PEPTIDE VARIABILITY EXISTS WITHIN α AND β SUBUNITS OF THE T CELL RECEPTOR FOR ANTIGEN

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To identify the antigen receptor on human T lymphocytes, clonal T cell populations of predefined specificities were utilized as immunogens for monoclonal antibody generation. Two sets of clonally unique (clonotypic) monoclonal antibodies resulted (1, 2). The first defined a surface molecule (Ti_1) on an MHC class I-specific cytolytic T lymphocyte (CTL) clone termed $CT8_{III}$ (1), whereas the second defined a clonotypic structure (Ti_2) on an MHC class II-specific CTL clone termed $CT4_{II}$ (2). The Ti_1 and Ti_2 structures were both 90-kdalton heterodimers composed of disulfide-linked 49–51-kdalton α and 43-kdalton β subunits (2). These in turn were noncovalently associated on the membrane with the monomorphic T3 molecule. It is likely that Ti_1 and Ti_2 represent the antigen receptor on $CT8_{III}$ and $CT4_{II}$, respectively since triggering with the relevant anti-Ti monoclonal antibody resulted in specific clonal proliferation and lymphokine production analogous to stimulation by antigen itself (3).

Although Ti_1 and Ti_2 are comparable in molecular characteristics and derived from T cells of the same individual, they express unique epitopes that can be detected by the above non–cross-reactive monoclonal antibodies (2). To define the structural basis for these differences, comparative biochemical analysis of the individual subunits of the Ti_1 and Ti_2 molecules was carried out. The results demonstrate that each subunit has a distinct pI. More importantly, 2-D peptide map comparison of Ti_1 and Ti_2 α chains, as well as the Ti_1 and Ti_2 β chains, indicates that they are comprised of both common and unique peptide fragments.

Materials and Methods

Cell Surface Radiolabeling and Immunoprecipitation Procedures

Clonal T cell populations were generated and maintained in culture as previously described (4). $CT8_{III}$ and $CT4_{II}$ were surface labeled under identical conditions with ^{125}I by lactoperoxidase technique, as previously described (2). Briefly, 60–70 × 10^6 cells from both clones were washed at room temperature twice with Hanks', once with phosphate-buffered saline (PBS) containing 1 mM of $MgCl_2$ and $CaCl_2$, respectively, and resuspended in the same buffer at 70 × 10^6/ml. To the cell suspension (viability >98%) the following reagents were added: 15 μl 0.5 M glucose, 6 μl 5 × 10^{-4} M NaI, 15 μl 2 mg/ml lactoperoxidase (Sigma Chemical Co., St. Louis, MO), 2 mCi ^{125}I (Amersham Corp., Arlington Heights, IL), and 30 μl glucose oxidase at 0.75 U/ml (Sigma). After 15 min

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incubation at room temperature, the reaction was stopped by adding 100 μl 1 M NaI. Cells were washed three times with ice cold Hanks' and then lysed at 50 × 10⁶/ml in RIPA buffer containing 1% T-X-100, 1 mM phenylmethylonatefluoride (Sigma) and 20 μg/ml ovomucoid trypsin inhibitor (Sigma). Cell lysates were ultracentrifuged for 20 min in a Beckman airfuge (Fullerton, CA) at 10⁵ g. To the supernatant 25 μl BSA (final concentration 1 mg/ml) and 50 μl of a 10% Staph A suspension were added. After 30 min of incubation at 4°C, the bacteria were removed by centrifugation and the cell lysate was incubated twice for 1 h each with an irrelevant monoclonal antibody coupled to Sepharose 4B beads followed by a 2-h incubation with the specific antibody coupled to Sepharose 4B. The latter were generated as described in detail previously (1–3). Beads were then washed once with RIPA containing 1% Triton X-100, 0.5 deoxycholate (DOC) and 0.5 M NaCl, once with the same buffer without NaCl but containing 10 mM EDTA and finally, with RIPA containing 0.1% SDS in addition to DOC and Triton X-100. Immunoprecipitates were then processed for 1-D and 2-D gel electrophoresis.

