

IDENTIFICATION OF SHARED ANTIGENIC DETERMINANTS OF THE PUTATIVE HUMAN T LYMPHOCYTE ANTIGEN RECEPTOR

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Monoclonal antibodies (mAb)¹ directed against a T lymphocyte membrane protein complex having characteristics predicted for the T lymphocyte antigen receptor have recently been described. A disulfide-linked, heterodimer (alpha [α] chain, M_r 41,000 and beta [β] chain, M_r 39,000) that appeared to be clone-specific was first identified on a murine T cell lymphoma and was suggested as a candidate for the T cell receptor (1). This protein complex was more firmly implicated as the putative antigen receptor on the basis of functional studies with mAbs against similar proteins on immunocompetent human T cell clones and murine T cell hybridomas. These mAbs could be shown either to block cytolytic function or to stimulate proliferation or interleukin-2 (IL-2) production in a clone-specific manner (2-5).

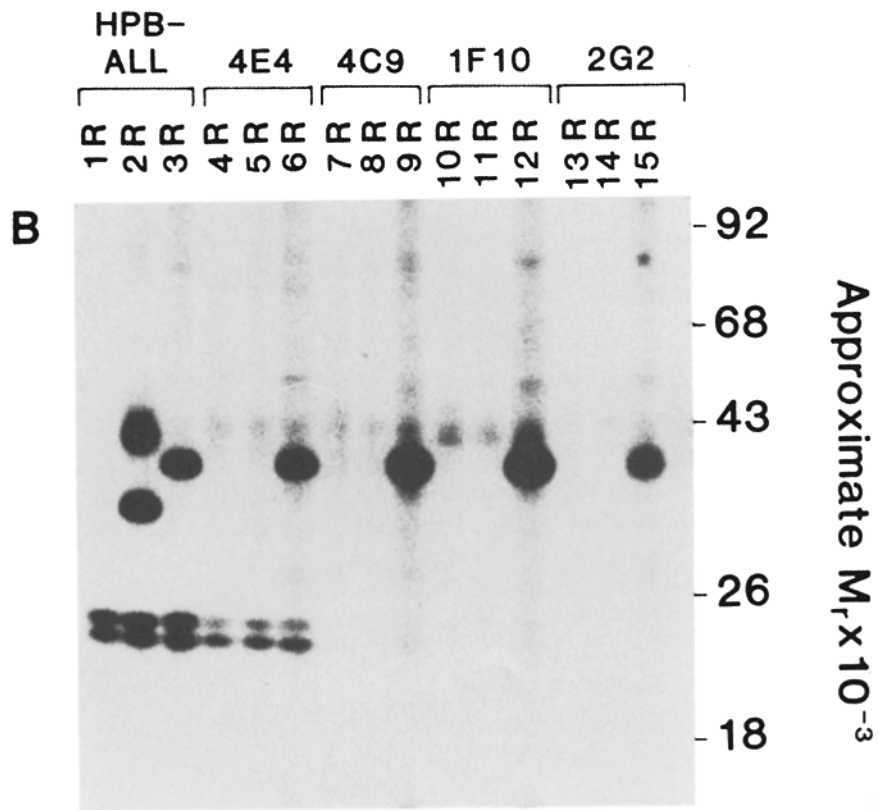
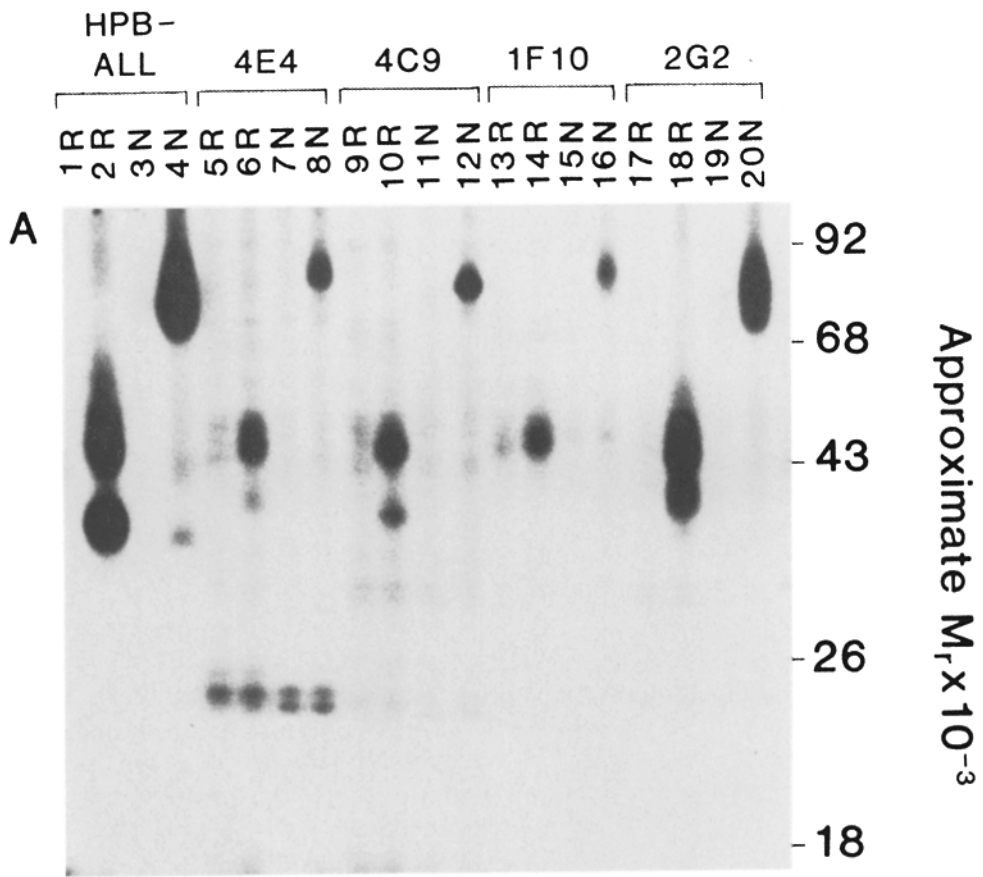
Biochemical characterization of the putative T cell receptor molecules from different cell lines by peptide mapping revealed both shared and unique peptides (6-8). Moreover, cDNA clones that may represent one of the chains of the T cell antigen receptor (since they detect a genomic DNA rearrangement in T cells) have been obtained and sequenced. Their encoded amino acid sequences suggest that this chain contains regions analogous to variable and constant regions of B cell immunoglobulin (9-11). In this report we demonstrate that the human T lymphocyte antigen receptors from different cloned cell lines share antigenic determinants, some of which can be shown to be on the α (heavy) subunit of the complex and none of which appear to be accessible at the cell surface.

