MOLECULAR ANALYSIS OF T Cell RECEPTOR (Ti) VARIABLE REGION (V) GENE EXPRESSION

Evidence that a Single Ti β V Gene Family Can Be Used in Formation of V Domains on Phenotypically and Functionally Diverse T Cell Populations

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T cell receptors for antigen and major histocompatibility complex (MHC) determinants have, using anticonalotypic monoclonal antibodies (mAb), been defined on inducer, suppressor, and class I and class II MHC-specific cytotoxic T lymphocytes as T3-associated molecules of 90 kilodaltons (kD) molecular mass (1–8). These clonotypic structures, termed Ti, are comprised of one 49–54 kD α and one 43 kD β subunit, which are disulfide linked. Peptide mapping analysis of isolated Ti α and β subunits from clones of differing specificities demonstrated that clonally unique peptides as well as shared peptides existed in each subunit, thus implying that variable (V) as well as constant (C) domains existed within Ti α and β molecules (5, 9). Subsequently, N-terminal amino acid sequencing and molecular cloning techniques led to identification of the Ti β gene structure, and showed that specific V-, D- (diversity), J- (joining), and C-like segments fuse to form an active β gene (10–16). These studies also indicated that the N-terminal V domains were encoded by nucleotides derived from V, D, and J segments. Similar characterization of Ti α subunits suggests that each contains a unique NH₂ V domain created by joining dispersed germline genes (17–21). In addition to the similarities between joining processes of T cell receptor and Ig gene segments, both structures manifest distant but clearcut homology at protein and DNA levels.

T cell receptors, like B cell receptors, must accommodate a myriad of different antigenic specificities in order to provide the organism with an efficient system for immunologic recognition. Moreover, since T cells generally recognize antigen in the context of polymorphic MHC determinants, it is reasonable to assume that V region structural diversity of the T cell population will be no less complex than that of B lymphocytes. In the case of B cells, five mechanisms account for...
the generation of diversity: (a) the presence of a multiplicity of germline V gene segments; (b) combinatorial diversity, created by association of different gene segments during active V gene formation (V, D, and J for heavy chain, and V and J for light chain); (c) junctional diversity, created by variation in the joining sites of V gene segments; (d) somatic mutation, created by one or more point mutations in assembled V region genes; and (e) association-mediated diversity, arising from association of different heavy and light chain V region polypeptides (22). Based on the homologies noted above, it is likely that T cell receptors will use similar mechanisms for generation of diversity. In fact, evidence has already been provided to indicate that combinational and junctional diversity must exist (15–20).

The heterogeneity of phenotypically and functionally defined T cell populations generated from separate sublineages of cells within the thymus has, however, no clearcut precedent in the B cell system. With regard to V gene usage, therefore, it is conceivable that there is either a complex set of rules restricting V gene usage to a given subset of T cells or, alternatively, that a given V gene can be used in the formation of any clonal T cell population. To address this issue, we have identified an anticlonotypic mAb that is reactive with the Ti β subunit of human T cell tumor REX and 2% of human peripheral blood T lymphocytes, and is herein shown to recognize an epitope expressed by members of the REX Ti β V gene family. Using this mAb and a subcloned complementary DNA (cDNA) fragment encoding the REX Ti β V gene as probes, we demonstrate that (a) these V gene products are not restricted to T4 or T8 subsets or functionally characterized inducer, suppressor, or cytotoxic T lymphocytes; and (b) T cell clones selected for expression of this set of V segment products use diverse Ti β D and/or JC segments, as well as different Ti α genes. The implications of these findings for creation of the T cell repertoire in general will be discussed.

Materials and Methods

Phenotypic Analysis of T Cell Surface Proteins. mAb anti-T3A, anti-T4A, anti-T6, anti-T8, anti-T11, and all anticlonotypic antibodies of the anti-Ti_6 series were produced and used in ascites form, as detailed elsewhere (1, 3, 6–9, 23). Phenotypic analysis was performed by means of indirect immunofluorescence with mAb and goat anti-mouse F(ab')2 fluorescein isothiocyanate (FITC) on an Epics V cell sorter (Coulter Electronics Inc., Hialeah, FL). Quantitative histogram comparisons were analyzed on a Coulter EASY system, programmed on the basis of mean channel fluorescence (23).

Derivation of Lymphoid Populations and T Cell Clones. Mononuclear cells were obtained by Ficoll-Hypaque density gradient centrifugation, and were from healthy donors. Postcentrifugation pellets were dextran-sedimented to fractionate granulocytes and red blood cells, whereas interface cells were rosetted with sheep erythrocytes to yield T (E+) and non-T (E−) cells. Portions of human thymus (age 2 mo to 2 yr) were taken at the time of corrective cardiac surgery, and used as a source of thymocytes, as previously described (24). The REX tumor line and Epstein-Barr virus–transformed B lymphoblastoid line, Laz 509, were maintained as previously described (3).

Generation of Anti-Ti3A-reactive T Cell Clones. Peripheral blood anti-Ti3A-reactive T cells were isolated from two normal individuals, using anti-Ti3A mAb in indirect immunofluorescence on an Epics V cell sorter. The Ti3A+ cells were then cloned by limiting dilution (at 10, 5, 1, or 0.5 cells/well) in V-bottom microtiter plates containing 0.1 ml of 5,000-rad irradiated feeder cells (0.50 × 10⁶ cells/ml autologous whole mononuclear cells
Culture medium was RPMI 1640 with 10% human serum, supplemented with glutamine and penicillin-streptomycin (Gibco Laboratories, Grand Island, NY), and containing phytohemagglutinin (PHA) at 0.25 μg/ml.

