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Immunoglobulin Synthesis and Secretion by Cells in the Mouse Thymus That Do Not Bear θ Antigen

(T and B cells/cytotoxic tests)

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ABSTRACT **The mouse thymus contains cells that synthesize and secrete minute amounts of immunoglobulin (Ig). These cells were studied by a combination of cytotoxic tests (with alloantisera prepared in congenic strains of mice) followed by pulse labeling of the surviving cell population with [^{3}H]tyrosine. The immunoglobulin-synthesizing cells constitute less than 2% of the total cell population and are θ^-, TL^-, Ly-B^-, Ig^+, H-2^+, and PC^+. Since this phenotype is not characteristic of cells of the thymocyte lineage, the Ig^+ cells are probably plasma cells and/or bone marrow-derived (B) lymphocytes.**

Similar studies of spleen cells indicate that immunoglobulin-synthesizing and secreting cells are θ^-.

Thymocytes and thymus-derived (T) cells have antigen-specific receptors demonstrable by the specificity of radioactive antigen-induced suicide (1), specific absorption by target cells (2), and direct binding of antigen (3–9). In the thymus, the immunocompetent cells are considered to be cortisone-resistant and represent less than 5% of the thymocytes (10, 11). In the peripheral lymphoid organs, T cells constitute 30–60% of the lymphocytes (12–15). There is considerable controversy as to whether the antigen receptor on T cells is an immunoglobulin (16–23), a fragment of it (17, 24–26), or a nonimmunoglobulin molecule (27–29). Our studies in which cells were enzymatically radioiodinated failed to demonstrate surface immunoglobulins on thymocytes or T cells, and suggest that thymocytes may have less than 250 molecules of immunoglobulin per cell (30).

The present report describes the synthesis and secretion of minute amounts of immunoglobulin by cells obtained from the thymus. These immunoglobulin-secreting cells constitute less than 2% of the total population and are θ^-.

METHODS

Antisera. The following sera were used in these experiments:

(*i*) AKR anti-C3H thymocytes and C3H anti-AKR thymocytes. These sera were a gift from Drs. Celso Bianco and Victor Nussenzweig, Department of Pathology, New York University Medical Center, New York. They had been prepared and assayed as described (31) and are referred to in the text as anti θ-C3H(i) and anti θ-AKR(i).

(*ii*) (C57BL/6xA) F_1 anti-A/θ-AKR thymocytes (anti-θ-AKR) and (A/θ-AKR x AKR/H-2^b) F_1 anti-A strain leukemia ASLI (anti-θ-C3H). Since A/θ-AKR is congenic with strain A (32), the only alloantibodies formed in these immunizations are anti-θ. They are referred to in the text as anti-θ-C3H(ii) and anti-θ-AKR(ii).

(*iii*) A/TL^- (congenic with strain A) anti-A strain leukemia ASLI (anti-TL.1,2,3) (anti-TL) (33).

(*iv*) (C3H/An x B6/Ly-B.1) F_1 anti-C57BL/6 (B6) leukemia ERLD (Ly-B.2) (B6/Ly-B.1 is congenic with B6) (anti-Ly-B.2).

(*v*) A.BY (H-2^b: congenic with A strain) anti-A strain leukemia (ASLI) (H-2^a) (anti-H-2^a).

(*vi*) DBA/2 (H-2^d) anti-BALB/c myeloma MOPC-70A (anti-PC.1) (34). This serum contains antibody to the differentiation alloantigen PC.1 (which is represented on plasma cells but not on T cells), but not to the IgG1 produced by the myeloma (34) (anti-PC.1).