Materials and Methods

Antibodies. mAbs used were mAb T40/25, anti-antigen receptor on HPB-MLT and HPB-ALL (8); mAb P3, used as a control for nonspecific immunoprecipitation (12); mAb UCHT-1, anti-human T3, kindly given by Dr. Peter Beverly (13); mAb T29/33, anti-human T-200 (14); and mAb W6/32, anti-HLA-A,B,C monomorphic determinant (15). Anti-gp40,49 is a mouse heteroantiserum prepared by immunizing BALB/c mice with an

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¹*Abbreviations used in this paper:* CTL, cytotoxic T lymphocyte; DTT, dithiothreitol; IAA, iodoacetamide; IL-2, interleukin 2; mAb, monoclonal antibody; NMS, normal mouse serum; NP-40, Nonidet P-40; PBL, peripheral blood lymphocyte; PBS, phosphate-buffered saline; SACI, *Staphylococcus aureus* Cowan I strain; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.



immunoprecipitate of gp40,49 (known to be a glycoprotein based on lectin binding and endoglycosidase sensitivity, unpublished observations). Briefly, 2×10^8 HPB-MLT cells were solubilized in 1% Nonidet P-40 (NP-40) and immunoprecipitated with 5 μ l of mAb T40/25 ascites. The immune complexes (mAb T40/25-gp40,49) were adsorbed to fixed *Staphylococcus aureus* Cowan I strain (SACI) and injected subcutaneously in phosphate-buffered saline (PBS) at multiple sites every 3 wk. 9–15 wk later, mouse heteroantiserum (anti-gp40,49) was obtained.