After 12–18 d, clones were transferred into round-bottom microtiter plates containing irradiated feeders, and grown in medium supplemented with 10% interleukin 2 (IL-2)-containing supernatants (1). Cells were expanded by feeding every 2–3 d with IL-2-conditioned medium, and restimulated every 2 wk with irradiated feeder cells and PHA. Cloning and recloning of various selected parent cultures were then performed at 0.3 cells/well, and subclones were grown for >10 mo.

Functional T Cell Assays. Individual mAb were purified from malignant ascites with protein A–Sepharose (Pharmacia Fine Chemicals, Piscataway, NJ). 5 mg of purified antibody was coupled to 1 ml of swollen CnBr-activated Sepharose 4 B in each case. Predetermined optimal concentrations of antibody-coupled beads were added to populations of 50,000 clonal T cells in a total volume of 200 μl of RPMI 1640 containing 10% human type AB serum (25). In other experiments, clones were stimulated by media alone or by media supplemented with affinity-purified IL-2 at 1 U/ml (kind gift of Dr. Kendall Smith, Dartmouth Medical School, Hanover, NH), with or without Sepharose-bound antibody. After a 24- or 72-h incubation at 37°C, the cultures were pulsed with 1 μCi [3H]thymidine ([3H]TdR) (Becton Dickinson and Co., Sunnyvale, CA)-and harvested 18 h later on a MASH II apparatus (M. A. Bioproducts, Walkersville, MD). [3H]TdR incorporation was then measured in a scintillation counter (Packard Instrument Co., Inc., Downers Grove, IL). Each value represents the mean of triplicates ±SD.

Induction of IgG secretion was determined with 5 × 10⁴ autologous or allogeneic B lymphocytes (E-rosette-negative peripheral blood mononuclear cells treated with anti-T4, anti-T8, anti-Mol, and rabbit complement). To these cells were added 2.5 × 10⁴ cloned cells and optimal concentrations of surface-bound mAb. In mixing experiments, 2.5 × 10⁴ cloned cells of each type were added. Culture conditions and IgG radioimmunoassay were identical to those described previously (8).

The cytotoxicity assay was a standard 4-h cell-mediated lympholysis (CML) system using 51Cr-labeled Laz 509, in which the effector/target ratio in all experiments was 20:1 (1). Concanavalin A (Con A) was used at a final concentration of 25 μg/ml, and added with the target cells at the beginning of the CML assay. This Con A-incubation did not affect spontaneous 51Cr-release. <20% killing was regarded as insignificant lytic activity.

One-dimensional Isoelectric Focusing (IEF). Radioactive bands from polyacrylamide gel slices, corresponding to ¹²⁵I-labelled α and β subunits, were eluted overnight at 37°C in 50 mM sodium bicarbonate with 0.05% sodium dodecyl sulfate (SDS), and trichloroacetic acid–precipitated by adding bovine serum albumin as a carrier. The pellet was then resuspended in the appropriate IEF sample buffer (one volume of “taken up” buffer and one volume of “lysis” buffer), as described by O’Farrell (26). IEF was performed in a horizontal electrophoresis apparatus (LKB model 2117 Multiphor, LKB Instruments, Inc., Gaithersburg, MD) as described by the manufacturer. Samples were loaded on chromatographic paper at the basic end of the gel, and run at 23 W until the potential reached 2,000 V. Gels were then fixed, dried, and exposed for autoradiography. For the β subunit, IEF sample buffer and gel mixture contained a ratio of 10:1:1 of pH 3.5–10, 5–7, and 6–8 ampholytes (LKB Instruments, Inc.), and for the α subunit, a 1:1 ratio of pH 3.5–5 and 4–6 ampholytes was used. For the latter IEF, the cathode solution contained 2% ampholytes at pH 5.7. pH gradients were measured in individual 5 mm gel slices, as described (26).

Immunoprecipitation and Peptide Maps. ¹²⁵I surface-labeling of ~2 × 10⁷ cells of each clone was performed by the lactoperoxidase method (3). Immunoprecipitation, SDS-PAGE (polyacrylamide gel electrophoresis), and peptide mapping were done as previously described (3). An anti-α C region heteroantiserum was utilized for immunoprecipitation of Ti α T cell clones (M. Fabbi, O. Acuto, A. Bensussan, C. B. Poole, and E. L. Reinherz, submitted for publication).

