PRODUCTS OF THE IgT-C REGION OF CHROMOSOME 12 ARE MATURATIONAL MARKERS FOR T CELLS

Sequence of Appearance in Immunocompetent T Cells Parallels Ontogenetic Appearance of $Tthy^d$, $Tind^d$, and Tsu^d*

By FRANCES L. OWEN

From the Tufts Medical School, Departments of Pathology and Cancer Research Center, Boston, Massachusetts 02111

The existence of multiple isotypes of T cell receptors has been hypothesized (1, 2). A biological role for individual isotypes could be linked to the effector function of their constant regions. A cytotoxic T cell may effectively use a different molecule than does a suppressor T cell. Alternatively, T cell isotypes may be characteristically distributed on cells at different stages in immune competence, i.e., a pre-T cell may use a different isotype than an antigen-triggered effector cell. The experiments described here use monoclonal antibodies, specific for products of the IgT-C region of chromosome 12 (G. M. Spurll, M. Frye, L. Riendeau, A. Finnegan, and F. L. Owen, manuscript in preparation), to address the later question.

Three T cell alloantigens linked to Igh-1, but separable by recombination from these genes, have been described (2, 3). These antigens may be allotypic determinants in the constant regions of antigen-specific receptors (1-5). $Tthy^d$, $Tind^d$, and Tsu^d are expressed preferentially on thymocytes or Lyt 2^+ peripheral cells or Lyt-1^+ cells, respectively. In this study, cells at different stages in immunological maturation were examined by cytotoxicity for expression of these three antigens. The order of appearance from the marrow to the antigen-triggered cell is $Tthy^d$, $Tind^d$, or Tsu^d. This parallels the order of appearance of these antigens in ontogeny. $Tthy^d$ is a thymocyte surface marker at days 1-2 after birth, $Tind^d$ is detectable at days 2-3 after birth, and Tsu^d appears at days 5-6 after birth.

These studies illustrate a maturational sequence for the appearance of Tthy, Tind, and Tsu but are also consistent with some functional compartmentalization of the receptor repertoire because none of these alloantigens are present on cytotoxic or mixed leukocyte-reactive (MLR)[1] cells (G. M. Spurll et al., see above). It seems probable that acquisition of isotypes is not the biological pressure for functional differentiation because all these antigens are acquired postnatally, and functional preT cells are already present in embryonic thymus (6).

These studies show that Tthy, Tind, and Tsu segregate independently in T cell hybrids, demonstrating that they are products of separate genes and not individual

* Supported by grants R01 AI-15262 and R01 AI-17442 from the National Institutes of Health.

[1] *Abbreviations used in this paper:* BAT, brain-associated thymocyte antigen; BSA, bovine serum albumin; CFA, complete Freund's adjuvant; FITC, fluorescein isothiocyanate; HAT, hypozanthine, aminopterine, thymidine; KLH, keyhole limpet hemocyanin; nude, athymic mouse; PEG, polyethylene glycol; TNBS, picrylsulfonic acid.

allotypic determinants on the same polypeptide. However, some hybrids (15%) show $Tthy^d$ coexpression with $Tind^d$ or Tsu^d. This stable dual expression is reminescent of the B cell that expresses both IgM and IgD (7). The data are consistent with a maturational model for T cells in which $Tthy^d$ antigen is expressed on a precursor cell for the antigen-triggered cells bearing Tsu^d and $Tind^d$; evidence for Tsu and Tind being separate lineages of cells is presented. Although the $Tthy^d$-bearing cell may be a precursor for the $Tind^d$ and Tsu^d-bearing cells, it is not a pre-T cell (8, 9).

Materials and Methods

Mice. C.AL-20 mice, bred at Tufts Medical School since 1978, originated from stock developed by M. Potter, National Institutes of Health, Bethesda, MD. A/J and AKR nude streaker mice (*nu/nu* and age-matched *nu/+*) were purchased from The Jackson Laboratory, Bar Harbor, ME. BALB/c AnN animals were purchased from Charles River Breeding Laboratories, Inc., Wilmington, MA.

Production of Monoclonal Cell Lines. Clones of cells producing monoclonal antibodies directed against T cell products were selected by fusion of BALB/c anti-C.AL-20-immune spleen cells with the BALB/c P.3U1 hypoxanthine, aminopterin, thymidine (HAT)-sensitive cell line (10). BALB/c AnN males were immunized with C.AL-20 blast cells. Donor cells were stimulated with 5 μg/ml concanavalin A (Con A) for 48 h in complete culture media, harvested, and separated on bovine serum albumin (BSA) discontinuous density gradients (5). Cells were washed five times in phosphate-buffered saline (PBS), resuspended, and injected intraperitoneally.

Spleen cells from immunized donors were tested between frosted glass slides, erythrocytes (RBC) were lysed with Tris ammonium chloride, and the cells were washed in serum-free media. One spleen equivalent was pelleted with 1.2×10^7 P.3U1 cells in serum-free Dulbecco's H-21. Polyethylene glycol (PEG) (Sigma Chemical Co., St. Louis, MO), 1,000 mol wt, in a 30% solution (0.5 ml), was added to the drained pellets. Cells were resuspended and immediately repelleted by 1,000 *g* centrifugation at room temperature. PEG was removed, and 10 ml of Dulbecco's H-21 was added slowly over 5 min. Cells were pelleted and resuspended in 60 ml of media, used to maintain the P.3U1 parent line, and incubated at 37°C overnight. On day 1, 1×10^{-4} M hypoxanthine, 4×10^{-7} M aminopterine and 1.6×10^{-5} M thymidine were added, and cells were aliquoted into 96-well microtiter plates (Costar Data Packaging, Cambridge, MA). On day 7, cells were fed media containing HAT. Clones were visible on day 8. The fusion frequency was ~50% but variable for each fusion.

Tissue culture supernatant was screened for clones secreting antibody by a cell surface radioimmunoassay. Con A was used to activate either BALB/c or C.AL-20 T cells grown in RPMI 1640 with 2% horse serum, not fetal calf serum, which was used in the growth of blast cells for immunization. Con A-activated blast cells were harvested in PBS and washed extensively in serum-free PBS before aliquoting (2×10^5 cells/100 μl) into each well of a 96-well polyvinyl chloride tray. Plates were centrifuged to increase the adherence of cells, and supernatant was removed. The plates were placed on ice, and 100 μl of 0.05% glutaraldehyde was added for 5 min and followed by addition of 0.05% ammonium chloride (100 μl) and washed four times with PBS. Horse serum (2% in PBS) (0.04 M NaN_3) was added overnight at 4°C. Plates containing either C.AL-20 or BALB/c cells were incubated with tissue culture supernatants (50 λ/well) for 2 h at room temperature. The plates were washed and incubated with 100 μg of ^{125}I goat anti-mouse IgG_1 or ^{125}I goat anti-mouse IgM overnight at 4°C. Labeled plates were washed in PBS and separated on a hot wire cutter for counting. Positive clones were selected on the basis of binding of the antibody to C.AL-20 cells and not to BALB/c cells.

