**Tthy\(^d\)**, A NEW THYMOCYTE ALLOANTIGEN LINKED TO Igh-1

Implications for a Switch Mechanism for T Cell Antigen Receptors*

BY FRANCES L. OWEN, GWENDOLINE M. SPURLL,† AND ELLEN PANAGEAS

Department of Pathology and Cancer Research Center, Tufts Medical School, Boston, Massachusetts 02111

T lymphocytes have been shown to react with antisera directed against cross-reactive idiotypes (1, 2). This observation lead to the hypothesis that T cells express Igh-1 linked gene products in association with antigen binding molecules (3). Failure to detect isotypic determinants characteristic of serum immunoglobulin (4) suggested that T cells may express unique immunoglobulin-like molecules that have their own constant regions. Cell lines secreting a monoclonal T cell product specific for sheep erythrocytes (5) and one specific for arsonate (Ars)\(^1\) (6) have supported the conclusion that some T cells use heavy chain-like molecules as antigen receptors.

A group of T cell-specific alloantigens are linked to the immunoglobulin locus on chromosome 12 in the mouse. The region encoding these antigens has been tentatively designated IgT-C (7). The existence of a gene complex, downstream from Igh-1, coding for a family of T cell isotypes has been proposed (7, 8). The first gene(s) described, Tsu\(^d\) (9), was mapped to this region using recombinant inbred and recombinant congenic lines (7). The Tsu\(^d\) antigen has been proposed to be a T cell constant region gene product because of the ability of antiserum directed against Tsu\(^d\) to polyclonally trigger suppressor cells for T-dependent antigens (10). The suppressor cells induced obey the rules of genetic restriction (11)\(^2\) established for idiotype-specific regulatory cells (12). Antiserum also blocks binding of anti-Ars IdX Ts2 cells (13) to their idiotype (14). The position of the gene complex distal to the heavy chain locus (7) is suggestive evidence that these may be T cell heavy chain-like molecules. A second T cell alloantigen, Tind\(^d\), was found in the course of attempts to make monoclonal anti-Tsu\(^d\). The use of a panel of Igh-1 recombinant mice limited the gene coding for this determinant to a region of chromosome 12 (15) closely linked to Tsu\(^d\). This study describes the use of two monoclonal lines secreting antibody against a third T cell-specific alloantigen, Tthy\(^d\). Tthy\(^d\), in contrast to Tsu\(^d\) or Tind\(^d\), is preferentially expressed on thymocytes. Use of Igh-1 recombinant lines shows the gene(s) coding for this antigen(s) is closely linked to Tsu\(^d\) (7, 15). The frequency of the cells in the thymus (30%) and periphery (1%), which express this antigen, stimulated the hypothesis that Tthy\(^d\) may be a third isotype for T cell receptors and that a switch mechanism from expression of Tthy\(^d\) to Tsu\(^d\) or Tind\(^d\) may parallel T cell maturation.

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† Fellow of the Medical Research Council of Canada.
\(^1\) Abbreviations used in this paper: Ars, arsonate; CI, cytotoxic index; Con A, concanavalin A.
\(^2\) Owen, F. L., R. Riblet, and P. D. Gottlieb. T suppressor cells activated by anti-Tsu\(^d\) serum are Igh-V restricted. Eur. J. Immunol. In press.
Materials and Methods

BALB/c AnN mice were purchased from Charles River Breeding Laboratories, Inc. (Wilmington, Mass). All other animals were bred in our laboratories at Tufts from lines developed elsewhere. C.AL-20 (Igh-1^a) animals, congenic to BALB/c except for an undetermined number of genes linked to immunoglobulin, were developed by M. Potter at the National Institutes of Health (Bethesda, Md.). BAB/14, developed by L. A. Herzenberg, Stanford University (Stanford, Calif.) are congenic to BALB/c and have recombination events in the cluster of IgH-V genes resulting in an IgH-V^a IgH-C^b haplotype. The C.B.AL-1 (Igh-V^d, IgH-C^b, Pre-1^b), C.B.AL-2 (Igh-V^d, IgH-C^b, Pre-1^b), and C.B.AL-4 (Igh-V^d, IgH-C^b, Tsu^a-, Pre-1^b) mice are recombinant Igh-1 strains derived from a cross of C.AL-20 and BAB/14 (16).