T Cell Lines and Clones. HPB-ALL and HPB-MLT are human T cell leukemia lines without known antigen specificity (16). Immunocompetent T cell lines and clones were generated in vitro from alloantigen-stimulated cultures propagated in conditioned media containing IL-2 activity (17). Each T cell line or clone was shown to have antigen specificity by cell-mediated cytotoxicity of target cells or specific proliferation responses to irradiated allogeneic stimulator cells. The lines chosen for study had non-overlapping specificities. 4E4 was cloned by limiting dilution (<1 cell/well) with ~3% of wells positive for growth. It is a cytolytic T lymphocyte (CTL) T3+, T8+, T4- functionally allospecific for HLA-A2. The other cell lines were derived from a different responder and have been subcultured at 5 cells per well. 2G2 is a T3+, T8+, T4- CTL line allospecific for HLA-B27. 4C9 is T3+, T8-, T4+, whereas 1F10 is T3+, 60%T8+, 40%T4+. Both these cell lines show moderate cytolytic activity and proliferative responses to different allogeneic cells but their specificities are not fully characterized.

Radiolabeling, Solubilization, and Immunoprecipitation. Cells were 125 I labeled using lactoperoxidase with either exogenously added hydrogen peroxide or glucose oxidase and glucose as previously described (18, 19). Briefly, cells were isolated by Ficoll-hypaque centrifugation and were typically >95% viable with trypan blue exclusion. The cells were washed three times and 10^7 cells were suspended in 1 ml of PBS, pH 7.4 containing 1 mM magnesium chloride. For the first procedure, 100 μ g of lactoperoxidase (80–100 U/mg, Sigma) was added, followed by 1 mCi of carrier free Na^{125}I (New England Nuclear). Then 20 μ l of a 0.03% hydrogen peroxide stock solution was added every 3 min for a total of six additions. This procedure was used for the immunoprecipitations shown in Figs. 1 and 3. For the second technique, exogenous hydrogen peroxide was omitted and generated endogenously with glucose oxidase (0.1 U/ml, Calbiochem) and glucose (5 mM). This procedure was used for the immunoprecipitations shown in Fig. 2. In both cases cells were labeled at room temperature. When viable cells are used, these procedures have been shown to selectively label cell surface proteins (18, 19). Immunoprecipitates were analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) as previously described (20, 21).

Fluorescence-activated Cell Analysis. One million HPB-MLT cells, PBL or 4E4 cells were incubated with saturating amounts of specific antibody (mAb T40/25 5 μ g/ml, anti-gp40,49 antiserum diluted 1:10, or UCHT-1 ascites diluted 1:100), washed, and then stained with fluorescein isothiocyanate-goat anti-mouse IgG or F(ab) $'_2$ as the second stage reagent. Analysis was performed using a Los Alamos design cell sorter as previously described (22).

FIGURE 1. SDS-PAGE analysis (10% acrylamide) of immunoprecipitates from 125 I-labeled T cell lines with mAb T40/25, anti-gp40,49 serum, and mAb W6/32. (A) Even numbered lanes were reduced (R) or nonreduced (N) immunoprecipitates utilizing mAb T40/25 for HPB-ALL cells and anti-gp40,49 for immunocompetent T cell lines 4E4, 4C9, 1F10, and 2G2. The odd-numbered lane immediately preceding each even numbered lane is a control immunoprecipitate, reduced or nonreduced, utilizing normal mouse serum (NMS). Reduced samples were incubated in 2 mM dithiothreitol (DTT) before boiling in SDS and all samples were incubated in an excess (15 mM) iodoacetamide (IAA) after boiling. (B) Immunoprecipitates from the same cell lines shown in A utilizing mAb P3 (negative controls, lanes 1, 4, 7, 10, and 13), mAb T40/25 (anti-receptor on HPB-ALL, lanes 2, 5, 8, 11, and 14), and mAb W6/32 (positive controls, lanes 3, 6, 9, 12, and 15). All samples were reduced in 5% 2-mercaptoethanol. The positions of M_r markers are shown to the right.