Preparation of α and β REX Probes. A cDNA clone encoding the complete Ti REX β subunit was isolated and subcloned into pBR322 (pβREX) as described (27). Segments
of the C (pCβREX) and V (pVβREX) region were inserted in pBR322 as follows. pβREX was digested with Eco RI, Hind II and Bgl II. Fragments were separated on a preparative agarose gel, and those representing the V region nucleotides (~400 basepairs in the Eco RI–Hind II fragment) and C region nucleotides (~800 basepairs in the Bgl II–Eco RI fragment) were purified by electrophoretic transfer to a NA 45 DEAE-nitrocellulose membrane (Schleicher and Schuell, Inc., Keene, NH). They were subsequently eluted with high-salt buffer (1 M NaCl) at 65°C, and ethanol precipitated. Vβ REX and Cβ REX fragments were ligated to Eco RI-cleaved and phosphatase-treated pBR322. Subsequently, the remaining Eco RI and Bgl II sites (pCβREX) were filled in with Klenow polymerase, and the blunt ends were ligated with T4 DNA ligase. For pVβREX, the Eco RI site of pBR322 was filled in, and the blunt end ligated to the already blunt-ended Hind II end of Vβ REX. Competent MC1061 E. coli cells were transfected, plated, and screened with Vβ REX and Cβ REX nick-translated probes (28).

**Northern and Southern Transfers, and Hybridizations.** DNA and RNA were prepared by standard techniques (28). DNA was digested with Eco RI or Bam HI and size-fractionated on agarose gels, transferred to Gene Screen Plus (New England Nuclear, Boston, MA) and hybridized to the 3²P-labelled V or C region probes. RNA was treated with formaldehyde and run on formaldehyde-containing agarose gels. Before transfer to Gene Screen Plus, gels were soaked in 20× standard sodium citrate for 30 min. After transfer, the membranes were hybridized (at 68°C, with 10⁶–10⁷ cpm/ml), essentially as suggested by the manufacturer. V and C region probes were prepared by nick-translation of pVβREX and pCβREX, respectively (28).

**Results and Discussion**

The structural nature of clonotypic epitopes has not been previously characterized. However, anticlonotypic antibodies could, in principle, be directed at epitopes representing any of several levels of complexity. One type of epitope would be encoded by a single V, D, or J segment on either the α or β TI subunit. A second, more complex epitope might represent either a sequential or conformational determinant, encoded by several TI α or β gene segments (i.e. V and D, D and J, etc.). The third and most complex clonotypic epitope would be combinational in nature, created at the sites where α and β V domains interact with each other. The latter, being the most highly ordered clonal specificity, would likely result in anticlonotypes reacting with epitopes represented very infrequently in a polyclonal T cell population. In contrast, “first” and perhaps “second” clonal epitopes might be found on T cell clones expressing related but nonidentical receptor structures, thus giving rise to more broadly distributed reactivities.

**Reactivity of Anticlonotypic Antibodies with Lymphoid Populations.** To investigate the possibility that some anticlonotypic epitopes might be directed at V gene-encoded specificities, we characterized 20 anticlonotypic mAb, 17 of which were directed against five IL-2-dependent T cell clones of differing specificity, and 3 of which were directed at the human T cell tumor line REX (3). For this purpose, we examined mAb reactivities on heterogeneous populations of peripheral blood T lymphocytes and thymocytes by indirect immunofluorescence assay with an Epics V cell sorter. This strategy was adopted on the assumption that “first order” clonotypic epitopes would be the most highly abundant in heterogeneous T lineage populations of all clonotypic epitopes, and that the V gene segment, being the largest of the gene segments responsible for the nature of the V domain, is most likely to encode clonotypic epitopes.
Of the 20 antibodies tested, 17 were unreactive (<0.1% reactivity) with T cells or thymocytes by this screening strategy. In contrast, all three mAb directed against the REX tumor detected 2% of peripheral blood T lymphocytes. Fig. 1A shows the profile of T cells with a representative unreactive anticlonotypic antibody (anti-Ti₃) (2T82F5) and a representative reactive anticlonotypic antibody (anti-Ti₃₅) (5REX9H5) in indirect immunofluorescence on an Epics V cell sorter. As controls, the thymocyte-specific antibody, anti-T6 (negative control), and a T cell–specific antibody, anti-T3, reactive with >95% of T cells (positive control), were used. Quantitative dot-plot analysis (Fig. 1A), as well as log integrated fluorescence analysis (Fig. 1B) indicate that a discrete population comprising ~2% of peripheral T lymphocytes is reactive with anti-Ti₃₅ (as well as anti-Ti₃₆ and anti-Ti₃₇) (data not shown). In contrast, <0.1% of T cells are reactive with anti-Ti₃ or anti-T6.

Further analysis of anti-Ti₃₅ reactivity is shown in Table I. Note that peripheral blood T lymphocyte populations derived from 30 unrelated individuals were all reactive with anti-Ti₃₅ antibody (2 ± 0.5%). In addition, each of 10 thymocyte preparations tested yielded detectable anti-Ti₃₅ reactivity (0.5 ± 0.2%). The lower level of surface reactivity on thymocytes is not surprising in view of earlier observations demonstrating that only a minor fraction of thymocytes (~1/5) express detectable levels of surface receptors (3). In contrast, non-T cell preparations from these same individuals were unreactive, demonstrating no more reactivity than the anti-T6 control (≤0.1). Furthermore, dual laser fluorescence analysis with directly FITC-labelled anti-Ti₃₅ and rhodamine isothiocyanate–bound to anti-T4 or anti-T8 indicated that this expression was not restricted to

![Figure 1](image_url)