Preparation of Labeled Thymocytes and Spleen Cells. Thymuses from 4- to 8-week-old BALB/c mice were removed and freed of parathymic nodes, capsules, and blood vessels under ×4 magnification. Spleens were perfused with cold phosphate-buffered saline, pH 7.3 (PBS) and teased into the same buffer. All cells were washed once in PBS and once in Eagle's minimal essential medium (MEM) containing 10% fetal-calf serum (Grand Island Biological) before treatment with antiserum and guinea pig complement. For cytotoxic tests, 5×10^7 thymocytes or 2×10^7 spleen cells were suspended in 2 ml of MEM containing 10% fetal-calf serum. Alloantisera or rabbit antisera to mouse immunoglobulins (30) (anti κ, λ, μ, and γ, used throughout) were added at concentrations that killed a maximum number of cells (usually 200–5000 μl of undiluted antiserum). 200–500 μl of fresh guinea pig serum (C) (which had been absorbed with 2×10^8 BALB/c spleen cells per ml) was added, and the cells were incubated at 37° for 30 min. [Control tubes received either complement alone, antiimmunoglobulin alone, or complement plus a rabbit antiserum to an unrelated antigen, or the anti-θ serum of reciprocal specificity (depending on the particular tests being performed).] For cytotoxic tests with anti PC.1 serum, both $PC.1^+$ and $PC.1^-$ mice were used, each with a control in which antiserum was omitted. After incubation, cell viability was determined with 0.05% trypan blue–PBS. Cells were then pelleted at $1000 \times g$ for 10 min, washed once in MEM lacking both tyrosine and calf serum, and resuspended at 10^7/ml in the same medium. Cells were labeled for 60 min at 37° with 20 μCi/ml of [*l*-^{3}H]tyrosine (25–50 Ci/mmol, New England Nuclear Corp.). After 1 hr,

Abbreviations: T and B cells, thymus- and bone marrow-derived cells, respectively.

TABLE 1. *Synthesis and secretion of protein and immunoglobulin by BALB/c thymocytes and splenocytes**

Cell source		Radioactivity: Acid precipitable (cpm × 10^{-6})	Radioactivity: Immunoprecipitable: Anti-immunoglobulin (cpm × 2† × 10^{-3})	Radioactivity: Immunoprecipitable: Anti-φX (control) (cpm × 2† × 10^{-3})	Ratio of radioactivity anti-immunoglobulin/anti-φX	Anti-immunoglobulin–anti-φX (cpm × 2 × 10^{-3})‡	Immunoglobulin precipitated/acid-precipitable radioactivity (%)
Thymus	Lysate	13.2	75.0	61.5	1.2	13.5	0.1
	Secretion	1.9	48.9	18.9	2.6	30.0	1.6
Spleen	Lysate	19.0	521.0	94.6	5.4	426.0	2.2
	Secretion	1.3	868.0	64.1	14.0	504.0	64.0

* 2.4 × 10^8 cells were labeled for 60 min at 37° with [^{3}H]tyrosine and incubated in complete medium for an additional 60 min. The lysates and secretions were prepared.

† Since the aliquot was divided equally, the cpm in each aliquot is multiplied by 2.

‡ Immunoglobulin precipitated.

the cells were again pelleted, resuspended in MEM containing 10% fetal-calf serum, and incubated at 37° for an additional 120–180 min. After the second incubation period, cells and medium (secretions) were separated by centrifugation; the cells were washed and lysed in 0.5% Nonidet P40 (NP40, Shell Chemical Co.) as described (35). Lysates and secretions were dialyzed overnight at 4° against PBS and centrifuged at 10,000 × *g* for 30 min. Aliquots of the supernatants were precipitated with trichloroacetic acid and the radioactivity was determined (35). Lysates and secretions were then divided into two aliquots and treated for 15 min at 37° with 20 μl of either rabbit antiserum to mouse immunoglobulins (containing specificities to μ, γ, κ, and λ chains) or rabbit antiserum to bacteriophage φX174 (containing a similar concentration of immunoglobulin). Complexes were then precipitated by the addition of an excess of goat antiserum to rabbit immunoglobulins for 30 min at 37° and 120 min at 4°. Precipitates were pelleted, washed, and dissolved (35). The supernatants of the anti-immunoglobulin precipitates were reprecipitated in order to demonstrate that all of the immunoglobulin had been precipitated in the first step. Small aliquots of the dissolved precipitates were counted in toluene containing 0.5% 2.5 diphenyloxazole (PPO) and 10% Bio-solv BBS-3 (Beckman Instruments) on a Beckman LS-250 liquid scintillation counter. Values for control precipitates were subtracted from those of the experimental precipitates. In several experiments, dissolved precipitates were electrophoresed on agarose–2.5% acrylamide gels (36) or reduced and alkylated (35) and electrophoresed on 5% acrylamide gels in sodium dodecyl sulfate (35).