Amplification of Antibody-mediated Cell Lysis by Fluorescein Isothiocyanate (FITC)-modified Monoclonal Antibody and Mouse Anti-FITC Immunoglobulin. Monoclonal antibody was collected from the ascites of mice primed with pristane and inoculated with hybrids of P.3U1 and BALB/c AnN anti-C.AL-20-immune cells.[1] Ammonium sulphate-precipitated protein (10 mg/ml) was modified by incubating with 0.5 M sodium carbonate (pH 9.5, containing 10 mg/ml FITC) for 1 h at 4°C. Labeled protein was eluted from a G-25 Sephadex column in 0.1 M sodium phosphate. Antibody was stored at 4°C in 0.04 M NaN_3. Limiting dilutions of FITC-modified

antibody were incubated with thymocytes from either C.AL-20 or BALB/c animals for 30 min at 37°C. Affinity-purified mouse antibody specific for FITC was incubated with washed cells for 30 min at 37°C without NaN_3, and pelleted cells were resuspended in selected rabbit complement. After incubating at 45°C for 30 min, cells were resuspended in PBS, pelleted, and stored at 4°C, while being scored by eosin red dye exclusion under visual microscopy. FITC anti-Tthy^d was used at 0.005 $\mu g/10^5$ cells. This is a 20-fold lower concentration than the unmodified antibody and permitted adsorption by fewer numbers of cells. Data are expressed as cytotoxic index. This represents the percent dead cells in samples treated with antibody and complement minus the percent dead cells in samples treated with complement divided by the percent dead cells in samples treated with rabbit anti-brain-associated thymocyte antigen (BAT) minus the percent dead cells in samples treated with complement times 100%.

Adsorption. Monoclonal antibody modified with FITC (0.005 μg) was incubated with limiting numbers (1×10^6 to 1.5×10^7) of lymphoid cells in an Oudin centrifuge tube (Fisher Scientific Co., Pittsburgh, PA) for 45 min at 4°C. Cells were centrifuged, and clarified supernatant was incubated with target cells to evaluate the extent of depletion of antibody.

BSA Gradient Separation of Thymocyte. The methods used have been described (5).

Cortisone-resistant Thymocytes. Adult (8–10 wk) male A/J animals were injected intraperitoneally with 2.5 mg of cortisone phosphate (Merck, Sharp & Dohme, West Point, PA) in suspension. Animals were killed at 48 h.

Antigen Priming of Lymph Nodes. Keyhole limpet hemocyanin (KLH; Calbiochem-Behring Corp., San Diego, CA) was modified with picrylsulfonic acid (TBNS). Antigen (50 μg in PBS) was emulsified in 50 μl of complete Freund's adjuvant (CFA; Difco Laboratories, Detroit, MI) and injected into footpads of adult animals on days 0 and 7. The draining popliteal nodes were removed at day 10 and used as targets for antibody- and complement-mediated lysis.

Selection of Fetal Thymocyte and Fetal Liver Hybrids. Thymocytes were removed microscopically from 17-d embryos (pregnancies were timed from the appearance of vaginal plugs). Thymocytes (2×10^6 cells/8 embryos) and fetal liver cells (10^7 cells/8 embryos) were fused with 10^7 BW5147 HAT-sensitive tumor cells by incubating in 0.1 ml of 40% PEG (Kochlight Laboratories, Inc., Coinbrook, England) for 1 min. Serum-free Dulbecco's high glucose minimal essential media (Microbiological Associates, Bethesda, MD) was used to dilute cells to 10 ml over a 10-min period. Cells were pelleted gently at 25°C and resuspended in media containing 10% FCS for 24 h in a 100-mm petri dish. At 24 h, cells were collected by centrifugation and resuspended in media containing HAT (1×10^{-4} HAT (1×10^{-4} M hypoxanthine, 1.6×10^{-5} M thymidine, and 4×10^{-7} M aminopterine)). Clones (4/150 wells per 8 embryos for thymocytes and 2/500 wells per 8 embryos for fetal liver) appeared at 14 d and were screened with antibody and complement to see whether they expressed the Thy-1.2, H-2^d, Lyt-1.2, and Lyt-2.2 antigens. Lines were used to adsorb anti-Tthy^d, anti-Tind^d, or anti-Tsu^d.

Selection of Hybrid T Cell Lines Originating from Antigen-primed Lymph Node Cells. Lymph nodes from C.AL-20 mice secondarily immunized in the footpads with KLH-trinitrophenyl (TNP) in CFA were removed and fused with BW 5147 cells (AKR HAT-sensitive T cell tumor) (10). Resulting hybrids were scored for surface markers characteristic of the C.AL-20 mice and not the AKR tumor parent (Thy-1.2, Lyt-1.2, Lyt-2.2, H-2^d). Hybrid lines were subcloned by limiting dilution analysis and screened for expression of Tsu^d, Tthy^d, and Tind^d alloantigens. The AKR tumor parent, BW5147, is surface negative for these antigens.

Results

Tthy^d Is expressed on Equal Numbers of Cortisone-sensitive and Cortisone-resistant Thymocytes. The observation (3) that only 35% of thymocytes can be lysed by anti-Tthy^d and complement suggested this T cell alloantigen might be a subpopulation marker in the thymus. Monoclonal antibodies specific for Tthy^d (clone 17IIC6, $\mu\kappa$), Tind^d (clone 9IIIA2, γ_1, κ), and Tsu^d (clone 13III B4, γ_1, κ) were modified with FITC (methods) and used with mouse anti-FITC antibodies and complement to estimate the frequency of positive cells in mice treated with 2.5 mg of cortisone phosphate 48 h before surgical removal of the thymus. These values were compared with the

percentages of cells expressing each T cell alloantigen in normal animals evaluated at the same time (Table I). $Tthy^d$ is expressed on 25–35% of cells, but anti-Tsu^d or anti-$Tind^d$ do not detect any positive cells in the thymus by direct lysis. The frequency of $Tthy^d$-positive cells is neither enhanced nor decreased in the cortisone-resistant population, which represents 20% of total thymocytes in the A/J animal. This suggests that an equal frequency of cells in the cortisone-sensitive and -resistant populations are positive (25–35%), although density of determinants per cell might limit the accuracy of frequency estimates. Fluorescence experiments (not shown) were unsuccessful, suggesting the number of surface determinants per cell might be limiting.

Cortisone-resistant Thymocytes Preferentially Express $Tind^d$ and Tsu^d. Anti-$Tind^d$ and anti-Tsu^d do not lyse thymocytes (Table I). However, adsorption of anti-$Tind^d$ with thymocytes depletes the lytic antibody tested on antigen-activated popliteal lymph node cells (Fig. 1). The absorption with spleen cells parallels that with thymocytes, in contrast to cortisone-resistant thymocytes, which are 10-fold enriched in adsorbing cells (frame A). Anti-Tsu^d could also be adsorbed by a comparable number of

TABLE I
Thymocytes Expressing $Tthy^d$ Are Not Restricted to a Subpopulation Defined by Cortisone Phosphate Sensitivity

FITC-modified antibody	Control thymocytes	Cortisone-resistant thymocytes
$Tthy^d$ (17IIC6)	23 ± 1*	15 ± 4‡
Tsu^d (13IIIB4)	2 ± 1	1 ± 1
$Tind^d$ (9IIIA2)	1 ± 1	1 ± 1
Thy-1.2	85 ± 5	90 ± 5

* Cytotoxic index.