One- and Two-dimensional Gel Electrophoresis

For one-dimensional sodium dodecyl sulfate gel electrophoresis (SDS-PAGE) (5), immune precipitates were resuspended in sample buffer, boiled for 5 min in the presence or absence of 5% ME or 0.1 M dithiothreitol (DTT) and electrophoresed on a 10% acrylamide gel for 15 h at 40 V, constant voltage.

Isoelectrofocusing (IEF) gels (2-D gels) were performed according to O'Farrell (6) using in the first dimension, a ratio of 10:1:1 of pH 3.5 to 10, 5 to 7, 6 to 8 ampholytes (LKB Instruments Inc., Gaithersburg, MD). Isoelectrofocusing gels were pre-run as described by O'Farrell and then run for 14 h at 300 V and for 2 h at 700 V. After equilibration with reducing Laemmli sample buffer, IEF gels were applied on top of a 10% acrylamide SDS-gel and run as described for one-dimensional SDS-PAGE. Gels were fixed and autoradiographed with intensifying screens (Cronex Lightning Plus, Dupont Co., Wilmington, DE) using Kodak X-R5 films (Eastman Kodak) at −70°C. ¹⁴C molecular weight markers were: phosphorylase b (97,400), ovalbumin (46,000), carbonic anhydrase (30,000), lactoglobulin A (18,300), and cytochrome c (12,300). pH gradient determinants were performed according to the procedure by O'Farrell (6).

Peptide Map Analysis of Separated ¹²⁵I Labeled α and β Chains of Ti

After 1-D SDS electrophoresis, gels were shaken for 1 h in 2% (wt/vol) mixed bed resin (Rexin 300, Fisher Scientific Co., Pittsburgh, PA) in H₂O, dried, and exposed for autoradiography. Bands corresponding to α and β chains of Ti were separately cut from dried gels, rehydrated in 0.7 ml 50 mM NH₄HCO₃ containing 0.05% SDS and incubated for 15 h at 37°C. After centrifugation for 10 min on an Eppendorf microfuge, the supernatants were removed and filtered through glass fiber filter (Whatman Laboratory Products Inc., Clifton, NJ) to remove residual pieces of acrylamide. 80 μg of the human IgG was added as a carrier and the solution was made 100 mM Tris-HCl pH 8.0, 50 mM NaCl, and 1 mM EDTA. Carboxymethylation was performed by incubating for 2 h at room temperature with 30 mM of DTT followed by alkylation with 60 mM of iodoacetamide for 30 min at room temperature in the dark.

Proteins were recovered by precipitation in 20% trichlorecetic acid at 4°C for 2 h and then washed three times with cold (−20°C) acetone. Trypsin digestion was performed essentially as described by Omary and Trowbridge (7). The dried protein pellet was resuspended in 150 μl of 50 mM NH₄HCO₃ and digestion with TPCK trypsin (Worthington Biochemical Corp., Freehold, NJ) was carried out for 20 h at room temperature by adding 40 and 20 μg of trypsin at 0 and 15 h incubation time, respectively. Samples were then diluted to 1 ml H₂O, lyophilized, resuspended in 1 ml H₂O and relyophilized.

Pepsin digestion of β chains was performed according to Corte et al. (8). After trichloroacetic acid (TCA) precipitation, samples were resuspended in 100 μl formic acid/acetic acid/water 1:4:45 (vol/vol) and pepsin at a 1:50 enzyme/protein ratio was added. Digestion was carried out for 18 h at 37°C. Samples were then dried under vacuum. Two-dimensional peptide maps on silica gels (20 × 20 cm) were obtained by spotting side by side two samples containing an identical number of cpm (∼7,000 cpm) to be compared

at one time. After electrophoresis in 15% formic acid, 5% acetic acid in H_2O pH 2.1, the plates were cut in half and chromatography was performed at right angles in n-butanol/ acetic acid/water/pyridine 75:15:40:50 (vol/vol).