**Figure 1.** Identification of peripheral T cells reactive with anti-Ti₃₅ anticlonotype. 50,000 T cells were mixed with anti-Ti₃ (2T82F5), anti-Ti₃₅ (5REX9H5), anti-T6, or anti-T3 antibody at a 1:250 dilution of ascites, and subsequently stained with goat anti-mouse–FITC. Analysis was performed by dot plot method. The abscissa represents fluorescence intensity, the ordinate, cell size, and density of dot pattern corresponds to cell number (A). Log integrated fluorescence analysis of anti-Ti₃₅-reactive cells is also shown (B) for this same population (X, fluorescence intensity, Y, cell number, and Z, cell size). Results are representative of 30 different individuals' T cells tested.
Reactivity of Anti-Ti3A mAb with a Small Fraction of T Lymphoid Cells

| mAb       | Reactivity of tested cells | Peripheral T* | Thymus | Non-T | REX |
|-----------|---------------------------|---------------|--------|-------|-----|
|           | %                         | ≤ 0.1         | ≤ 0.1  | ≤ 0.1 | < 0.1 |
| Control   |                           | 2 ± 0.5       | 0.5 ± 0.2 | 0.1       | > 95 |
| Anti-Ti3A |                           | 95 ± 5        | 30 ± 12 | 1 ± 0.1 | > 95 |

Reactivity was determined by quantitative analysis on an Epics V cell sorter by previously described methods. Samples tested: peripheral T cells (n = 30); thymus (n = 10); non-T cells (n = 5); REX (n = 3).

* T4 and T8 subset determinations using dual laser analysis with directly FITC-labelled anti-Ti3A, and biotinylated anti-T4 or anti-T8 antibody followed by RITC-avidin incubation showed that 3 ± 1% of T cells were T4+, Ti3A+ and 1 ± 0.3% were T8+, Ti3A+ (n = 3).

Phenotype and Function of Anti-Ti3A-reactive Clones

| Clone | Donor | Phenotype | Function* |
|-------|-------|-----------|-----------|
| A     | 2     | T3+, T4+, T8-, T11+ | Inducer   |
| B     | 2     | T3+, T4+, T8-, T11+ | Inducer   |
| C     | 2     | T3+, T4+, T8-, T11+ | Inducer   |
| D     | 2     | T3+, T4+, T8-, T11+ | Cytotoxic |
| E     | 2     | T3+, T4+, T8-, T11+ | Inducer   |
| F     | 2     | T3+, T4+, T8-, T11+ | Inducer + cytotoxic |
| G     | 2     | T3+, T4+, T8-, T11+ | Inducer   |
| H*    | 1     | T3+, T4+, T8-, T11+ | Inducer   |
| I*    | 1     | T3+, T4+, T8-, T11+ | Suppressor |

* Functional analysis used T cell–dependent B cell Ig assay and Con A–lectin approximation studies with 31Cr-labelled LAZ 509, an EBV-transformed B lymphoblastoid line.

either the T4 or the T8 subset of T cells (T4+, Ti3A+, 3 ± 1%; T8+, Ti3A+, 1 ± 0.3%).

Biochemical Analysis of Anticlonotypic Reactivities on T4+ and T8+ T Cell Clones. To understand the basis of these reactivities at the molecular level, anti-Ti3A-reactive cells were obtained by fluorescence-activated cell sorting, cloned by limiting-dilution techniques, and subsequently recloned at 0.3 cells/well to insure clonality of individual populations. Since these clonal populations were not selected on the basis of their antigenic specificities, initial and subsequent stimulation of cells employed the T cell mitogen, PHA. Table II shows the results of phenotypic analysis of nine clones derived from two unrelated donors. Eight of the nine clones express the T3+,T4+,T8−,T11+ phenotype, whereas one clone expressed the T3+,T4−,T8+,T11+ phenotype. The reason for the predominance of T4+ cells in this sampling is not clear. However, it may reflect either an intrinsic difficulty in cloning human T8+ cells or, alternatively, a random sampling artifact due to the small number of clones tested. Because T4 cells are
approximately twice as frequent as T8 cells in the peripheral T lymphocyte population, the dual fluorescence analysis noted above does not suggest any tendency for TisA expression to be significantly greater in the T4 population.

To characterize the molecule reactive with the anti-TisA antibody on these T cell populations, individual clones were surface labeled by the lactoperoxidase technique, and after solubilization of membranes, anti-TisA-reactive molecules were precipitated and analyzed by SDS-PAGE (polyacrylamide gel electrophoresis). As shown in Fig. 2, under reducing conditions two bands of molecular mass 49–52 kD and 43–44 kD were detected in the radioautograph of these gels. These species correspond in size to previously defined Ti α and β subunits, respectively. In addition, although not shown, nonreducing gels of anti-TisA-precipitable material identified a single species of ~90 kD in all cases. From these results, it is clear that anti-TisA reacts with a disulfide-linked heterodimer similar in structure to receptor molecules that have been previously characterized on individual clonal populations. Furthermore, modulation, with anti-T3 antibodies, of the T3 molecular complex on these cells resulted in selected loss of anti-TisA reactivity, as previously described (7) for T3-associated clonotypes on all other human T cell clones tested (data not shown).

Functional Analysis of Anti-TisA-reactive T Cell Clones. To next determine whether the surface structure recognized by the anti-TisA antibody served as receptor for the individual clonal populations in triggering cell function, we characterized the ability of anti-TisA bound to Sepharose to induce clonal activation (25). The latter was initially assessed by examining the effect of anti-TisA-Sepharose on T cell proliferation and subsequent regulatory activities.