The method of typing for Tsu^a has been described (7, 8).

Monoclonal anti-Tthy^a (Clone 1711C6) was produced using a modification of the immunization procedure described for Tind^a (15). Briefly, BALB/c animals were immunized with C.AL-20 T cells activated with concanavalin A (Con A). Spleen cells that formed the least dense bands from a discontinuous bovine serum albumin gradient were used for immunization (9). Animals received 10^7 cells in a single immunization 3 d before killing. Spleen cells were fused (17) with P.3U1 tumor cells (18) using polyethylene glycol and plated in selective media supplemented with 1 × 10^{-4} M hypoxanthine, 4 × 10^{-5} M aminopterine, and 1.6 × 10^{-5} M thymidine (19). The frequency of fusion was 10% and cells were subcloned into nonirradiated C.B~20 thymocytes (10^6/well) with an efficiency of 65%. Screening for positive clones used a cell surface radioimmunoassay (Sprull, G. M., L. Riendeau, A. Finnegan, and F. L. Owen, manuscript in preparation). Briefly, either BALB/c or C.AL-20 Con A-activated T cells were plated into 96-well microtiter plates, and cell monolayers were fixed with glutaraldehyde. Positive clones were those that secreted antibody binding to C.AL-20 and not to BALB/c when a second step 125I-labeled goat anti-mouse IgM was added as a developing reagent (20).

Monoclonal anti-Tthy^b (b, from clone 208IE6, arose as a deliberate effort to produce antibody against thymocytes. An alternate immunization procedure was initiated. BALB/c nu/nu mice (Charles River Breeding Laboratories, Inc.) were immunized intraperitoneally with 10^7 C.AL-20 thymocytes on days 10 and 3 before fusion of immune spleens with P.3U1 tumor cells. The fusion frequency was 3%, and the one selective clone (208IE6) was subcloned by limiting dilution into C.AL-20 thymocyte feeder cells. The subcloning efficiency was 2% when two cells per well were plated. The resulting IgGlk-secreting line was obtained by screening in the cellular immune assay described above using either BALB/c or C.AL-20 thymocytes as targets and 125I-goat anti-IgG1 (MOPC-21) as the developing reagent. The expression of Tthy^b on thymocytes was confirmed in a visual dye exclusion cytotoxicity test. A two-stage antibody and complement-mediated lysis was performed by incubating 5 × 10^5 thymocytes from either BALB/c or C.AL-20 animals at 37°C for 30 min with 10 µl of tissue culture supernatant from monoclonal lines. This was followed by an incubation at 37°C for 45 min with 25 µl of 1:12 diluted selected rabbit complement. Dead cells were evaluated by the inability to exclude eosin red. Data are expressed as cytotoxic index (CI); the arithmetic mean ± SE of duplicate determinations was adjusted for complement controls. CI is equal to the percent of dead cells with antibody-C'/percent of dead cells with anti-BAT-C'. Experiments were executed in a double blind study to minimize bias. Data are expressed as percent of lysis with antibody and complement minus percent of lysis with complement alone. The complement background varied with the strain used and was 25 ± 5% on the C.AL-20 thymocytes.

Absorption experiments were used to estimate the Lyt phenotype of the cells expressing the antigen. Tissue culture supernatant from clone 1711C6 (40 µl) was incubated with limiting numbers of thymocytes, bone marrow cells, lymph node cells, or antibody-treated cell populations. Anti-Lyt-1.2 was a monoclonal antibody purchased from New England Nuclear (Boston, Mass.), and monoclonal anti-Lyt-2.2 (21) was the generous gift of P. D. Gottlieb, Austin, Tex.