‡ A/J (3-wk-old males, six experimental and six control) were treated with 2.5 mg cortisone phosphate (i.p.) 48 h before killing. Thymocytes surviving (2–4 × 10^7 cells/mouse) were 20% of the yield from normal age-matched mice.

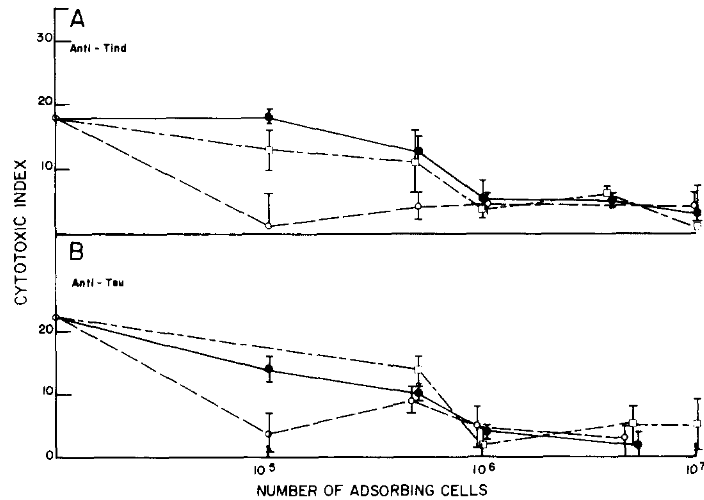

FIG. 1. Thymocytes from cortisone-treated (○) or control (●) mice were used to adsorb anti-Tsu^d or anti-$Tind^d$. Residual antibody was tested on antigen-activated lymph node cells. The ability of splenic T cells to adsorb these antibodies is compared with thymocytes (□).

cortisone-resistant thymocytes (frame B). Therefore, Tsu^d and $Tind^d$ are both preferentially expressed on the cortisone-resistant population.

Thy-1.2-sensitive Cells in Bone Marrow Express $Tthy^d$. Cells in the bone marrow absorb anti-$Tthy^d$ monoclonal antibody with an efficiency 15–25-fold lower than that of thymocytes (3). Pretreatment of the adsorbing cell with anti-Tthy-1.2 monoclonal antibody (11) and complement or anti-rabbit anti-brain-associated antigen (BAT) (12) antibody and complement depletes an adsorbing cell (Table II). Pretreatment of bone marrow cells with complement and adsorption of anti-$Tthy^d$ does remove the lytic activity (reduced from C.I. 17 to C.I. 5), showing that complement above does not lyse this cell. The use of cytotoxic index to display data corrects for spontaneous lysis of thymocytes by complement (5%). Four experiments showing that both anti-BAT and anti-Thy-1.2 lyse the Tthy-bearing cell in marrow were done in double-blind experiments. In one experiment, anti-Thy-1.2 alone did not block this adsorption. These results might imply that the positive cell in marrow is a recirculating cell because prothymocytes are reputed to be Thy-1.2 negative (8).

BSA Gradient Fractionation of $Tind^d$- and Tsu^d-bearing Thymocytes. Discontinuous sedimentation of thymocytes on BSA gradients separates the populations into characteristic groups (13), which correlates with ability to respond to phytohemagglutinin (PHA) (14). These groups show differences in the levels of H-2 antigens and Thy-1.2 per cell (15), and a decrease in surface density might be associated with more "mature" cell populations on the basis of surface characteristics. These characteristic bands suggest there are a small number of thymocytes (2%) that are low density cells expressing Tsu^d and/or $Tind^d$ but not $Tthy^d$ (Table III).

Antigen-activated Lymph Node T Cells Express $Tind^d$ and Tsu^d Antigens but Not $Tthy^d$. Monoclonal antibodies specific for $Tthy^d$, $Tind^d$, and Tsu^d do not lyse popliteal lymph node cells from virgin mice. However, secondary immune lymph node cells from footpad-challenged mice can be lysed with antibody and complement (Table IV). The frequency of Tsu^d- or $Tind^d$-expressing cells is not <15% of viable cells on day 3 after a secondary immunization with KLH-TNP in CFA. $Tthy^d$ cannot be detected on these cells. When CFA alone was used to inject the mice, increased frequencies of cells expressing Tind or Tsu could not be detected (data not shown). Although CFA contains mycobacterium, a potential antigen, the quantity of antigen might be too low to stimulate T cells.

Spleen cells activated with Con A adsorbed antibody nonspecifically to an extent that did not permit examination of the expression of Tsu^d, $Tind^d$, or $Tthy^d$ on

TABLE II

Bone Marrow Adsorption of Anti-$Tthy^d$ Is Dependent on a T Cell That is BAT and Thy-1.2 Surface Positive

Pretreatment adsorbing bone marrow cells	Number adsorbing cells	Antibody	C′	Thymocyte target cells cytotoxic index
—	0	Thy-1.2	+	78 ± 1
—	0	$Tthy^d$	+	17 ± 3
—	10^7	$Tthy^d$	+	1 ± 1
Anti-Thy-1.2 + C′	10^7	$Tthy^d$	+	21 ± 3
Anti-BAT + C′	10^7	$Tthy^d$	+	13 ± 3
C′	10^7	$Tthy^d$	+	5 ± 1

TABLE III
Absorption of Tthy^d, Tind^d, and Tsu^d by Thymocytes Separated on BSA Discontinuous Gradients

Percent BSA-separated adsorbing cells	Cytotoxic index		
	Tthy^d	Tind^d	Tsu^d
None	15 ± 2	28 ± 5	25 ± 5
23	11 ± 1	24 ± 1	16 ± 1
26	>1	10 ± 1	7 ± 3
29	>1	9 ± 1	7 ± 2
35	>1	15 ± 2	26 ± 1
Unfractionated	>1	24 ± 2	29 ± 4

TABLE IV
Enhanced Expression of Tsu^d and Tind^d on Antigen-activated T cells

FITC-modified antibody	Normal lymph node	Immune lymph node*
Anti-Tind^d (9IIIA2)	1 ± 1‡	19 ± 2
Anti-Tthy^d (17IIC6)	1 ± 1	2 ± 1
Anti-Tsu^d (13IIIB4)	1 ± 1	12 ± 3

* A/J (8-wk-old males) were immunized in the footpad with 50 μg of KLH-TNP in CFA (1:1 in PBS) on days −10 and −3. Popliteal nodes from three animals were pooled for each group.
‡ Cytotoxic index on direct visual cytotoxicity experiment.

mitogen-activated cells. C.AL-20 or BALB/c cells grown for 96 h in complete culture media with 5 μg/ml Con A failed to express these T cell alloantigens in a way detectable by direct cytotoxicity. Adsorption of antibody with either allelic form of antigen (C.AL-20 or BALB/c) removes all activity, suggesting Fc-nonspecific adsorption has obscured any results.