As shown in Table III for clone H, IL-2, anti-T3, and more importantly, anti-TisA-Sepharose induced the clones to proliferate, as measured by [3H]TdR incorporation. In contrast, anti-T4-Sepharose and anti-T8-Sepharose failed to

![Figure 2](image-url)
TABLE III

Clonal Proliferation Induced by Anti-Tis Antibody Bound to Sepharose

| Stimulus                  | [\(^{3}\text{H}\)]Tdr incorporation in clones of: |
|--------------------------|-----------------------------------------------|
|                          | H(T4\(^{+}\),Tis\(^{+}\)) | I(T8\(^{+}\),Tis\(^{+}\)) | a(T4\(^{+}\),Tis\(^{-}\)) |
| Media                    | 55*                           | 309               | 43               |
| IL-2                     | 6,502                         | 24,759            | 8,242            |
| Anti-T3 Sepharose        | 13,753                        | 594               | 27,533           |
| Anti-T4 Sepharose        | 73                            | 825               | 94               |
| Anti-T8 Sepharose        | 56                            | 959               | 88               |
| Anti-Ti4 Sepharose       | 102                           | 865               | 65               |
| Anti-Ti\(_{8}\) Sepharose| 17,799                        | 493               | 107              |
| Anti-Ti\(_{8}\) Sepharose + IL-2 | 18,001                  | 1,294             | 8,652            |

* Numbers represent the mean of triplicate samples. SD \(\leq 10\%\) in all cases tested.

result in a proliferative response. Similar findings were obtained with all eight different T4\(^{+}\),Ti\(_{8}\)\(^{-}\) clonal populations tested. These results are in keeping with other data (25) indicating that crosslinking of T3–Ti complexes by Sepharose-bound mAb or antigen-MHC leads to growth via an IL-2-dependent mechanism of most T cell populations.

In the case of the T8\(^{+}\),Ti\(_{8}\)\(^{+}\) clone I, only exogenous IL-2 induced clonal proliferation. That neither anti-T3 nor anti-Ti\(_{8}\)–Sepharose triggered a proliferative response is of interest in light of earlier studies (7) indicating that T8\(^{+}\) suppressor T cells fail to proliferate after triggering of their T3–Ti complex. This finding suggested that clone I was derived from a suppressor T cell population. Also consistent with this notion was the observation that anticonotypic triggering abrogated proliferation of clone I in response to exogenous IL-2 during the subsequent 72-h culture period (1,294 vs. 24,739 cpm).

Table III also shows that the ability of anti-Ti\(_{8}\)–Sepharose to mediate induction of proliferation is restricted to those clones expressing the anti-Ti\(_{8}\)–reactive heterodimer. Thus, anti-Ti\(_{8}\) bound to Sepharose has no effect on proliferation of the T4\(^{+}\),Ti\(_{8}\)\(^{-}\) clone a (medium alone, 43 cpm; anti-Ti\(_{8}\)–Sepharose, 107 cpm). Note, however, that antibodies against the monomorphic T3 subunits of the antigen-MHC–receptor complex do induce [\(^{3}\text{H}\)]Tdr incorporation (27,533 cpm).

To next determine whether anti-Ti\(_{8}\)–Sepharose was capable of triggering clonal regulatory activities, its capacity to induce help or suppression of B cell Ig was assessed. Control stimulation with anti-T4–Sepharose, known to be incapable of activating T cell regulatory activity (8), was examined in parallel. Table IV shows the results of a representative experiment. As indicated, anti-Ti\(_{8}\)–Sepharose and anti-T4–Sepharose induced neither B cells nor T cell clones to secrete Ig by themselves. The two representative T4\(^{+}\),Ti\(_{8}\)\(^{+}\) clones, A and H, specifically provided help to B cells in the presence of anti-Ti\(_{8}\)–Sepharose but not anti-T4–Sepharose. In contrast, T8\(^{+}\),Ti\(_{8}\)\(^{+}\) clone I failed to provide help. More importantly, as shown in the mixing experiments, addition of clone I to the mixture of clone H and B lymphocytes abrogated IgG production induced by anti-Ti\(_{8}\) bound to Sepharose. This effect was not due to cell crowding, since addition of the T4\(^{+}\),Ti\(_{8}\)\(^{+}\) clone A to the clone H plus B cell–mixture did not inhibit IgG
Table IV

| Cell population | Anti-T4- | Anti-Ti3- |
|-----------------|----------|-----------|
|                 | sepharose| sepharose |
| B cells alone*  | <50      | <50       |
| Clone A alone   | <50      | <50       |
| Clone H alone   | <50      | <50       |
| Clone I alone   | <50      | <50       |
| Clone A + B cells| 120     | 4,615     |
| Clone H + B cells| 95      | 3,522     |
| Clone I + B cells| <50    | <50       |
| Clone H and clone I + B cells| <50 | 120 |
| Clone A and clone H + B cells| 88       | 4,888     |

* Data is shown for B cells derived from donor 2. Similar data was obtained in parallel experiments with B cells from donor 1. Results are representative of three separate experiments. IgG in supernatants was quantitated by means of solid-phase radioimmunoassay.

production. Thus, clones A and H are T4⁺ inducer clones, whereas clone I is, as expected, a T8⁺ suppressor clone.