TABLE 2. *Immunoglobulin synthesis and secretion by BALB/c thymocytes and splenocytes**

Cell source	cpm specific precipitate / cpm control precipitate: Lysate	cpm specific precipitate / cpm control precipitate: Secretion	% Total protein that is immunoglobulin,†: Lysate	% Total protein that is immunoglobulin,†: Secretion
Thymus	0.7–1.2	2–3	0–0.1	1.5–2.5
Spleen	5–6	10–15	1–3	60–80

* 1 to 2 × 10^8 cells were labeled for 1 hr with [^{3}H]tyrosine, washed, and incubated again for 2 hr. 2 to 19 × 10^6 acid-precipitable cpm were obtained from the cell lysate. The results of six individual experiments are summarized above.

† $\frac{\text{Specific precipitate–control precipitate}}{\text{Total acid-precipitable radioactivity}} \times 100$.

RESULTS

Immunoglobulin Synthesis by Thymocytes. A preliminary experiment suggested that ^{3}H-labeled thymocytes secrete minute amounts of immunoglobulin into the medium (Table 1). As seen in Table 2, in six such experiments the ratio of radioactivity in the specific precipitate to that in control precipitate in the lysates was 0.7–1.2. However, the same ratio in the secretions varied from 2–3. Thus, the large background of ^{3}H-labeled nonimmunoglobulin molecules in the lysate prevented recognition of the trace amount of synthesized immunoglobulin that was still intracellular. The immunolgobulin was detectable in the secretions because of the lower background of nonimmunoglobulin molecules due to

TABLE 3. *Effect of treatment with anti-θ sera prepared in noncongenic mice and complement on immunoglobulin synthesis by BALB/c (θ-C3H) thymocytes**

Treatment (complement + antiserum)	% Cells killed	Radioactivity†: Acid-precipitable cpm × 10^{-4}	Radioactivity†: Immunoprecipitable cpm‡	% Loss in radioactivity: Acid-precipitable	% Loss in radioactivity: Immunoprecipitable
Anti-θ-C3H(i)	98	8.22	2952	97	51
Anti-θ-AKR(i)	6	200.00	3994	13	36
Medium§	2	230.00	6238	—	—

* This is a representative experiment of six performed.

† Lysate + secretion.

‡ 2 (Anti-immunoglobulin–anti-φX).

§ Medium + complement and normal AKR serum + complement gave similar results, so only the former was used routinely.

TABLE 4. *Effect of treatment with anti θ-sera prepared in congenic mice, and complement on immunoglobulin synthesis by BALB/c (θ-CH3) thymocytes**

Treatment (complement + antiserum)	% Cells killed	Radioactivity† Acid-precipitable cpm × 10^{-5}	Radioactivity† Immunoprecipitable cpm‡	% Loss in radioactivity Acid-precipitable	% Loss in radioactivity Immunoprecipitable
Anti-θ-C3H(ii)	98	2.1	12,660	95	0
Anti-θ-AKR(ii)	4	40.0	13,208	4	0
Medium	2	42.0	12,052	—	—

* This is a representative experiment of three that were performed.

† Lysate + secretion.

‡ 2 (Anti-immunoglobulin–anti-φX).

the selectivity of the secretory process. The thymus cells secreted less than 3% of the radioactive immunoglobulin secreted by equal numbers of spleen cells. Acrylamide gel electrophoresis of unreduced, and also of reduced and alkylated specific precipitates from secretions indicated that thymus cells secreted 19S IgM, 8S IgM, and IgG (Fig. 1).