Expression of Tsu^d, Tthy^d, and Tind^d in the Homozygous AKR "Nude Streaker" and Heterozygous AKR Littermates. Because Tthy^d is expressed on a cell in marrow, it was possible that a pre-T cell also expressed this determinant. The nude animal is a rich source of pre-T cells. An animal that had the Igh-1^d or Igh-1^e type and a nude gene on the same genetic background was required to complete this experiment. The AKR (Igh-1^d) animal has been bred for expression of the *nu/nu* streaker phenotype. The streaker mutant genetic phenotype arose as a spontaneous mutation event separate from the original nude mutation. The genetic trait is in the same complementation group as the original *nu/nu* gene (16). In breeding experiments, this gene was shown to be allelic with nude. The phenotypic and development characteristics of the nu^{str}/nu^{str} animals are currently inseparable from those of the *nu/nu*.

Bone marrow cells from the AKR $+/nu^{str}$ or nu^{str}/nu^{str} animal were used to adsorb FITC-modified anti-Tthy^d monoclonal antibody (Fig. 2). Cells from the thymus of the heterozygous animal ($+/nu^{str}$) were used as a positive control. Cells from the nude animal (nu^{str}/nu^{str}) failed to adsorb anti-Tthy^d in contrast to cells from the heterozygote ($+/nu^{str}$). These data suggest that Tthy^d is not a marker for pre-T cells present in the nude animal. Splenic cells from both animals were also used for adsorption; both were negative (data not shown).

Expression of Tind^d, Tthy^d, and Tsu^d Alloantigens on Hybrid T Cell Lines Constructed

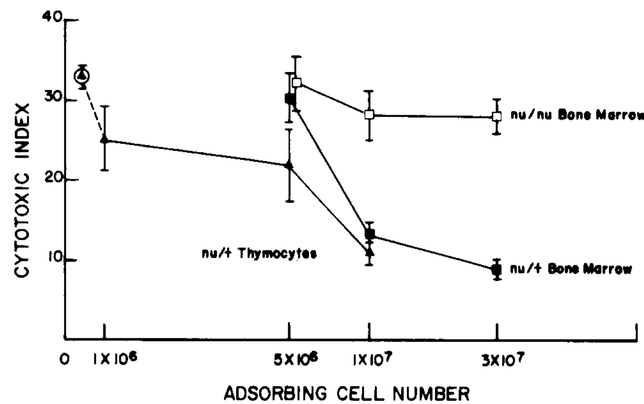

FIG. 2. Bone marrow cells from AKR *nu/nu* (□) or littermate heterozygous (*nu*/+) (■) animals were used to adsorb FITC-modified anti-Thy^d monoclonal antibodies. AKR *nu*/+ thymocytes, used as adsorbing cells (▲), are shown for comparison. Adsorbed antibody was used for cytotoxicity on A/J (5 wk) thymocytes. Unabsorbed antibody (●) was the positive control.

between BW5147 and C.AL-20 Antigen-activated Lymph Node Cells. Hybrid T cell lines express Tsu^d, Tthy^d, and Tind^d alloantigens (Table V). The lines express these three determinants independently, supporing the concept that these antigens are carried on separate structural products, in contrast to the possibility that they might be three antigenic sites on the same molecule. The 27 lines typed showed random association between Lyt-1 and Lyt-2 and these antigens. We had reported earlier (2, 5) that Tind^d was preferentially associated with Lyt-1-bearing cells and that Tsu^d was associated with Lyt-2$^+$ cells. Those determinations were made by adsorbing antibody with virgin cell populations. The cells used for fusion originated from the lymph node of KLH.TNP CFA secondarily immunized mice. The immune cell populations could be vastly different from the virgin, and/or fusion to the tumor cell (BW5147) could result in gene inactivation or activation so that an unexpected product is expressed on the surface. These data, however, lend little support for a maturation pathway in which Tsu^d- or Tind^d-bearing populations parallel the Lyt differentiation schemata. No lines coexpress both Tsu^d and Tind^d, suggesting these are distinct and possibly nonoverlapping lineages. In contrast, we found four (15%) lines where Tthy^d was coexpressed with either Tsu^d or Tind^d, which is in agreement with but does not prove that the Tthy^d-bearing populations could be precursor populations for both the Tind^d- and Tsu^d-bearing cells. It is also possible that double expressing cells could be the result of triple cell fusions and not be characteristic cells in the developmental pathway. The presence of multiple copies of chromosome 12 per cell could identify trisomatic hybrids. Those karotyping experiments have not been done. The frequency of positive cells in any line varied from 10–50% and fluctuated in response to addition of new culture media. This might indicate that the antigens are expressed with a low surface density per cell, and the unsynchronized growth pattern of each line could lead to disparate densities from cell to cell.

Tthy^d, Tind^d, and Tsu^d Appear Sequentially in Ontogeny. Thymocytes from neonatal mice (1–27 d) were used as targets for direct cytotoxicity with anti-Tthy^d FITC (Fig. 3, frame A), anti-FITC, and complement. Thymocytes from 3-d-old mice express Tthy^d, but the adult frequencies of cells are not detected until after day 19. When

TABLE V
Independent Expression of Tthy^d, Tind^d, and Tsu^d in T Cell Hybrids

Clone	Thy-1.2	Lyt-1.2	Lyt-2.2	T cell phenotype*		
				Tthy^d	Tind^d	Tsu^d
JTφ3	−	−	+	−	+	−
JTφ4	−	+	−	−	−	+
JTφ6	+	−	+	+	−	−
JTφ7	−	+	−	−	+	−
JTφ8	−	+	+	+	−	−
JTφ9‡	+	+	+	+	−	+
JTφ11	+	−	+	−	+	−
JTφ13	+	−	−	−	−	+
JTφ14	−	+	−	−	−	+
JTφ15	−	−	−	+	−	−
JTφ16‡	−	−	−	+	+	−
JPD	−	−	+	−	−	+
JPE‡	+	+	−	+	+	−
JTφ19	−	−	−	+	−	+
JTφ21	+	+	−	−	−	+
JTφ22	−	−	−	+	−	−
JTφ23	−	−	−	−	−	+
JTφ24	−	+	−	+	−	−
JTφ26	−	+	+	+	−	−
JTφ27	−	+	+	−	−	+
JTφ28	+	−	+	−	+	−
JTφ30	−	+	+	+	−	−
JTφ31‡	−	+	+	+	−	+

* Phenotyping was executed with antibody and complement. The percentage of positive cells in each hybrid line varied from 10–50% and may result from surface antigen density variations in unsynchronized cell populations.