Results

Tthy^d is expressed on thymocytes or recombinant mice with the Tind^a+ phenotype. Thymocytes of the C.AL-20 strain (Igh-1^a) can be lysed with anti-Tthy^d (1711C6) monoclonal (IgMk) antibody and selected rabbit complement. BALB/c (Igh-1^a) and
BAB/14 (Igh-1b) thymocytes are not sensitive to the effect of anti-Tthy\(^d\). Three Igh-1 recombinant strains, constructed by crossing C.AL-20 and BAB/14 animals, also failed to express the Tthy\(^d\) antigen in a detectable way. Since the immunoglobulin genes of the three recombinant animals, C.B.AL-1, C.B.AL-2, and C.B.AL-4, have been characterized and have been typed as negative for Tsu\(^d\) (7), the failure to express Tthy\(^d\) indicates that the gene(s) coding for Tthy\(^d\) is closely linked to Tsu\(^d\). The recombinant mice limit the position of the gene coding for this determinant to chromosome 12. It must be downstream from Igh-Dex and upstream from a recombination event between Tsu\(^d\) and H(Igh) (see map inset on Table I). Antibody and complement lyse 15–35% of thymocytes from C.AL-20 animals (Table I) but do not lyse a detectable number of T cells from lymph node or spleen (data not shown). Estimates of the total frequency of cells expressing this antigen may be a reflection of cell surface density, and the number of cells that actually are Tthy\(^{d+}\) could be much higher (Table I). Other strains not listed in Table I were typed Tthy\(^{d+}\), A/J, AKR/J, or Tthy\(^{d-}\), C57BL/6J, and CBA/Tu.

The Tthy\(^d\) antigen is restricted to the T lymphocyte lineage and is preferentially

### Table I

**Strain Distribution of Tthy\(^d\) Limits the Gene Coding for This Determinant to IgT-C**

| Strain   | Anti-Tthy\(^d\) C\(^\dagger\) | Anti-BAT C\(^\dagger\) | Anti-BSA C\(^\dagger\) | IgH-V | Igh-C | Strain Phenotype* | H(Igh) | Pre-1 |
|----------|-----------------|-----------------|-----------------|-------|-------|------------------|--------|-------|
| C.AL-20  | 23\%            | 94              | 1               | d     | d     | d (d,d)          | −      | a     |
| BALB/c   | 3               | 83              | 1               | a     | a     | (−,−)            | +      | 0     |
| BAB/14   | 1               | 79              | 1               | a     | × b   | − (−,−) ×        | −      | c     |
| C.B.AL-1 | 1               | 78              | 1               | d     | × b   | − (−,−) −        | −      | a     |
| C.B.AL-2 | 3               | 69              | 1               | d     | × b   | − (−,−) −        | −      | c     |
| C.B.AL-4 | 2               | 85              | 1               | d     | × b   | − (−,−) −        | −      | c     |

* Monoclonal antibody from tissue culture supernatant (10 µl/5 × 10\(^6\) thymocytes) was incubated with cells in a 96-well microtiter plate at 37°C for 30 min, followed by a 45-min incubation at 37°C, followed by a 45-min incubation at 37°C with 1–12 dilute selected rabbit complement. Duplicate samples were observed visually.

\(\dagger\) The data shown are for the cytotoxic index of one experiment. The grand arithmetic mean of the difference between C\(^\dagger\) and anti-Tthy\(^d\) was determined for 10 separate experiments for C.AL-20 (25 ± 2%) and BALB/c (2 ± 1%), \(P < 0.001\).