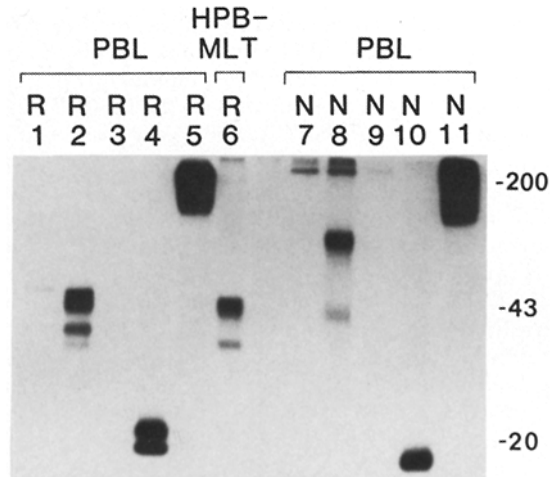


FIGURE 2. SDS-PAGE analysis (10% acrylamide) of ^{125}I -labeled immunoprecipitates from Ficoll-Hypaque separated human PBL and HPB-MLT lymphoma cells. Anti-gp40,49 serum immunoprecipitates from PBL are shown reduced (R) in lane 2 and nonreduced (N) in lane 8. For comparison the anti-gp40,49 immunoprecipitate from HPB-MLT cells is shown in lane 6 (R). mAb T40/25 did not precipitate a specific band (lanes 3 and 9) from PBL. Nonspecific precipitates with NMS are shown in lane 1 (R) and lane 7 (N). Positive control precipitates from PBL with mAb UCHT-1 (anti-T3) are shown reduced (lane 4) and nonreduced (lane 10) and for T29/33 (anti-T200) reduced lane 5 and nonreduced lane 11. The positions of M_r markers are shown to the right $\times 10^{-3}$.

Results

mAbs against the putative T cell receptors on human and murine T cell lines that have been reported are all clone-specific, with the exception of one mAb that appeared to recognize 2–3% of peripheral blood lymphocytes (23). To investigate whether antibodies against common determinants of the human T cell receptor could be obtained, mice were immunized with the putative T cell receptor dimer, gp40,49, isolated by immunoprecipitation from HPB-MLT cells with the clonotypic mAb T40/25. Reactivity of the antiserum obtained was investigated in immunoprecipitation studies using a panel of normal, immunocompetent T cell lines. Mouse anti-gp40,49 serum specifically precipitates labeled molecules from each of four different immunocompetent T cells surface-labeled by the lactoperoxidase technique (Fig. 1A). Under reducing conditions, two labeled bands were isolated from 4E4, 4C9, and 2G2 cells, whereas only one labeled band was obtained from 1F10 cells. However, it could be shown that each cell line yielded disulfide-linked dimers; the α subunit (M_r 46,000–49,000) and β subunit (M_r 40,000–45,000) appeared as a single band (M_r 70,000–85,000) under nonreducing conditions. Despite the biochemical similarity of the products isolated from the four lines, each was readily distinguished from the others and from the complex recognized by mAb T40/25 on HPB-ALL cells (lanes 2 and 4) by differences in relative molecular mass. In Fig. 1A the β (light) subunits appear the most variable in M_r , but on other gels of higher acrylamide percentage and on three additional lines the α (heavy) subunit was also observed to vary in M_r . The reactivity of mouse anti-gp40,49 serum can be contrasted with that of mAb T40/25, which did not precipitate the receptor complex from any of the

immunocompetent T cell lines (Fig. 1B). Sequential immunoprecipitation studies showed that mAb T40/25 and anti-gp40,49 serum recognize antigenic determinants on the same molecules from HPB-ALL cells. In further immunoprecipitations, anti-gp40,49 did not react with several non-T cells, including the B lymphoblastoid cell lines JY and HOM-2, erythroid leukemia cell line K562, and the histiocytic lymphoma cell line U937.