‡ Four lines were positive for both Tthy^d and Tsu^d or Tind^d. This dual expression in JTφ9 has been maintained for 9 mo in vitro and appears to be a stable characteristic of the cell.

neonatal thymocytes were used to adsorb anti-Tthy^d and the residual antibody was scored on adult thymocytes, Tthy^d could be detected on days 1–2 (Fig. 3, frame B). It is possible that Tthy^d is expressed on fetal thymocytes as a surface determinant but our assay is too insensitive to detect these determinants. One might predict a gradual increase in surface density as the positive cells appear in ontogeny. There is a linear increase in the percentage of positive cells between day 2 and day 5 (Fig. 3, frame A). Extrapolating that line back to the origin would suggest the frequency in fetal cells should be <0.1%. It seemed of interest to look for surface expression of Tthy^d on fetal thymocytes because functional studies by Ceredig et al. (17) have shown that day 14 fetal cells have antigen receptors for histocompatibility antigens. To overcome the problems of low yields of fetal cells from dissection, coupled with an expected low frequency of positive cells, hybrid T cell lines were constructed by fusing BW5147 HAT-sensitive cells with 17-d fetal thymocytes (Table VI). Three lines were established, all of which failed to adsorb these antigens. Statistcally, one would have to examine 10,000 lines, if the frequency of positive cells is 0.1%, to conclude that day-17 thymocytes do not express Tthy^d as a surface antigen. It is, however, apparent that some fetal cells that express Thy-1.2 do not express Tthy^d. Two fetal T lines originating

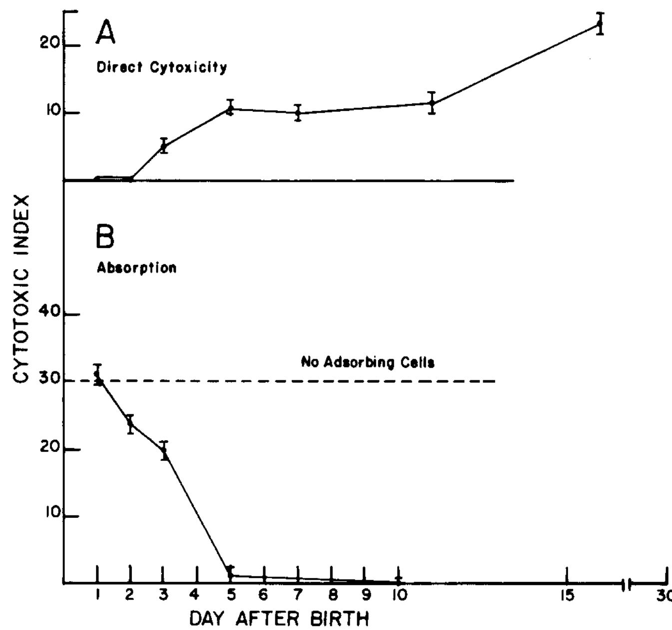

FIG. 3. Ontogeny of surface expression of Tthy^d detected by direct cytotoxicity on neonatal thymocytes or adsorption of antibody onto neonatal thymocytes. Cells from neonatal thymus (5 × 10^5 cells/microtiter well) were incubated with 10 μl of tissue culture supernatant containing monoclonal anti-Tthy^d modified with FITC. Percent dead cells was enumerated after an incubation with complement (frame A). In contrast, 5 × 10^6 neonatal thymocytes were used to adsorb 40 μl of monoclonal anti-Tthy^d (frame B). Residual antibody was scored by cytotoxicity on 4-wk-old thymocytes.

TABLE VI
Tthy^d, Tind^d, and Tsu^d Are Not Expressed on Day-17 Fetal T Cell Hybrids

Clone	Thy-1.2	Lyt-1.2	Lyt-2.2	Tthy^d	Tind^d	Tsu^d
FTT 17-1	+	–	–	–	–	–
FTT 17-2	+	–	–	–	–	–
FTT 17-3	+	+	–	–	–	–
FTL 17-1	+	–	–	–	–	–
FTL 17-2	–	–	–	–	–	–

* Surface phenotyping was done with antibody and complement. All are surface immunoglobulin negative.

from fusion of BW5147 with fetal liver cells are reported in Table VI. It is possible that these cells are precursor T cells because one line expresses Thy-1.2 with high densities at all times in the cell cycle, one is negative for Thy-1.2, but both fail to have detectable levels of Lyt-1 and Lyt-2. However, the ability to express Lyt-1 and Lyt-2 could be dependent, either directly or indirectly, upon the expression of chromosomes that are lost in these hybrid lines. All the fetal lines experienced 4-wk crisis periods early in establishment. Similarly, only one of the three lines that originated from fetal thymus fusion with BW5147 expressed Lyt-1.2. Because Thy-1.2 and Lyt-1.2 should be expressed on fetal thymocytes at 17 d, it is unclear why our hybrids do not display these antigens. This might be a result of the statistical frequency of positive cells, BW5147 might have a preferential fusion partner, or Lyt-1.2 might be on a chromo-

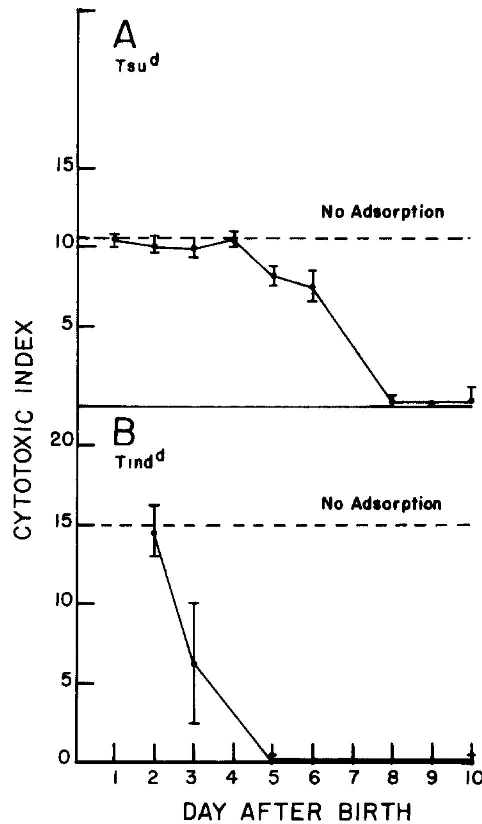

FIG. 4. Ontogeny of surface expression of Tsu^{d} and Tind^{d}. In frame A, monoclonal anti-Tsu^{d} (0.005 μg), FITC modified, was adsorbed with 5 × 10^6 spleen cells. Residual antibody was tested by direct cytotoxicity on antigen-primed lymph node cells. In frame B, 0.05 μg of monoclonal anti-Tind^{d}-FITC in 40 μl was absorbed with 5 × 10^6 unfractionated spleen cells. Residual antibody was incubated with KLH-TNP-primed lymph node cells. Previous experiments determined the optimum number of adsorbing cells and the best concentration of antibody.

some that segregates and is easily lost.

Tind^{d} is expressed on neonatal splenic cells at days 2–3 (Fig. 4, frame A). Anti-Tind^{d} can be adsorbed by 5 × 10^6 neonatal spleen cells, and the lytic activity of that antibody on antigen-activated popliteal node cells is depleted by day-2 cells. In contrast, anti-Tsu^{d} could not be adsorbed by splenic T cells (5 × 10^6) until days 5–6 postnatally (Fig. 4, frame B).

