens were synthesized by allosensitized T cells.

This was accomplished by immunoprecipitation and SDS polyacrylamide gel electrophoresis of a radioactive p29,34 complex from the plasma membranes of MLC-primed T cells which had been labeled with [35S]methionine. The presence of significant numbers of viable stimulator B cells or monocytes was excluded by the demonstration that a heterologous antiserum specific to T cells reacted with greater than 98% of the cells recovered from MLC as determined by indirect immunofluorescence on an FACS 1. In addition to anti-p23,30 serum, a rabbit anti-p44,12 serum which recognized HLA-A and -B antigens (44,000 daltons) and β_2 microglobulin (12,000 daltons) was employed to precipitate these antigens as reference proteins. As shown by an autoradiogram of a representative gel in Fig. 3, both p29,34 and p44,12 antigens were precisely defined by this technique. Although the radioactivity of the HLA-D complex was appreciably less than that of the HLA-A or -B antigens, the former is clearly demonstrated to be synthesized by allosensitized T cells.

It should be mentioned that HLA-D determinants were also found on T cells transformed in cultures containing either specific antigens, Con A, or phytohemagglutinin. However, the requirement of monocytes to induce T-cell activation in these cultures would have obviously frustrated our experimental intent. We therefore sensitized highly purified T cells in MLC, because the possibility that mitomycin-C-treated B cells or monocytes survived 6 days under these conditions was unlikely, and was in fact excluded by examining the purity of the recovered T cells.

Finally, these studies raise a number of interesting questions related to the role of murine Ia antigens in mediating T-cell immunoregulatory functions. As human analogues of Ia antigens, HLA-D related T-cell products may be part of the network

through which cell-cell signals are conveyed by functional subsets of T cells. This notion is supported by the recent demonstration that an antigen-specific T-cell helper factor is structurally associated with determinants recognized by B-cell alloantisera specific to HLA-D antigens (R. Geha, personal communication). In this regard we would emphasize that these studies should not be taken as proof that MLC-primed cytotoxic T cells actually synthesize the HLA-D antigens which they express, because these structures may be received by T-cell blasts as regulatory signals from a subset(s) of T cells. It would therefore be of interest to define structural distinctions of p29,34 which are unique to T cells or to functional subsets of T cells (10).

Summary

We have studied the modulation of Ia-like antigens on the surface membrane of human T cells responding in a one-way mixed leukocyte culture. A heterologous antiserum, (anti-p23,30), which is specific to HLA-D-related antigens and which is unreactive with normal peripheral T cells or thymocytes, was found to bind significantly to all T cells transformed in mixed leukocyte culture (MLC) as determined by indirect immunofluorescence on a fluorescence-activated cell sorter 1. Furthermore, cytotoxic T cells responsible for cell-mediated lympholysis were shown to react with anti-p23,30, whereas their unactivated progenitors did not. Immunoprecipitation and sodium dodecyl sulfate polyacrylamide gel electrophoresis of a radioactive 29,000 and 34,000 dalton complex from MLC-primed T cells labeled with [^{35}S]methionine indicated that allosensitized T cells synthesized these HLA-D-related antigens.

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