**Fig. 1.** Absorption of anti-Tthy\(^d\) with thymocytes (○) is 10-fold more efficient than absorption with lymph node (●), or marrow (□) cells. Absorption was done by incubating limiting dilutions of cells with 40 µl of tissue culture supernatant from clone 17III(C at 4°C for 1 h. Cells were removed by centrifugation and remaining tissue culture supernatant containing antibody was used to treat C.AL-20 thymocytes as described in Table I.
FIG. 2. Anti-Tthy\textsuperscript{d} recognizes a thymocyte with the Lyt-1\textsuperscript{+}2\textsuperscript{+}3\textsuperscript{+} phenotype. Thymocytes (5 × 10\textsuperscript{7}/500 ml) were divided into three parallel cell groups. Group A was treated with tissue culture media (○); group B with 5 μl of monoclonal anti-Lyt 2.2 (○); and group C with 20 μl of monoclonal anti-Lyt-1. Cells were incubated for 30 min at 37°C, pelleted by centrifugation, and resuspended in 1–12 dilute rabbit complement for 45 min at 37°C (percent of dead cells was A, 12%; B, 48%; C, 67%). Washed cells were used at limiting dilutions to absorb anti-Tthy\textsuperscript{d} from tissue culture supernatant of clone 17IIIC6. Controls, using anti-Tthy\textsuperscript{d} from tissue culture supernatant of clone 17IIIC6. Controls, using anti-Tthy\textsuperscript{d} and C', lysed 64% of the cells, C' alone, 30%, and rabbit anti-BAT and C', 93%. The graph is plotted as cytotoxic index (see Materials and Methods). Standard deviations on duplicate determinations were <2% and were not plotted.

**TABLE II**

Anti-Tthy\textsuperscript{d} Is a New Specificity Not Identified by Monoclonal Anti-Tind\textsuperscript{d} or Anti-Tsu\textsuperscript{d}

| Antibody | C' Preblocking reagent* | Percent dead cells ±SD |
|----------|-------------------------|------------------------|
| –        | –                       | 13.7 ± 1               |
| Anti-Tthy\textsuperscript{d} (17IIIC6)\textsuperscript{f} | +                         | 36.0 ± 3               |
| Anti-Tthy(b)\textsuperscript{a} (208IE6) | +                       | 22.3 ± 1               |
| Anti-Tthy\textsuperscript{d} (17IIIC6) | + Anti-Con A (7IA7) | 37.5 ± 4               |
| Anti-Tthy\textsuperscript{d} (17IIIC6) | + Anti-Tind\textsuperscript{d} (9IIIA2) | 39.0 ± 2               |
| Anti-Tthy\textsuperscript{d} (17IIIC6) | + Anti-Tsu\textsuperscript{d} (13IIIB4) | 33.0 ± 1               |
| Anti-Tthy\textsuperscript{d} (17IIIC6) | + Anti-Tthy\textsuperscript{d} (17IIIC6) | 46.0 ± 11              |
| Anti-Tthy\textsuperscript{d} (17IIIC6) | + Anti-Tthy(b)\textsuperscript{a} (208IE6) | 43.0 ± 8               |

* Antibody used for preblocking was 20 μl of tissue culture supernatant. Antibody was preincubated with cells for 1 h at 37°C and left in during a second incubation with anti-Tthy\textsuperscript{d} and complement.

\textsuperscript{f} Tissue culture supernatant (10 μl/5 × 10\textsuperscript{6} thymocytes) was determined to be at the concentration for optimum lysis (experiments not shown).

expressed on thymocytes. Limiting dilution absorption experiments of Tthy\textsuperscript{d} with thymocytes, lymph node cells, and splenic T and B cells (Fig. 1) suggest that thymocytes express this marker with a cell density and/or total frequency 10 times greater than that of other lymphoid cells. In contrast, bone marrow cells do remove some antibody, suggesting the antigen may be on a very immature T cell. This confirms our negative attempts to lyse peripheral cells with antibody and complement.

The Lyt phenotype of thymocytes expressing this determinant was examined by absorption. Thymocytes were treated with monoclonal anti-Lyt-1.1 or anti-Lyt-2.2
thymocytes were used to absorb anti-Tthy. Limiting dilutions of cells (0.5–5.0 × 10^6) were used to absorb tissue culture supernatant. Absorbed or unabsorbed antibody was used to lyse thymocytes. We conclude that the Lyt-1^+2^+3^+ thymocyte expresses Tthy because either anti-Lyt-1.2 or anti-Lyt-2.2 and complement deplete the cell capable of absorbing anti-Tthy antibody (Fig. 2). It is possible that Lyt-1^+2^+3^-, Lyt-1^+2^+3^+, and Lyt-1^-2^+3^+ cells all express Tthy. Our frequency estimates are based on the use of a complement that is cytotoxic for 25% of thymocytes. If that cell lysis was nonspecific, then the actual number of positive cells could be significantly greater.