The immediate question to arise was whether the thymus cells that secreted immunoglobulins were θ^+ cells. To answer this question, thymus cells from BALB/c (θ-C3H) mice were pretreated with either anti-θ-AKR(i) (control) or anti-θ-C3H(i) and complement, or with complement and medium alone. In six such experiments, the specific anti-θ serum killed 98–99% of the cells. The other anti-θ serum (control) or complement alone killed only 0–5% of the cells. When the residual viable cells were pulsed with [^{3}H]tyrosine, the incorporation of isotope into protein was reduced proportionately to the number of cells killed. Table 3 shows a representative experiment. The total incorporation of [^{3}H]tyrosine into immunoglobulins was not markedly different between the anti-θ-AKR and the anti-θ-C3H preparations, even though the latter contained a barely detectable number of viable cells. However, both values were 44 ± 8% lower than those obtained when cells were incubated with medium and complement alone. These results suggest that (*i*) θ-bearing cells (98–99% of the total) are not responsible for immunoglobulin synthesis and secretion, and (*ii*) both anti-θ sera contain specificities against immunoglobulin-synthesizing cells of BALB/c origin, but the specificity is not to the θ antigen.

To confirm these tentative conclusions, the experiment was repeated with anti-θ sera prepared in congenic mice. As seen in Table 4, in such an experiment, the anti-θ-C3H(ii) killed 98% of the cells and reduced protein synthesis proportionately. Synthesis and secretion of immunoglobulins, however, was now *identical* in the cell aliquots treated with complement and medium, anti-θ-C3H(ii), or anti-θ-AKR(ii). Hence, these sera appeared to contain specificities only to the θ antigen, and the θ^- population synthesized and secreted *all* the immunoglobulins attributable to the total population. This experiment was repeated twice and the results were similar.

The immunoglobulin-synthesizing cells in the thymus were examined for other cell-surface markers in the same manner as for θ antigen. As summarized in Table 5, these cells are θ^-, TL^-, Ly-B^-, H-2^+, and Ig^+, and the majority are PC.1^+.

Immunoglobulin Synthesis by T Cells. In similar experiments, spleen cells were treated with complement and anti-θ-AKR(i), anti-θ-C3H(i), or no antiserum. In three such experiments, treatment with anti-θ-C3H killed 28–38% of the cells, whereas the controls showed a loss of about 0–10% (Table 6). When the residual viable cells were labeled with [^{3}H]tyrosine, the immunoglobulin synthesized and secreted by the treated and control populations was identical. These results indicate that θ^- cells synthesized virtually all of the immunoglobulin made by the total cell population. It is of interest that protein synthesis by anti-θ-treated cells was reduced by a much smaller percentage than the cell viability. One possible explanation is that T cells are biosynthetically less active than B cells and thymocytes. Antiserum to immunoglobulin and complement killed the majority of the B cells in the

TABLE 5. *Effect of treatment with alloantisera or antisera to immunoglobulin on synthesis and secretion of immunoglobulins by BALB/c thymocytes**

Treatment (antiserum)	% Cells killed	% Loss Acid-precipitable radioactivity	% Loss Immunoglobulin synthesis and secretion
Anti-TL	28	28†	0
Anti-Ly-B.2	58	53†	0
Anti-H-2^a	84	97†	94
Anti-immunoglobulin	1–2	1–2‡	85
Ant-iPC.1	5–10	0§	67

* BALB/c thymocytes are TL.2, Ly-B.2, and H-2^d (which includes the strong component H-2.4 and other components shared with H-2^a; this is why the anti-H-2^a serum could be used in place of anti-H-2^d, which was not available at the time). Since the cells lack TL.1 and 3, there is a relatively low % lysis with anti-TL.

† Based on a control value obtained from cells treated with complement + medium (see†, Table 1).

‡ Based on a control value obtained from cells treated with either antiserum to immunoglobulin alone or with rabbit antiserum to an unrelated antigen and complement.

§ Based on both* and values obtained from thymocytes of PC.1-negative mice (C57Bl/6).

TABLE 6. *Effect of treatment with anti-θ sera, prepared in noncongenic mice, or antiserum to immunoglobulin on immunoglobulin synthesis by BALB/c (C3H) splenocytes**

Treatment (antiserum)	% Killed	% Loss† Acid-precipitable radioactivity	% Loss† Immunoglobulin synthesis and secretion
Anti-θ-C3H(i)	28–38	3–17	0
Anti-θ-AKR(i)	0–10	6–24	0
Anti-immunoglobulin	42–52	51–74	42–60

* Average of four experiments.