Comparable analysis with the remaining anti-Ti3A-reactive clones led to the results summarized in Table II. Note that all T4⁺ clones except clone D provided help for B cells in this assay, implying that the majority were inducer clones. As indicated, this latter clone was found to mediate cytotoxic effector function when tested in a lectin-approximation cytotoxic T lymphocyte assay, as described (see Materials and Methods). In addition, clone F provided help for B cell IgG production, and was also found to mediate cytotoxic activity in this same assay system. That a single clone could provide both inducer and cytotoxic effector function is not without precedent, and implies that clonal T cells can both produce inducer lymphokines and lytic effector molecules. In summary, functional analysis of the anti-Ti3A-reactive clones defined six inducer clones, one suppressor clone, one cytotoxic effector clone, and one clone mediating both cytotoxic effector function and inducer function. Together, the above data imply that the heterodimer detected by anti-Ti3A is the T cell receptor on these clones.

Anti-Ti3A Defines an Epitope on the REX V Gene Product(s). Immunoprecipitation studies from biosynthetically labelled REX tumor cells had previously shown that a partially glycosylated intracellular form of the Ti β subunit (41 kD) that was not associated with the α subunit was always precipitated by anti-Ti3A under both reducing (3) and nonreducing conditions (our unpublished observation). These results suggested that the antibody was recognizing an epitope expressed on the Ti β subunit. In addition, the fact that anti-Ti3A reacted with 2% of peripheral T cells of 100% of the individuals tested (n = 30) made it unlikely that it recognized an allotypic determinant expressed on the V or C domain of the β subunit.

Based on these observations, it appears plausible that anti-Ti3A might detect a Ti β V region epitope shared by the REX tumor line and a peripheral T cell
population. To test this hypothesis, a REX β V gene probe was generated (as described in Materials and Methods), 32P-labelled, and used as a probe for Northern analysis with total cytoplasmic RNA derived from individual clonal populations. Fig. 3 shows the results of such an analysis. Note that REX V β cDNA hybridized to an RNA of ~1.3 kilobases (kb) in all anti-Tiα-reactive clones (clones A–G). In contrast, no RNA species were detected in anti-Tiα-unreactive clones (clones a–d) from the same donor. Nevertheless, these same anti-Tiα-unreactive clones express receptor RNA, as indicated by positive hybridization with a Ti β C region probe, Cβ REX. In the latter case, the 1.3 and 1.0 kb RNA are similar in size to those found in the representative Tiα+ clones tested, F and G. This correlation between Tiα expression and the presence of Vβ REX RNA implies that the Tiα epitope is encoded by one or more of the REX Ti β V genes known to exist in the germline.

The presence of two molecular species of RNA in the Northern blots of clones F and G RNA probed with Cβ REX contrasts to the single species identified with the V β REX probe in these same clones. This result is not unexpected, due to

![Figure 3](image-url)
the fact that the 1.0 kb RNA species in T cells is known to be a transcript resulting from DJ joining in the absence of VD joining (29). Fig. 3 also indicates that V β REX-related message is easily detected in REX (Fig. 3, 1), as well as in the unselected peripheral blood T cells (Fig. 3, 2) and thymocyte population (Fig. 3, 3), despite the fact that only a fraction of cells in the latter two is anti-Ti3A-reactive. The greater abundance of V β REX-related RNA transcripts in thymus as opposed to peripheral T cells contrasts with the significantly lower level of surface expression of Ti3A in the former, and suggests that a sizable fraction of RNA has not been translated and expressed in the form of surface protein. In this regard, the presence of both a 1.3 and 1.0-1.1 kb messenger RNA (mRNA) species in thymocytes probed with a V β REX suggests that perhaps aberrant splicing or premature termination of β-message RNA may exist. Although the Cβ REX probe hybridizes with a component of the 1.0-1.1 kb mRNA species (data not shown), it is possible that some of the lower molecular weight species represent primary V β transcripts (±D). The precise correlation of V β REX RNA expression and anti-Ti3A surface reactivity also strongly suggests that the Ti3A epitope is encoded in the REX V gene family, rather than, or in addition to D and/or J segments.

**Heterogeneity of Ti α and β Subunits on Anti-Ti3A-reactive Clones.** Given that the majority of T4+,Ti3A clones were phenotypically indistinguishable by existing mAb reactivities, the possibility that each represented daughter clones derived from the same parent cell had not been excluded. It was thus of importance to characterize any similarities and differences of the Ti α and β subunits of the various clonal populations. Moreover, this would provide further information about the relatedness of Ti β V gene products detected by the Vβ REX probe. As a first approach, we immunoprecipitated Ti molecules from surface-labelled clonal populations, separated Ti α and β subunits from seven T4+,Ti3A clones (derived from a single donor) by SDS-PAGE analysis, and, after elution, subjected them to IEF analysis. Fig. 4 shows the results of parallel studies with both α and β subunits. The ranges of pI for α (4.2–4.6) and β (5.6–6.6) were consistent with previous observations (9).

It is evident that although some clones had either an α or β chain that focused in a similar fashion with this set of ampholytes, no two clones were identical in both. Thus, it is highly unlikely that the various populations were derived from the same parent clone. Note in addition that the sizable difference in pI value (up to 1 pH unit) among β chains derived from distinct clones, as uncovered by this analysis, strongly suggests that such differences in net charge were due either to use of different members of the Vβ REX gene family, or, alternatively, to different D or J segments.