Tthy is a unique specificity not recognized by monoclonal antibodies specific for Tind (9IIIA2) (15) or Tsu (13IIIB4) (unpublished observation). Thymocytes were treated with anti-Tthy (clone 17IIC6) or anti-Tthy(b) (clone 208IE6) and complement (Table II). Pretreatment of thymocytes with nonlytic IgG1K monoclonal antibodies against Con A (71A7) (21), Tind (9IIIA2) (16), or Tsu (13IIIB4) did not block lysis with anti-Tthy (17IIC6). Clone 208IE6 must recognize a similar specificity to that recognized by 17IIC6. The cytotoxicity observed with the two antibodies is not additive and the cell populations seem identical (data not shown). Clone 208IE6 has tentatively been named anti-Tthy(b) pending biochemical identification. Antibody from clone 208IE6 has been typed IgG1k but is a complement-fixing molecule.

Discussion

Evidence for the expression of antigen binding receptors on the surface of thymocytes is indirect. Most of the lymphocytes in the thymus are nonfunctional prothymocytes (22). A few cells corresponding to the low density cortisone-resistant populations do respond to lectins and alloantigens when the unresponsive population is removed (23). The most convincing argument that antigen receptors exist for thymocytes has arisen from allogeneic radiation chimeric mice. It has been shown (24, 25) that T cells, before their exit from the thymus, are influenced to recognize self histocompatibility antigens in association with nominal antigen. Many hypotheses have attempted to explain this apparent “imprinting” involving either thymic epithelium (26), the thymic nurse cell (27), or a nonthymic hematopoietic cell (28). Some studies would suggest this imprinting may actually occur earlier in hematopoiesis. When nu/nu lymphocytes were transferred into recipients, the same restrictions (29) as those attributed to influence of a thymic microenvironment were observed. If early thymocytes or prothymocytes in the bone marrow can recognize self- or alloantigens (30), then it is probable that antigen-specific receptors modulate this recognition. However, in contrast to mature, post-thymic T cells (31–33), thymocytes have never been shown to directly bind to cell monolayers or antigen.

We have previously reported two alloantigens, Tsu^d^ and Tind^d^, that may represent isotypes of T cell receptors (13). If this is the case, it is surprising that neither Tsu^d^ (9) nor Tind^d^ (15) is expressed in a detectable way on the majority of thymocytes. Tsu^d^ can be detected in the thymus only when cells are separated by density sedimentation to enrich for the least dense cells (9). Tind^d^ in contrast has not been found on this cell population or any other in the thymus in our visual fluorescent microscopy experiments. The apparent lack of these two antigens in the thymus gave rise to the experiments summarized here. Tthy appears to be another T cell-specific alloantigen coded for by a gene(s) closely linked to Tsu^d^ and Tind^d^ but preferentially expressed
on thymocytes (Table I). It seems possible that Tthy\textsuperscript{d} represents a third isotype of an antigen-binding moiety, and that selective pressures during T cell maturation induce an isotype switch from Lyt-1\textsuperscript{+}2\textsuperscript{+}3\textsuperscript{+} thymocytes and the Lyt-1\textsuperscript{+}2\textsuperscript{+}3\textsuperscript{−} or Lyt-1\textsuperscript{−}2\textsuperscript{+}3\textsuperscript{+} splenic cells. The frequency of Tthy\textsuperscript{d}-positive cells (estimated by antibody and complement-mediated lysis) is a minimum of 30\% of the total thymocytes. This implies that many, if not all, of the Lyt-1\textsuperscript{+}2\textsuperscript{+}3\textsuperscript{+} thymocytes express Tthy\textsuperscript{d}. However, absorption experiments with lymph node or splenic cells indicate Tthy\textsuperscript{d} is undetectable on Lyt-1\textsuperscript{−}2\textsuperscript{+}3\textsuperscript{−} or Lyt-1\textsuperscript{−}2\textsuperscript{+}3\textsuperscript{+} “mature cells.” In contrast, Tsu\textsuperscript{d} is present on a limited number of thymocytes corresponding to the low density, functionally reactive (9) population and also on the Lyt-1\textsuperscript{−}2\textsuperscript{+}3\textsuperscript{+} splenic T cells (7, 9, 10). In comparison, Tind\textsuperscript{d} is present on a low frequency of Lyt-1\textsuperscript{−}2\textsuperscript{−}3\textsuperscript{−} splenic T cells (15).