The ability of mouse anti-gp40,49 serum to recognize the receptor complex on unstimulated peripheral blood lymphocytes (PBL) was also tested. Anti-gp40,49 serum precipitated three bands from PBL under reducing conditions (Fig. 2, lane 2), including a more diffuse α chain than that of HPB-MLT (lane 6) and two distinct light chains. The amount of radioactivity precipitated by anti-gp40,49 serum from PBL was similar to that associated with the T3 glycoprotein precipitated by mAb UCHT-1, suggesting that the antiserum was reacting with a significant fraction of T cell receptor molecules present in the PBL lysate. mAb T40/25 did not precipitate detectable amounts of the T cell receptor complex from the PBL lysate (lanes 3 and 9).

To determine whether the common antigenic determinant(s) on the T cell receptor complex recognized by anti-gp40,49 serum could be localized to the α or β subunit of the molecule the following experiment was performed. The T cell receptor complex was isolated from the T cell line 2G2 and the α and β subunits dissociated by heating in the presence of SDS and dithiothreitol. After alkylation and dilution of the sample in buffer containing NP-40, a second immunoprecipitation was carried out with anti-gp40,49 serum. As shown in Fig. 3 (lanes 3 and 4), the isolated α (heavy) subunit but not the β (light) subunit was still reactive with the antiserum.

When the reactivity of the anti-gp40,49 serum with PBLs was tested by surface binding in fluorescence-activated cell analysis, no specific staining was detected (Fig. 4C) (compared to the positive control with anti-T3, Fig. 4D), even though >95% of HPB-MLT cells were brightly stained with the antiserum (Fig. 4B) as with mAb T40/25 (Fig. 4A). This result with PBL was unanticipated given the data presented in Fig. 2, which suggested that the anti-gp40,49 serum was capable of precipitating a substantial fraction of the T cell receptor molecules expressed on PBL. It seemed unlikely, therefore, that the explanation for the discrepancy between fluorescence staining and immunoprecipitation with PBL could be that the anti-gp40,49 serum was only recognizing the antigen receptors of a small subpopulation of normal T cells. To investigate this question further, the clonal T cell line 4E4, from which the T cell receptor complex could be isolated by immunoprecipitation with anti-gp40,49, was also subjected to fluorescence-activated cell analysis. Essentially no staining of 4E4 cells with anti-gp40,49 was observed (Fig. 4E) (compared to the positive control with anti-T3, Fig. 4F). These results suggest that the staining of HPB-MLT cells with the antiserum (Fig. 4B) was due to the fraction of antibodies in the antiserum that were clone-specific.

Discussion

mAb T40/25 recognizes a disulfide-linked glycoprotein heterodimer (gp40,49) on HPB-MLT and HPB-ALL cells and displays characteristics similar to those

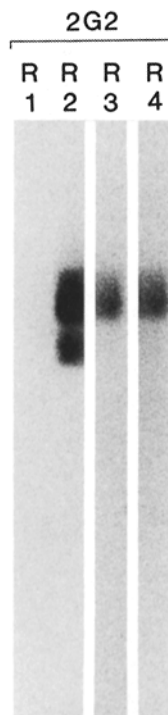


FIGURE 3. SDS-PAGE analysis (10% acrylamide) of ^{125}I -labeled immunocompetent T cell line 2G2 immunoprecipitated with anti-gp40,49 serum before and after separation of the α and β subunits. Lane 1 is a NMS negative control. Lane 2 is the reduced immunoprecipitate with anti-gp40,49. In lanes 3 and 4 immunoprecipitation was performed after separation of the α and β subunits. To accomplish this a first precipitation was performed as usual and the immunoprecipitate adsorbed to SACI was heated, either at 56°C for 15 min (lane 3) or at 100° for 3 min (lane 4), in 2 mM DTT and 1.5% SDS which separates the subunits as shown in lane 2. After heating, the SACI were removed by centrifugation and the supernatants were diluted in a fivefold excess of 1% NP-40, 10 mM Tris saline, and 15 mM IAA. A second immunoprecipitation was then performed with anti-gp40,49 and SACI.