Discussion

If multiple isotypes of T cell receptors exist (1), then the expression of distinct isotypes might be characteristic of the maturational level of the cell and not the functional subpopulation (18). B lymphocytes have been shown to undergo a differentiation pathway from IgM → IgM + IgD → IgD or IgG (19) in response to antigenic pressures. It is possible that T cells undergo a similar pathway in which a primitive receptor-bearing cell is the precursor (pre-T) for all T cells. As that cell matures ontogenetically and functionally, it might acquire new isotypes that make it

functionally more efficient (20).

Fetal thymocytes express receptors for alloantigens. At day 14 of gestation, thymocyte precursors for CTL- or MLR-alloreactive cells exist, and these cells can mature functionally in organ cultures (21, 22). These are the earliest known T cell functions (6). Because alloreactivity parallels the development of the thymic rudiment (23) and the earliest expression of Thy-1.2 (24), the antigen receptor on alloreactive T cells could be considered the primitive T cell μ analogue. In contrast to these data, we found that Tthy^d, Tind^d and Tsu^d, all of which are expressed postnatally, are markers on late-maturing cells. It is, therefore, not surprising that monoclonal antibodies specific for Tthy, Tsu, and Tind do not react with cytotoxic effector cells for alloantigens or precursors of MLR-reactive cells. (G. M. Spurll, L. Riendeau, A. Finnegan, and F. L. Owen, manuscript in preparation).

Congenitally athymic mice (BALB/c *nu/nu*) have pre-T cells that can be induced to mature to functional cytotoxic T cells in the presence of T cell growth factor (TCGF) (9). One would expect that they have antigen receptors and, therefore, the presence of a thymus is not a prerequisite for expression of antigen receptors. Because the Tthy^d alloantigen is expressed preferentially on thymocytes and marrow cells and lost on functionally mature T cells, it seemed a likely candidate for a pre-T cell antigen receptor. This is not the case; adsorption studies with marrow and spleen cells show that the AKR *nu/nu* streaker does not have the Thy^d-bearing cell population. The data showing that the marrow cell bearing Tthy^d is a Thy-1.2, BAT-positive cell also suggest that this cell is a more mature recirculating T cell and not a pre-T cell (8). In addition, experiments depleting Tthy^d by in vivo injection at birth with μg quantities of monoclonal anti-Thy^d do not produce a T-cell-depleted mouse, although Tthy^d-, Tsu^d- and Tind-bearing cells do not develop (S. K. Keese and F. L. Owen, manuscript in preparation). Other immunization protocols have been initiated to look for a pre-T cell receptor coded for by a gene linked to Tsu, Tind, and Tthy on chromosome 12. One would predict that a fetal T cell should express that isotype by day 15 in gestation and that the precursor for the cytotoxic T cell in nude animals might retain that isotype.

That T cell receptor isotypes are characteristic of functionally diverse cells has been hypothesized (1, 25). This speculation is based on experimental data showing that Tsu and Tind are preferentially expressed on either Lyt-2 or Lyt-1 cells (1, 2) and on the finding that antisera made with a similar immunization protocol distinguishes between antigen:specific augmenting and suppressing factors (26). Contrasting data from the studies of Binz and Wigzell (27) show that alloreactive antigen receptors on Lyt-1- and Lyt-2-bearing cells are identical. These data are compatible when functional diversification occurs before receptor isotype acquisition. It is possible that the T cell lineage is branched; a pre-T cell could mature into a cytotoxic precursor (Tpc) and, from that line, a group of regulatory cells (Ts, Th, Tind) could diverge. If Ts, Tc, Th develop in ontogeny much later than Tc, the receptor isotypes could be acquired after Lyt-1- and Lyt-2-bearing cells functionally diversify (28). The isotypes of the late-maturing population would appear to be distinctive of the Lyt functional type, whereas an early maturing alloreactive cell would use the same isotypes for Lyt-1 or Lyt-2 cells. If one isotype is more efficient for a particular functional cell, antigenic pressures would expand the most appropriate clone. One would expect to see T suppressor cells with a spectrum of isotypes, one of which might predominate

because these clones have been selectively expanded. In nature, one might expect to see most suppressor receptors associated with the Tsu isotype, whereas individual clones, some of which would be functionally more competent, would have random isotypes.

The latter interpretation fits the data obtained with our T cell hybrids. In the 27 clones examined, cells express either Tsu or Tind. No clone showed simultaneous expression of both antigens, although 3 of 27 clones showed $Tthy^{d}$ co-expressed with Tsu or Tind. These data are consistent with our initial observations that Tsu-bearing cells and Tind cells represent two separate lineages of cells and that the Tthy population may be a precursor for both these cells. Tsu and Tind show random association with Lyt-1 or Lyt-2 in the hybrids. No functional data is available for these clones. It is possible that association of an appropriate T cell isotype with an appropriate I-A or I-J phenotype is necessary for function (29–30). I region-encoded products may contribute to the quaternary structure of these polypeptides. If this is the case, many of our hybrids may be nonfunctional; only a clone that has the appropriate random association of Tind, I-A, and/or Lyt-1 chains would be biologically functional. This model necessitates waste of many precursor clones that might have the appropriate variable region specificity but does account for the observed Ia-associated restrictions on T cell factors.

Some of our "T cell" hybrids (Table V) do not express Tthy-1.2. It is possible that segregation of chromosomes takes place, independently leading to loss of Thy-1.2, Lyt-1.2, and Lyt-2.2. These clones were selected for expression of Tsu, Tind, or Tthy. None express surface immunoglobulin. Mature T cells are frequently low theta dense, and the hybrids might have few alloantigens per cell, escaping detection by direct lysis. The surface expression of Tsu, Tind, and Tthy is a stable phenotype; chromosome 12 may be vital for growth of the cell and, therefore, would not be deleted in viable cells.

The maturational schemata for T cells (Fig. 5) illustrates the acquisition of Tthy, Tind, or Tsu at characteristic points in development. Many thymocytes express $Tthy^{d}$ (35% C.I.), although it is a subpopulation marker. Studies of Keese and Owen show that this is not a determinant on all cells in the thymus. Both cortisone-sensitive and -resistant cells express $Tthy^{d}$, although a low density thymocyte, which is Thy-1.2 sparse and H-2k dense, lacks this antigen. Cortisone-resistant thymocytes, in contrast, express Tind and Tsu. Tsu is preferentially expressed on thymocytes in the least dense BSA fraction. As cells exit the thymus, Tthy is lost. We know it must be present genetically in some of the cells in the periphery because our hybrids, which originate from lymph node, rescue this specificity. This might be due to genetic complementation, assuming $Tthy^{d}$ is not deleted, and $Tthy^{d}$ need not be assumed to be a surface marker in lymph node in vivo for the results to be consistent. Resting spleen cells can adsorb Tsu and Tind but the frequency of cells and/or density of antigen per cell prevents visualization of direct killing. In contrast, 15–20% of popliteal node cells from antigen-primed, footpad challenged mice express Tind or Tsu. The populations appear to be numerically nonoverlapping. Tthy-, Tind-, and Tsu-bearing populations might represent a progression of cells undergoing differentiation pressures, which parallel acquisition of immunocompetence.