An isotypic switching mechanism might be an adaptive mechanism for control of self-recognition interactions. An early, nonfunctional precursor T cell in the thymus may be impotent because its isotype is one that is not released effectively, is enzymatically degraded easily, and/or is characterized by low affinity for antigen. This would be an advantage intrathymically if low affinity for self-recognition is a prerequisite for extrathymic trafficking. A post-thymic shift to an isotype associated with higher avidity for antigen and a functionally distinct role would allow a functional cell to release a product capable of more efficiently homing to its target. If there are multiple isotypes of T cell receptors and if those receptors are released in either an identical or post-translationally modified form to serve as soluble mediators, then it is probable that different isotypes may have different effector functions. The ability to bind antigen and to express molecules serologically cross-reactive with the major serum cross-reactive idiotypes is a property associated with both helper factors (33) and suppressor factors (34). The antigen-binding site alone should therefore not be enough to determine functionality of a T cell product. The isotype switch model would provide a mechanism for preservation of the same T cell “idiotype” on helper and suppressor cell isotypes, permitting regulation of the balance of the immune response by a closed but fluid loop of idiotype anti-idiotype interactions (35). The switch mechanism proposed here has many similarities with the change in surface immunoglobulin isotypes characteristic of virgin- and antigen-triggered B lymphocytes (36). A sequential genetic rearrangement of the DNA in developing cells (37) has provided a mechanistic answer to this phenomenology. A similar mechanism might be used by T cells. The close proximity of the genes coding for Tsu\textsuperscript{d}, Tind\textsuperscript{d}, and Tthy\textsuperscript{d} to the immunoglobulin locus could allow these T cell genes to combine with B cell antigen-specific variable region genes. Alternatively, T cells may have their own unique but evolutionarily related variable region genes, utilizing a similar genetic rearrangement. The finding that B cell J segments are not rearranged (8, 38), and that the g gene is not deleted (38) in T cell lines, makes the second possibility attractive. However, other post-translational mechanisms for conservation of B cell variable region genes on T cell receptors are equally plausible.

The model outlined above suggests that the most mature T effector cells should express nonoverlapping isotypes. Our observations show that Tsu\textsuperscript{d} and Tind\textsuperscript{d} are preferentially expressed on cells with different Lyt phenotypes. One might expect to see a spectrum of cells expressing a progression of T cell isotypes as the cells move further away from the thymic influence.

The foregoing hypothesis makes the assumption that Tsu\textsuperscript{d}, Tind\textsuperscript{d}, and Tthy\textsuperscript{d} are
antigen-binding gene products. That fact has been documented for the monoclonal
Ars Ts1 factor. It is possible that this series of T cell alloantigens is a group of
differentiation antigens parallel to the Lyt series, but the association of Tind with a
suppressor factor makes this unlikely. In either event, the region of chromosome 12
tentatively designated IgT-C appears to encode a series of T cell-specific gene products
of fundamental importance to T cell activation and maturation.

Summary

Tthy is an alloantigen coded for by a gene(s) near the immunoglobulin locus on
chromosome 12 in the mouse. This T cell-specific antigen may be the third member
of a family of antigen receptors on T cells encoded by a cluster of genes in the IgT-C
region. This antigen is preferentially expressed on thymocytes in contrast to Tind or
Tsu that are expressed on peripheral T cells. The hypothesis that T cell receptors
undergo a switch in surface isotype upon maturation is discussed.

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