described for the proteins recognized by clonotypic mAb directed against functional human and murine T cells (8). The gp40,49 was therefore isolated and used to immunize mice to obtain a conventional antiserum. When analyzed by immunoprecipitation and SDS-PAGE, the anti-gp40,49 serum was shown to react with a family of closely related protein complexes on PBL and immunocompetent T cell lines and clones. Each of these complexes was a disulfide-linked dimer and shared the determinant(s) recognized by anti-gp40,49. However, consistent with the heterogeneity expected for T cell antigen receptors, there were differences in M_r of the complexes isolated from individual cell lines exhibiting different antigen specificities. Taken together, these observations suggest that it is possible to obtain antibodies against common antigenic determinants of the putative human T cell receptor by immunization of mice with gp40,49 isolated from HPB-MLT cells and confirms the relationship of this protein complex to the antigen receptor on normal human T cells. A rabbit antiserum against the putative antigen receptor of a murine T cell lymphoma

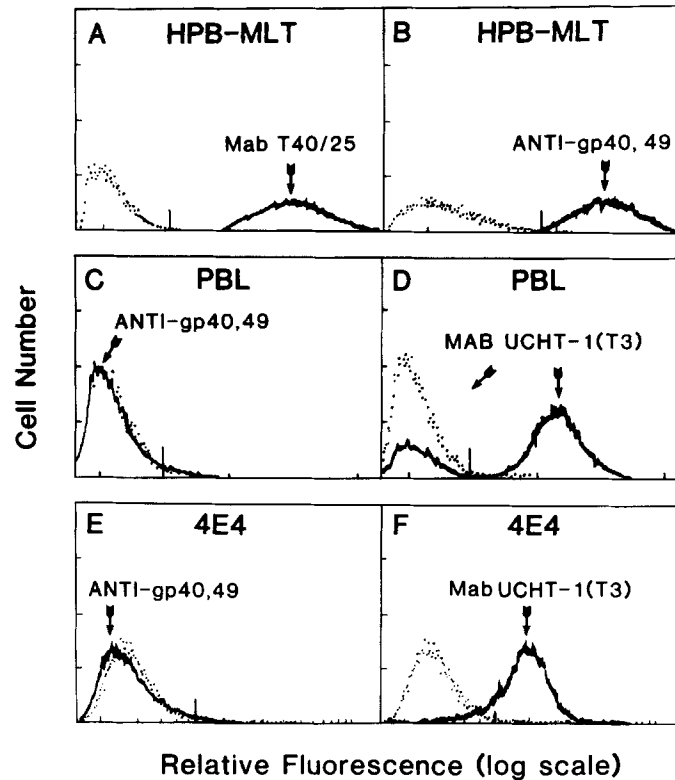


FIGURE 4. Fluorescence-activated cell analysis of T cell lymphoma HPB-MLT, PBL, and immunocompetent CTL clone 4E4. For each analysis a negative control was obtained (dotted profile) utilizing NMS for all frames except *A* in which the negative control was obtained by omission of the FITC second stage reagent. The FITC-conjugated second antibody used was intact goat anti-mouse IgG for the analysis on HPB-MLT and 4E4 cells and the F(ab)₂' fragment was used so as to minimize background staining on PBL. The lower threshold for positive cells was arbitrarily set at a fluorescence intensity of two standard deviations higher than that of the mean fluorescence of the control and is indicated in each frame by a vertical bar. At this setting, 6.8% of PBL were positive with anti-gp40,49 compared to 6.2% for NMS and 71% with mAb UCHT-1. Similarly, 4.8% of clone 4E4 were positive with anti-gp40,49 compared to 3.5% with NMS and 84% with mAb UCHT-1.

has recently been obtained that also reacts with the receptors of normal murine T cells (7).

Co-immunoprecipitation of the T cell receptor and the T3 glycoprotein has been reported (24, 25) but was not observed in several recent reports (26, 27) or in this study (Figs. 1 and 2). Differences in the methods of solubilization and immunoprecipitation could account for the variable co-immunoprecipitation of these two protein complexes.