Results and Discussion

Given the exquisite specificity of CTL clones and the lack of cross-reactivity of the anticlonotypic monoclonal antibodies, it was anticipated that T cell antigen receptor structures directed at unrelated specificities would differ from one another in their biochemical composition. To directly compare the molecular nature of the Ti_2 structure on $CT4_{II}$ with Ti_1 on $CT8_{III}$, solubilized membrane preparations were obtained from the externally ^{125}I-labeled $CT4_{II}$ and $CT8_{III}$ clones and antigens defined by anti-Ti antibodies (both of IgG1 isotype) precipitated. Subsequently, precipitates were electrophoresed and analyzed in one-dimensional 10% SDS polyacrylamide gels or by 2-D IEF gels. For peptide map analysis, individual bands corresponding to ^{125}I-labeled α and β chains (Fig. 1) were eluted from SDS-polyacrylamide gels, subject to digestion with proteolytic enzymes and peptides separated by electrophoresis and chromatography. By utilizing clones derived from the same donor, it was likely that the heterogeneity of surface receptors could be reduced in complexity.

As shown in Fig. 1, the molecule precipitated by anti-Ti_{1A} (lane A) from ^{125}I-labeled $CT8_{III}$ cells appears as two bands of apparent molecular weight 49 kdaltons and 43 kdaltons under reducing conditions. These are termed α and β chain, respectively. Similarly, anti-Ti_{2B} (lane B) precipitated material from the ^{125}I-labeled $CT4_{II}$, which appears as two bands of apparent molecular weight 51 kdaltons (α) and 43 kdaltons (β) on SDS-PAGE analysis under reducing conditions. The difference in molecular weight of the Ti α chains was a consistent finding and in agreement with earlier results (2). It should be noted that the 10% polyacrylamide gel used here allowed for better separation of the Ti_1 and Ti_2 α and β subunit than the previously employed 12.5% polyacrylamide gel.

To determine whether there were differences in pI (isoelectric point) among the α and β chains of Ti_1 and Ti_2, anti-Ti_1 and anti-Ti_2 immunoprecipitates were subject to 2-D gel analysis using IEF in one dimension and SDS-PAGE in the second. As shown in Fig. 2, there is a series of specific spots at 51 kdaltons migrating to the acidic side of the gel (mean pI 4.4) in the anti-Ti_2 precipitate from $CT4_{II}$. In contrast, the β subunit at 43 kdaltons is a more basic protein (mean pI 6.0) than Ti_2 α. A similar relationship is noted between the more acid α and more basic β subunits of Ti_1 on $CT8_{III}$. However, the α subunits of Ti_1 and Ti_2 have distinct pI's (pI 4.4 vs. 4.7), as do the β subunits (pI 6.0 vs. 6.2).

The resolution of the α and β subunits from Ti_1 and Ti_2 into a series of spots is likely secondary to differing numbers of siliac acid residues and strongly suggests that these are glycoproteins. The molecular basis for the slight differences in size and labeling intensities among the Ti α chains of the two clones is unclear but consistently demonstrated in both one- and two-dimensional gel analyses. Fig. 2 also provides strong evidence for the purity of the immunoprecipitated Ti_1 and Ti_2 material.