† Based on a control value obtained from cells treated with complement and medium (see†, Table 1).

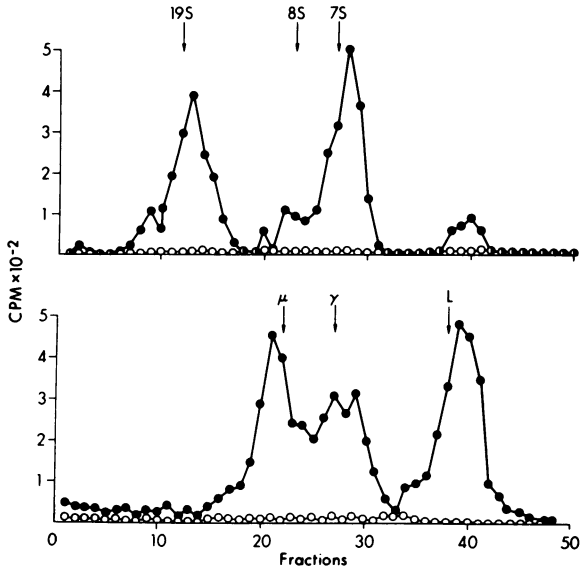

FIG. 1. [³H]Tyrosine-labeled immunoglobulin secreted by BALB/c thymocytes. Dissolved precipitates were electrophoresed on sodium dodecyl sulfate–agarose acrylamide gels (*upper panel*) or reduced and alkylated and electrophoresed on sodium dodecyl sulfate–acrylamide gels (*lower panel*) with appropriate markers. ●——●, antiserum to immunoglobulin; O— - O, control.

spleen (12–14) and reduced immunoglobulin synthesis and secretion by 42–60%. Failure to completely obliterate immunoglobulin synthesis by complement and antibodies to immunoglobulin may have been due to the relative insensitivity of a proportion of plasma cells to such treatment (15).

DISCUSSION

The present studies indicate that θ-bearing cells in the thymus and spleen do not synthesize and secrete detectable amounts of immunoglobulin after short-term incubation with [³H]tyrosine. By themselves, these experiments do not exclude the possibility that the immunoglobulin is the antigen receptor on T cells, because in contrast to B cells (36), the turnover of immunoglobulins in T cells might be very slow and immunoglobulins might not be secreted. However, these results taken together with our failure to demonstrate immunoglobulins on thymocytes and T cells after surface radioiodination (30) constitute a formidable argument against the claim that there are equivalent amounts of immunoglobulin on thymocytes and B cells (19, 20). We therefore continue to regard the nature of the T-cell receptor as an unanswered question.

In addition, these studies have identified the cellular source of immunoglobulin synthesis and secretion in thymocytes from normal BALB/c mice as a distinct population comprising less than 2% of the cells. If we assume maximum killing by anti-θ, -TL, and -Ly-B, the phenotype of the immunoglobulin secreting cells is θ^-, TL^-, $Ly\text{-}B^-$, $H\text{-}2^+$, Ig^+, and in the majority of cells, PC^+, which is different from cells in the T-cell lineage as currently defined (37–40). The simplest explanation is that these cells are plasma cells and B lymphocytes from parathymic nodes, blood, or the thymus itself. Their presence may explain the results of others showing immunoglobulin associated with cells in thymus preparations (16, 18–21).

It is likely that the immunoglobulin $(Ig)^+$ cells are irrelevant to the differentiation of thymocytes into T cells. Another possibility is that these Ig^+ cells play a major role in T-cell function. In particular, they could be responsible for antigen recognition by T cells either by directly interacting with antigen and nonspecifically stimulating T cells (the converse of current concepts of T and B cell interactions) or by secreting antibody and passively sensitizing neighboring T cells. Since B cells require sensitized T cells for cell cooperation, the above hypothesis implies that the Ig^+ cells in the thymus are not conventional B cells or plasma cells. In this regard, it is of interest that Perkins *et al.* (41) have described a minor population of cells in the thymus having cell-surface immunoglobulins and a morphology distinct from B lymphocytes or plasma cells. The hypothesis could be tested by determining separately the specific and nonspecific contributions of θ-bearing cells to T-cell function.

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