It was shown that after reduction and alkylation of the protein complex in the presence of SDS anti-gp40,49 serum specifically reacts with the isolated α subunit obtained from one of the immunocompetent T cell lines (Fig. 3). The α subunit of the human receptor must, therefore, express one or more shared determinants that are immunogenic in the mouse. Previously, peptide mapping studies have shown that both subunits of the human T cell receptor isolated from T cells with

different antigen specificities share common peptides (6) and similar results have been obtained in the mouse (8). It is possible that the regions of homology between the β subunits of human T cell receptors with different antigen specificities are less immunogenic in the mouse than those of the α subunits. Alternatively, the lack of reactivity of the anti-gp40,49 serum with the isolated β subunit of the 2G2 cell line may result from the conditions used to separate the two subunits.

Antibodies against the clonotypic determinants of the gp40,49 complex of HPB-MLT cells could be readily detected in the anti-gp40,49 serum by quantitative fluorescence-activated cell analysis. However, in contrast to the results of the immunoprecipitation studies, antibodies against common determinants of the human T cell receptor were not detected by this cell surface staining technique. One possible explanation for this observation is that antibodies against clonotypic determinants predominate even if mice are immunized with the purified human T cell receptor and that the immunoprecipitation techniques detect the fraction of antibodies directed against shared determinants more efficiently than fluorescence-activated cell analysis. In our experience, however, antisera precipitating the amounts of radioactivity associated with the surface-iodinated T cell receptors shown in Figs. 1 and 2 would be expected to give detectable surface staining. It, therefore, seems more likely that the common determinant(s) recognized by the anti-gp40,49 serum may not be accessible on the cell surface. Such determinants could be shielded from reaction with antibody at the cell surface by having an orientation internal to the membrane, or because of tertiary folding or interaction with another molecule such as the T3 glycoprotein.

Assuming that the sequences deduced from the cDNA clones are those for one chain of the T cell receptor, the structure for that chain appears to have two Ig-related domains (presumptively V and C) which are N-terminal and exterior to a putative membrane spanning peptide (9, 11). To account for the apparent failure of polyclonal anti-gp40,49 serum to react with these cell surface shared determinants, one must postulate that (a) this human chain was poorly immunogenic in the BALB/c mouse under the immunization conditions or (b) the genome encodes a number of such T cell receptor specific V and C regions which, like immunoglobulin V and C region isotypes, fail to cross-react either immunologically or by DNA hybridization. Thus, the immunizing antigen, gp40,49, may have been of a different isotype than that found on the T cell lines examined, and the precipitating antibodies may have been directed against determinants not accessible on the surface and present on the other chain. The screening methods previously used to identify T cell receptor molecules required some form of cell surface binding whether radioimmunoassay, fluorescence staining, stimulation, or blocking of function and, therefore, would not be expected to detect the antibodies against the shared determinants described here, which are not accessible on the cell surface. This has important practical implications for the screening techniques that may be necessary to identify mAbs to shared T cell receptor determinants.

Summary

A mouse antiserum, anti-gp40,49 was obtained by immunizing BALB/c mice with the putative T cell antigen receptor isolated from HPB-MLT cells. This antiserum reacted with peripheral blood lymphocyte (PBL) and a panel of immunocompetent T cell lines and clones in each case precipitating from lysates of cells labeled by surface iodination, a disulfide-linked dimer consisting of an α subunit M_r (46,000–49,000) and a β subunit M_r (40,000–45,000). Variability in M_r of the two subunits, particularly of the β (light) subunit, was observed when the receptors of immunocompetent T cell lines with different antigen specificities were compared. Anti-gp40,49 serum reacted selectively with the α subunit after reduction and alkylation of the protein complex. These results confirm the relationship between the gp40,49 protein complex of HPB-MLT cells and the putative T cell antigen receptor on normal immunocompetent T cells and indicate that the α subunit of the human receptor expressed shared determinant(s) that are immunogenic in the mouse. Some features of the T cell antigen receptor appear to be unusual in that even with a xenoantiserum against the purified molecule, only antibodies against clonotypic determinants could be detected at the cell surface by quantitative immunofluorescence analysis.

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