B lymphocytes express surface isotypes after antigenic maturation in a preprogrammed order that follows the order of the constant region genes on chromosome 12

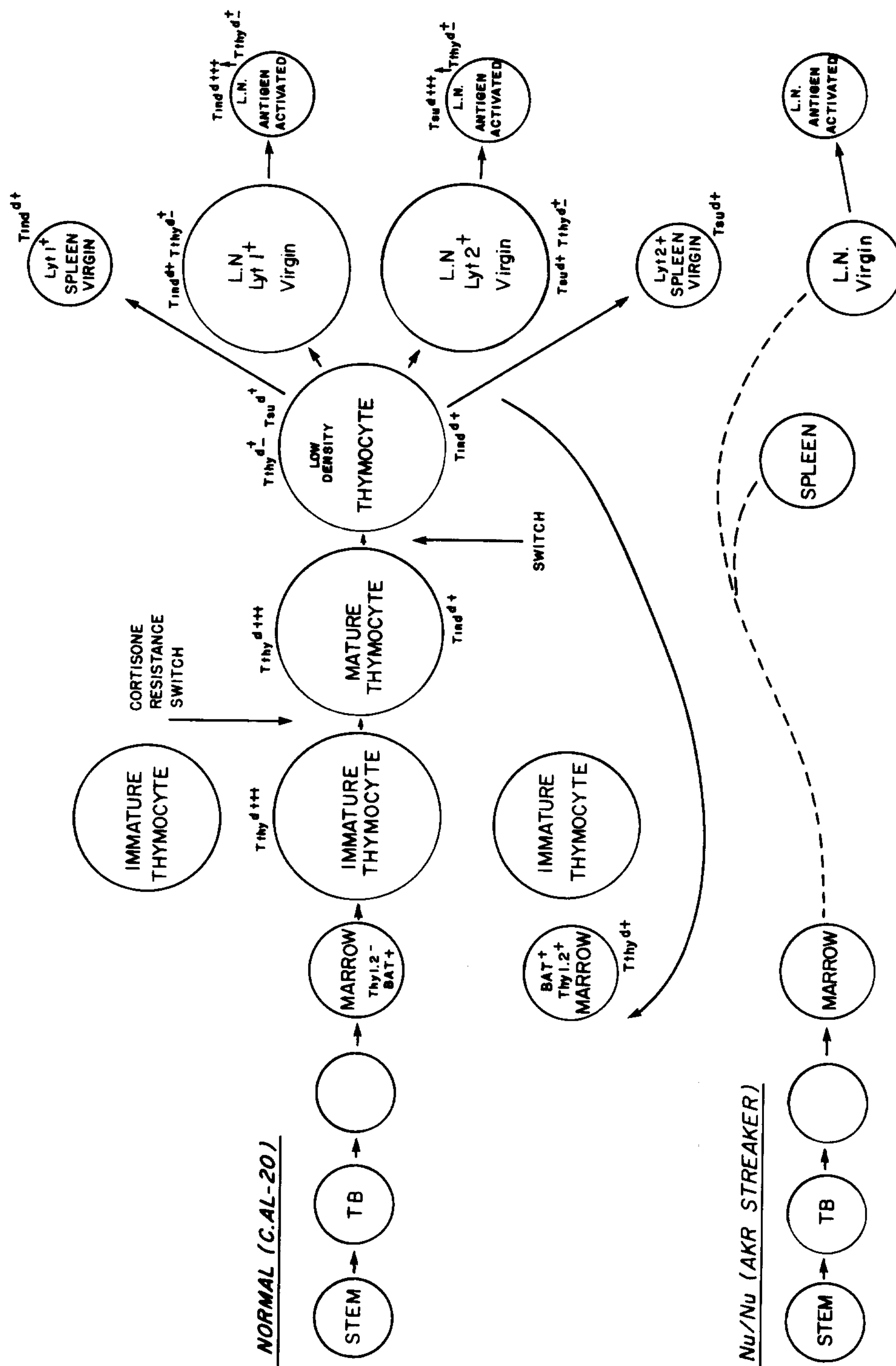

FIG 5. The differentiation spectra of T cells expressing Tthy, Tind, and Tsu in normal vs. athymic mice.

($\mu\ \lambda\ \gamma_3\ \gamma_1\ \gamma_{2b}\ \gamma_{2a}\ \alpha\ \epsilon$) (31). That same order is followed in the ontological expression of the isotypes (7). It is possible that variable region genes must be translocated sequentially by inserting into characteristic switch sites rather than being randomly translocated to a constant region gene downstream from μ. The order of expression of $Tthy^d$, $Tind^d$, and Tsu^d on maturing T cells (not proven to be antigen triggered) also parallels the ontological order of appearance of these antigens. This suggests a similar organization of genes within a T cell-receptor gene complex. Because cDNA probes do not yet exist for T cell-constant regions, one can only speculate that a mechanism for biological preservation of V_H genes could dictate the use of characteristic switch mechanism for T cell antigen receptors.

Summary

Monoclonal antibodies specific for three T cell alloantigens linked to the immunoglobulin complex on chromosome 12 were used to establish the order of expression of these antigens on immunocompetent cells and in ontogeny. Modification of monoclonal antibodies with fluorescein isothiocyanate (FITC) and use with anti-FITC and complement has amplified lytic capacity of the monoclonals and allowed us to complete a distribution study of Tsu^d, $Tind^d$, and $Tthy^d$ alloantigens on immunocompetent cells. $Tthy^d$ is expressed on both cortisone-sensitive and cortisone-resistant thymocytes; Tsu^d and $Tind^d$ are on "mature" cortisone-resistant cells. $Tthy^d$ is also expressed on a Thy-1.2-bearing recirculating marrow cell but is undetectable in the peripheral T cell pool. In contrast, resting spleen and lymph node T cells express Tsu^d and $Tind^d$; antigen-activated populations express these two cells in high frequencies. These antigens must be markers for relatively differentiated cells because "nude" animals, which have pre-T cells, fail to express these determinants. All three antigens segregate independently in our T cell hybrids, arising from adult peripheral node cells, supporting the hypothesis that these are three separate structural products of a gene complex. In contrast, fetal T cell hybrids fail to express these antigens. The appearance of all three antigens on the cell surface in ontogeny is postnatal; $Tthy^d$ is expressed at days 1–2, $Tind^d$ at days 2–3, and Tsu^d at days 5–6. If the T cell isotype genes are organized similar to the immunoglobulin loci, then the parallels in maturational expression on immunocompetent cells and in ontogeny would lead one to speculate a gene order of $Tthy^d \rightarrow Tind^d \rightarrow Tsu^d$. Orientation with respect to the centromere is unknown.