To determine whether Ti_1 and Ti_2 were different with regard to their peptide structure, comparative peptide maps were performed on isolated ^{125}I labeled subunits (Fig. 1) following digestion with proteolytic enzymes. As shown in Fig. 3, the tryptic peptide maps of the α chains of these two cell types appear very
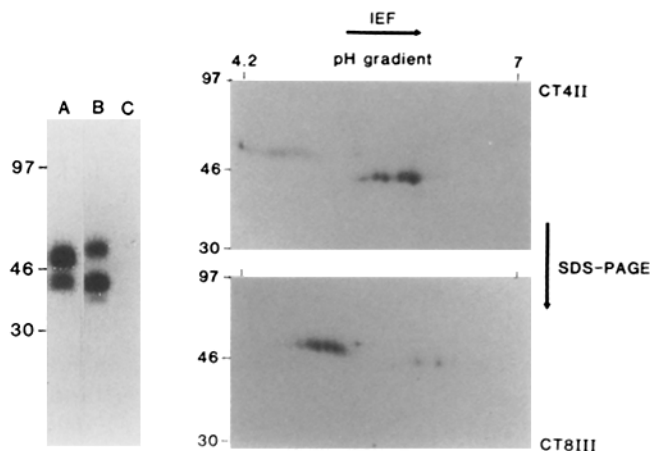
FIGURE 1 (left). SDS-PAGE of ^{125}I-labeled Ti immune precipitates from CT8$_{III}$ and CT4$_{II}$ clones. T-X-100 lysates from ^{125}I-lactoperoxidase-catalyzed surface labeling of CT8$_{III}$ and CT4$_{II}$ were reacted with anti-Ti monoclonal antibodies as described under Materials and Methods. A, anti-Ti$_{1A}$ on CT8$_{III}$; B, anti-Ti$_{2B}$ on CT4$_{II}$; C, anti-T6 (control).

FIGURE 2 (right). 2-D gel analysis of Ti$_2$ and Ti$_1$ immunoprecipitated from CT4$_{II}$ and CT8$_{III}$, respectively. Ti$_2$ and Ti$_1$ were immunoprecipitated with the respective anticlonotypic antibodies coupled to Sepharose and analyzed in a 2-D gel system as described by O'Farrell (7). pH gradient was detected in a separate IEF gel run in parallel in the same experiment.

similar. At least one major peptide and one minor peptide (arrow) migrate to an identical position. Mixing experiments supported the conclusion that these two peptides were shared in common (Fig. 3C). In addition, note that a cluster of several peptides with minimal mobility in the chromatographic dimension are likely related. In contrast, the remaining (unmarked) peptides are clearly distinct. Peptide maps made from pepsin digests of the two α clones also supported the notion that the subunits were similar, but not identical (data not shown).

The β chains, unlike the α chains, were not well digested by trypsin. Therefore, to make an adequate comparison of the β chains of CT8$_{III}$ and CT4$_{II}$, proteolysis with pepsin was required. As demonstrated in Fig. 3 (D and E) and in contrast to the similarity of the Ti α chain, the peptide maps of the two β chains were dissimilar to one another in overall appearance and shared only two minor common spots (Figs. 3, D and E). This was again confirmed in mixing experiments (Fig. 3F). It is also important to note that parallel analysis of pepsin digestion of Ti$_1$ and Ti$_2$ α and β chains indicated that the individual subunits were comprised of totally distinct peptides and thus, bore no precursor-product relationship (data not shown).