To acquire more direct information on the relatedness of the V regions utilized by the Ti3A+ clones, two-dimensional peptide mapping analysis was performed on Ti α and β subunits from several representative T4+,Ti3A+ clones, and, for comparison, a T4+,Ti3A+ clone derived from the same donor. To this end, soluble membrane preparations were obtained from externally 125I-labelled clones and immunoprecipitated with anti-Ti3 and with the anti-Ti α C region H36 heteroantisera (Fabbi et al., submitted for publication). Individual bands corresponding to 125I-labelled α and β chains were eluted from one-dimensional
FIGURE 4. IEF analysis of $^{125}$I-labelled Ti $\alpha$ and $\beta$ subunits from Ti$_{3A}$ clones (A–G). Numbers indicate pI values determined as described in Materials and Methods.

SDS-polyacrylamide gels, subjected to digestion with proteolytic enzymes, and the peptides were separated by electrophoresis and chromatography.

As shown by peptide maps of $\beta$ chains from anti-Ti$_{3A}$–reactive clones A, E, and B in Fig. 5, each of these subunits is clearly related. The Ti $\beta$ chains of clones A and E are strikingly similar, although a few reproducible peptide differences were noted. A third clone, B, was less similar to A and E, but nevertheless shared three distinct peptides (arrows). Note that the assignment of identity of these peptides was based on additional studies (not shown) where peptide maps of the Ti $\beta$ chains from clones E and B were run on the same plate. Collectively, these data would argue that the V gene used by clones A and E in formation of the Ti $\beta$ subunit is most likely the same, whereas, clone B may use a different V$\beta$ REX family member. Fig. 5 also shows that, as expected, a representative anti-Ti$_{3A}$–unreactive clone (a) gave a Ti $\beta$ peptide pattern which was totally unrelated to that seen in clones A, B, or E.

The similarity of the $\beta$ subunits of clones A and E supports the idea that these clones may have related antigenic specificity. Further support for this possibility
FIGURE 5. Two-dimensional peptide map analysis of $^{125}$I-labelled $\alpha$ and $\beta$ subunits isolated from Ti3A+ clones (A, E, and B) and one Ti3A- clone (a). Ti molecule from clone a was isolated by means of a rabbit antiserum (H36) that reacts with constant determinants on the Ti $\alpha$ chain (see text). Isolated $\alpha$ and $\beta$ chains were digested with trypsin and pepsin, respectively, and peptides separated by electrophoresis (—) and chromatography (---), as previously described (9). Arrows indicate common peptides. The identification of peptides common to B, A, and E was made possible by running the $\beta$ map of clones E and B in parallel.
comes from analysis of the α subunits of these two clones, which demonstrates that they are highly related. That there is no requirement for relatedness between α subunits of clones defined by anti-Ti3A, however, is evident from the peptide map analysis of the Ti3A+ clone B. Note that a single peptide migrating in both chromatographic and electrophoretic dimensions to the middle of each plate is a peptide derived from the C region previously shown to be present in all T cell clones regardless of specificity (9).

*The Ti3A Epitope Is Present on Products of Several REX Vβ Gene Family Members.* Since it is known that the REX V gene family consists of four or more members, it was of interest to use restriction analysis of DNA derived from Ti3+ and Ti3− clones, in conjunction with the Vβ REX probe, to determine whether more than one pattern of gene segment rearrangement could be detected (13). As shown in Fig. 6 for granulocyte DNA, the germline pattern of the Vβ REX genes in Southern blot analysis contains four bands in Eco RI digest (two weakly hybridizing bands, at 8.2 and 6.1 kb, and two strongly hybridizing bands, at 4.7 and 2.0 kb, respectively). Presumably, one of the two more strongly hybridizing bands contains the Vβ REX gene. Six of seven Ti3+ clones (all but clone D) showed obvious alterations in the germline pattern. Thus, for example, a new band appears below the Vβ REX germline 6.1 kb band in DNA digests from clone A, above the Vβ REX 8.2 kb band in DNA from clone B, whereas in clone C, the Vβ REX 2.0 kb band is deleted. Furthermore, the loss of the 2.0 kb Vβ REX band is concomitantly associated with an increase in intensity of the 8.2 kb band, perhaps as a result of recombination events that lead to a new overlapping restriction fragment and deletion of the other 2.0 kb allele. Based on these observations, it is likely that anti-Ti3 defines an epitope on more than one member of the Vβ REX V gene family, thus consistent with differences in peptide

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**FIGURE 6.** Southern hybridization of DNA from Ti3A+ and Ti3A− T cell clones with Vβ REX. DNA from T cell clones A–G, a–d, and autologous granulocytes was digested with Eco RI; fragment size separated by agarose gel electrophoresis, transferred to Gene Screen Plus, and hybridized with Vβ REX probe. The size of the germline granulocyte Vβ REX fragments was determined by comparison with λ Hind III markers, and found to be 8.2, 6.1, 4.7, and 2.0 kb, respectively.
maps of Tiβ subunits from clones A and B, for example. Although not shown, rearrangements of the Vβ REX bands were detected in Southern analyses of Bam HI digests of clone D. Fig. 6 also demonstrates that DNA from Ti3 clones a–d is preserved in the germline pattern, further supporting the notion that anti-Ti3 recognizes products of the Vβ REX family.