The technical assistance of John Fontana and Lauren Riendeau is appreciated.

Received for publication 30 March 1982 and in revised form 17 May 1982.

References

1. Owen, F. L., and G. M. Spurll. 1981. Evidence for a T cell constant region gene family: characterization of cell surface antigens by immunoprecipitation with alloantisera and monoclonal antibodies. *In* Immunoglobulin Idiotypes. C. A. Janeway, E. E. Sercarz, and H. Wigzell, editors. Academic Press, Inc., New York. 419.
2. Spurll, G. M., and F. L. Owen. 1981. A Family of T-cell alloantigens linked to Igh-1. *Nature (Lond.)*. **293:**742.
3. Owen, F. L., R. Riblet, and B. A. Taylor. 1981. The T suppressor cell alloantigen Tsu^d

maps near immunoglobulin allotype genes and may be a heavy chain constant-region marker on a T cell receptor. *J. Exp. Med.* **153:**801.

4. Owen, F. L., G. M. Spurll, and E. Panageas. 1982. Tthy^{d}, a new thymocyte alloantigen linked to Igh-1. Implications for a switch mechanism for T cell antigen receptors. *J. Exp. Med.* **155:**52.
5. Owen, F. L., A. Finnegan, E. R. Gates, and P. D. Gottlieb. 1979. A mature T lymphocyte subpopulation marker closely linked to the Ig-1 allotype C_H locus. *Eur. J. Immunol.* **9:**948.
6. Owen, J. J. T., T. Andrew, R. K. Jordan. P. McElroy, J. H. Robinson, U. Singh, and H. N. A. Willcox. 1977. Factors Influencing the Primary Differentiation of Lymphocyte Populations. J. D. Soloman, and J. D. Hortan, editors. Elsevier/North-Holland Biomedical Press, Amsterdam, Holland. 139.
7. Lawton, A. R., and M. D. Cooper. 1974. Modification of B-lymphocyte differentiation by anti-immunoglobulins. *Contemp. Top. Immunobiol.* **3:**193.
8. Basch, R. S., and J. L. Kadish. 1977. Hematopoietic thymocyte precursors II. properties of the precursors. *J. Exp. Med.* **145:**405.
9. Hunig, T., and M. Bevan. 1980. Specificity of cytotoxic T cells from athymic mice. *J. Exp. Med.* **152:**688.
10. R. Hyman. 1973. Studies on surface antigen variants. *J. Natl. Cancer Inst.* **50:**415.
11. Marshak-Rothstein, A., P. Fink, T. Gridley, D. H. Raulet, M. J. Bevan, and M. L. Gefter, 1979. Properties and applications of monoclonal antibodies directed against determinants of the Thy-1 locus. *J. Immunol.* **122:**2491.
12. Golub, E. S. Brain associated theta antigen. 1971. *Cell. Immunol.* **2:**353.
13. Steinman, R. M., S. J. Blumencranz, B. G. Machtinger, J. Fried, and Z. A. Cohn. 1978. Mouse spleen lymphoblasts generated *in vitro*: their replication and differentiation *in vitro*. *J. Exp. Med.* **147:**297.
14. Stobo, J. D., and W. E. Paul. 1973. Functional heterogeneity of murine lymphoid cells. III. Differentiation responsiveness of T cells to phytohemagglutinin and Con A as a probe for T cell subsets. *J. Immunol.* **10:**362.
15. Cantor, H., and I. Weisman. 1976. Development and function of subpopulations of thymocytes and T lymphocytes. *Progress in Allergy.* **20:**1.
16. Schultz, L. D., H.-J. Heiniger, and E. Eicher. 1978. A remutation to nude in the AKR/J strain, *In* Animal Models of Comparative and Developmental Aspects of Immunity and Disease. E. L. Gershwin, and E. L. Cooper, editors. Pergamon Press, New York. 211.
17. Ceredig, R. H., E. J. Jenkinson, H. R. MacDonald, and J. J. T. Owen. 1982. Development of cytolytic T lymphocyte precursors in organ-cultured mouse embryonic thymus rudiments. *J. Exp. Med.* **155:**617.
18. Cantor, H., and E. A. Boyse. 1975. Functional subclass of T lymphocyte-bearing Ly antigens. *J. Exp. Med.* **141:**1376.
19. Black, S. J., W. van der Loo, M. R. Loken, and L. A. Herzenberg. 1978. Expression of IgD by murine lymphocytes. Loss of surface IgD indicates maturation of memory B cells. *J. Exp. Med.* **147:**984.
20. Owen, F. L. 1982. Acquisition of diverse T cell isotypes. *Immunology Today.* **3:**95.
21. Owen, J. J. T., R. K. Jordan, J. H. Robinson, U. Singh, and H. N. A. Wilcox. 1977. *In vitro* studies on the generation of lymphocyte diversity. *Cold Spring Harbor Symp. Quant. Biol.* **41:**129.
22. Chakravartz, A., L. Kubai, Y. Sidky, and R. Auerbach. 1975. Ontogeny of thymus cell function. *In* Thymus factors in immunity. H. Friedman, editor. *Ann. N. Y. Acad. Sci.* **249:**34.
23. Rugh, R. 1968. The Mouse. Burgess Publishing Co., Minneapolis, Minnesota.
24. Owen, J. J. T., and M. C. Raff. 1970. Studies on the differentiation of thymus-derived lymphocytes. *J. Exp. Med.* **132:**1216.
25. Kronenberg, M., M. M. Davis, P. W. Early, L. E. Hood, and J. D. Watson. 1980. Helper

and killer T cells do not express B cell immunoglobulin joining and constant region gene segments. *J. Exp. Med.* **152:**1745.

26. Tokuhisa, I., and M. Taniguchi. 1982. Two distinct allotypic determinants on the antigen-specific suppressor and enhancing T cell factors that are encoded by genes linked to the immunoglobulin locus. *J. Exp. Med.* **155:**126.
27. Binz, H., and H. Wigzell. 1981. T cell receptors with allo-major histocompatibility complex specificity from rat and mouse. Similarity of size, plasmin susceptibility, and localization of antigen-binding region. *J. Exp. Med.* **154:**1261.
28. Mathieson, B. J., S. O. Sharrow, P. S. Campbell, and R. Asofsky. 1979. An Lyt differentiated subpopulation of thymocytes detected by flow microfluorometry. *Nature (Lond.).* **227:**478.
29. Hiramatsu, K., S. Miyatani, M. Kim, S. Yamada, K. Okumura, and T. Tada. 1981. Unique T cell Ia antigen expressed on a hybrid cell line producing antigen-specific augmenting T cell factor. *J. Immunol.* **127:**1118.
30. Taniguchi, M., I. Takei, and T. Tada. 1980. Functional and molecular organization of an antigen-specific suppressor factor from a T-cell hybridoma. *Nature (Lond.).* **283:**227.
31. Honjo, T., and T. Katavka. 1978. Organization of immunoglobulin heavy chain genes and allelic deletion model. *Proc. Natl. Acad. Sci. U. S. A.* **75:**2140.