The peptide map comparisons provide unequivocal evidence that the Ti$_1$ and Ti$_2$ structures are analogues of one another, since they share several peptide fragments in common. In addition, these results imply that constant domains exist within the Ti α and Ti β subunits. The presence of unique peptides following proteolysis of different Ti molecules isolated either directly by anticlonotypic monoclonal antibodies as shown here, or indirectly by anti-T3 as reported previously (9), supports the notion that variable regions also exist. Based upon the present data, the most likely location of such a "V-region" equivalent would be within in the β subunit. Nevertheless, it cannot, as yet, be ruled out that a
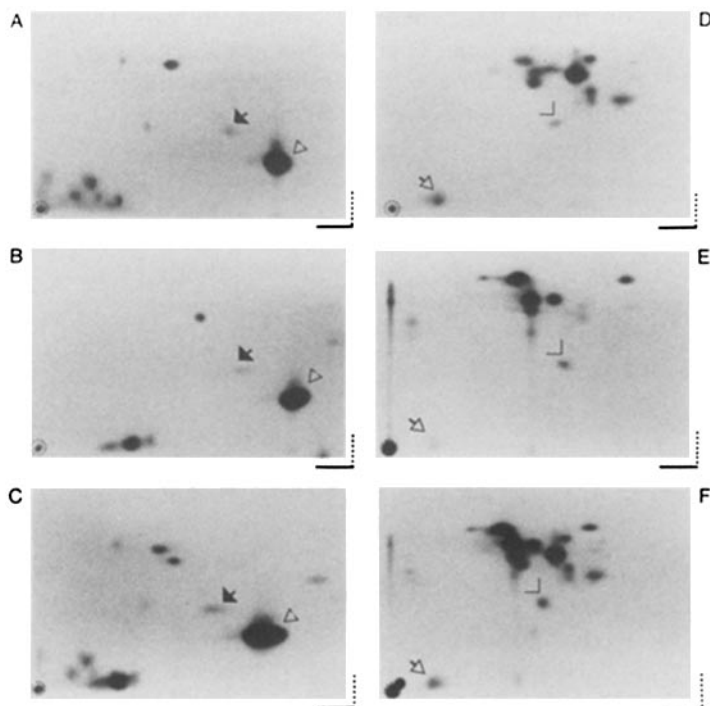
FIGURE 3.   2-D peptide map comparison of ^{125}I-labeled α and β chains of Ti$_1$ and Ti$_2$. α chains of Ti$_1$ (A) and Ti$_2$ (B) were digested with trypsin. C = mix of $A + B$. β chain of Ti$_1$ (D) and Ti$_2$ (E) were digested with pepsin. F = mix of $D + E$. Each sample was spotted on a silica gel plate (origin: O) and peptides separated by electrophoresis (horizontal dimension: ———) and chromatography (vertical dimension: – – –). Note that 7,000 cpm were applied per individual sample in A–E. In mixing samples, an equal number of cpm was added from each digest.

second variable region might exist within the α subunit.

The extent of the peptide differences in the antigen receptor of these two clones is considerable but not surprising in view of their different specificities and the fact that they react with non–cross-reactive monoclonal antibodies. Given that both clones were derived from the same donor, one cannot easily account for the above molecular differences on the basis of genetic polymorphism, unless they are of a allotypic nature. Nor is it likely that the differences are due to the equivalent of "isotype" variation since both Ti$_1$ and Ti$_2$ are expressed by cytotoxic effector cells.

The information that is emerging about the Ti structure in man and, more recently, the mouse (10) is intriguing and suggests that there may be similarities to the immunoglobulin molecule vis-a-vis variable and constant regions of light and heavy chains (11–13). It now becomes necessary to identify the genes that encode the α and β subunits in order to understand the mechanism by which T cell receptor diversity is generated, and the relationship of Ti α and β genes to the immunoglobulin genes.

Summary

The T cell receptor for antigen (Ti) has recently been identified as a 90-kdalton T3-associated clonotypic structure composed of one 49–51-kdalton α

and one 43-kdalton β subunit, which are disulfide linked. Here, Ti molecules from two alloreactive CTL clones derived from the same donor but of differing specificities (CT8$_{III}$ and CT4$_{II}$) are directly compared following isolation with anticlonotypic monoclonal antibodies. Isoelectric focusing shows that the α subunits (pI 4.4–4.7) are more acidic than the β subunits (pI 6.0–6.2) but that each glycoprotein species is distinctive. More importantly, two-dimensional peptide maps of ^{125}I-labeled surface receptors indicate that the β chains of Ti$_1$ and Ti$_2$ appear unique and share only two peptides in common. In contrast, peptide maps of Ti$_1$ and Ti$_2$ α chains are more related although not identical. These results suggest that the human T cell receptor is composed of constant as well as variable regions and that at least one of the latter is located within the β subunit.

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