Implications for T Cell Receptor Structure and Function. Although clones A and E yielded identical results when their DNA was probed with Vβ REX after digestion of DNA with Eco RI (Fig. 6), or Bam HI (data not shown), the observed differences in IEF analysis, and small but reproducible differences in two-dimensional peptide map analysis of their Ti α and β chains suggest that they do not express identical receptors. Aside from the overall similarity of restriction patterns of clones A and E, the heterogeneity in Southern analysis is consistent with the idea that there are probably few, if any restrictions on the mechanisms that generate combinational and junctional diversity. This is probably also true for chain association–mediated diversity as well, given that related Ti β V gene segments form V domains in association with related and unrelated Ti α subunits (Fig. 4). The dissimilarities among Ti α and β subunits of the various clones demonstrated by IEF and peptide map analysis make it very unlikely that such cells have been primed in vivo against a single antigen. Rather, it suggests that these clonal populations, which use the REX Ti V gene segment family and possibly other different D, J, and C segments, are directed at multiple unrelated specificities. To date, studies have failed to provide evidence for somatic mutation (11). Thus, it is improbable that the observed differences in Ti α and β chains were generated by this mechanism.

Presumably, the constraints placed on Tiβ-bearing molecules expressed by clones that have been selected by immunization with a specific antigen, as opposed to the unprimed T cells used here, will yield a much more restricted and homogeneous V domain, perhaps derived from a single set of Ti β, V, D, and J segments. Assuming that the Ti α domain is involved in antigen-MHC binding, we predict that structural constraints are also placed on the latter. Such a view has precedent in the B cell system, where the generation of antibody responses to p-azophenylarsonate has been studied at the molecular level (29). In this regard, the present system may provide a useful model to examine the contribution of one V gene family to the immune repertoire directed against a defined antigen.

The finding that one Ti β V gene family contributes to the V domain of one in every 50 nonimmune peripheral blood T lymphocytes implies that either the germline V gene repertoire is extremely limited, or that this single family is frequently utilized, analogous to the Vk 21 family in the B cell system (30). Support for the first view comes from recent studies (31) indicating that five different V gene probes identified ~30% of the total Ti β cDNA clones in a mouse thymocyte library. In addition, at least one other anticonnotropic antibody, specific for a Sezary tumor cell, reacts with a fraction of peripheral blood T lymphocytes as well (32). While the specific epitope it recognized has not been linked to any particular V gene segment, such an association appears likely. We cannot, however, rule out the possibility that there are many other V genes that are infrequently used. Nevertheless, given the various mechanisms available to
T cells for generating receptor diversity, the finite nature of the Ti β V gene pool, like that of V gene pools for Ig light and heavy chains, would pose no major constraints on receptor heterogeneity.

The functional analysis of TiβA+ clones presented herein demonstrates that V segments of the REX family can be used by T4 or T8 sublineages, or for that matter, functionally defined distinct regulatory and effector populations. Assuming that this family is a paradigm for other Ti β V gene families, these data imply that V gene usage is independent of phenotype and function. If these notions are correct, it is unlikely that class I and class II MHC preferences of T8 and T4 subsets, respectively, are due to usage of different sets of V gene pools. Rather, ancillary recognition structures, such as T4 and T8 molecules themselves, may give rise to these observed MHC preferences by increasing overall avidity for MHC (33–35). Moreover, it is certainly possible that one set of V gene segments is used in recognition of multiple distinct antigens.

The ability to make mAb against members of a particular V gene family should aid in understanding V gene usage and heterogeneity. What additional restrictions antigen places on selection of D and J elements in conjunction with a given set of V genes will also be of interest.

The reactivity of all three anti-Tiβ clonotypes against the REX tumor, along with 2% of peripheral T cells and the lack of reactivity of 17 additional anticlonotypes directed against other Ti molecules, implies that the former define much more frequent V gene families than the latter. Nevertheless, we cannot rule out the possibility that the lack of reactivity of some of these anticlonotypes is due to the hierarchal nature of clonotypic epitopes, giving rise to higher degrees of "uniqueness." The reactivity of anti-TiβA with T cells from each of 30 individuals tested, however, excludes the possibility that this antibody defines an allotypic epitope (36). Such anti-V domain probes should be useful in understanding the normal and abnormal immune response.

Summary

We examine the rules governing Ti β variable (V) gene segment usage in the formation of T cell antigen-MHC receptors in diverse regulatory and effector T lymphoid subpopulations. To this end, a single Ti β V gene family and its products were analyzed. A monoclonal antibody, termed anti-TiβA, which was shown to be reactive with an epitope encoded by members of the REX cell line Ti β V gene family, and which was expressed on 2% of human T lymphocytes was used in selection of clones from unprimed peripheral T lymphocytes. Both T4+, as well as T8+ T cell clones with inducer, suppressor, and/or cytotoxic function were defined. Southern analysis, isoelectric focusing and two-dimensional peptide mapping indicated that individual members of the REX V gene family were linked to different Ti β diversity and/or joining and constant region segments. Moreover, the Tia chains of such clones were distinct. These results imply that Ti β V gene usage is not restricted to any functionally or phenotypically defined T cell subsets, and there is presumably little, if any, restriction on the mechanisms that generate combinational, junctional or chain association-mediated diversity.

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