ROLES OF T AND B LYMPHOCYTES IN THE TERMINATION
OF UNRESPONSIVENESS TO AUTOLOGOUS THYROGLOBULIN
IN MICE*

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(Received for publication 17 December 1973)

That a central immunological unresponsive state exists to our own body constituents is widely accepted. The precise role played by the various cell types of the lymphoid system in the maintenance or abrogation of this unresponsiveness to self remains unclear. A large number of nonself-antigens require the collaboration of thymus-derived (T) and bone marrow-derived (B) lymphocytes (1, 2). Although both T and B cells may be made unresponsive, only one cell type need be in order that the intact animal remain unresponsive (3). In a rabbit model, the cellular events in the termination of unresponsiveness to bovine serum albumin (BSA),1 by the injection of cross-reacting albumins, were best explained by assuming that the T cells, but not the B cells, were unresponsive to BSA and to cross-reacting determinants on other albumins (4). Such a situation may explain the apparent ease with which unresponsiveness to self components may be abrogated in autoimmune disorders such as thyroiditis (5–7) and rheumatic fever (8).

The present investigations were undertaken to directly assess by adoptive transfer, antigen-binding and antigen-suicide techniques, the interactions of T and B cells during the induction of autoantibody to syngeneic thyroglobulin (Tg) and during the production of thyroid lesions.

Materials and Methods

Immunization of Mice with Heterologous Tg.—6-8-wk old female A/J mice (Jackson Laboratories, Bar Harbor, Maine) were injected intraperitoneally with 0.5 mg of an equal mixture

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* Publication no. 788 from the Department of Experimental Pathology, Scripps Clinic and Research Foundation, La Jolla, Calif. Supported by U.S. Public Health Service Grant AI-07007, Atomic Energy Commission Contract, AT (04-03)-410 and American Cancer Society Grant IC-S8B.

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§ Recipient of U.S. Public Health Service Research Career Award 5-K6-GM-6936.

1 Abbreviations used in this paper: ABC, antigen-binding capacity test; ABL, antigen-binding lymphocytes; BSA, bovine serum albumin; D-PFC, direct PFC; FCS, fetal calf serum; HoRBC, horse red blood cells; IEP, immunoelectrophoresis; I-PFC, indirect PFC; PBS, phosphate-buffered saline; PFC, plaque-forming cells; Tg, thyroglobulin; Tx, thymectomized.
of soluble human, equine, and bovine Tg's daily for 6 days. A second and third series of injections were given with a 2-wk interval between series.

Isolation of Tgs.—Bovine and equine thyroids were obtained from local slaughterhouses, and murine thyroids were removed from normal female A/J mice. Human thyroids removed from autopsy specimens were kindly provided by Dr. Robert Nakamura, University of California at Irvine. The thyroids were minced and extracted in the cold with normal saline. Cell and tissue debris were removed by centrifugation in the cold at 10,000g for 30 min. The supernatant material was precipitated in the cold with ammonium sulfate as described by Rose and Stylos (9). The Tg-rich fraction (1.7 M) was redissolved in phosphate-buffered saline (PBS), pH 7.2, and chromatographed on a 2.5 × 90 cm Sephadex G-200 column (Pharmacia Fine Chemicals, Inc., Piscataway, N. J.). The excluded peak was isolated and shown to contain at least 90–95% Tg and some 5–10% serum proteins by immunoelectrophoresis (IEP). Small samples of each Tg were further purified by DEAE-cellulose chromatography as described previously and were found to be free of serum protein by IEP. The DEAE-purified Tg's were used in autoradiographic and antigen-binding capacity assays.

Detection of Plaque-Forming Cells (PFC) to Heterologous Tg.—Heterologous Tg was covalently coupled to goat erythrocytes essentially as described previously (10). The plaque assay was a modification (11) of the method of Jerne to assay for direct PFC (D-PFC) (12). For detection of indirect PFC (I-PFC), a goat antimouse IgG antiserum was appropriately diluted in a complement source of guinea pig serum. Appropriate controls were included in each plaque assay to determine the background to the target erythrocyte.

Histology.—Thyroids were removed from exsanguinated mice and placed in Bouin's solution for 8–24 h. The hematoxylin-eosin and periodic acidic-Schiff sections were examined and graded for increased cellularity. Sections were scored from 0 to 4+. A normal thyroid was given a 0 score. A score of 1+ was given when there was interstitial accumulation of inflammatory cells distributed between two or more follicles. Where there were one to two foci of inflammatory cells equivalent in size to a follicle, a rating of 2+ was given. A 3+ score indicated that about 10–40% of the thyroid was replaced by inflammatory cells and a score of 4+ indicated that greater than 40% was replaced by inflammatory cells.

Iodination of Tg.—DEAE chromatographically purified Tg's were iodinated as described by McConahey and Dixon (13); for the autoradiographic and antigen-suicide studies, the specific activities ranged from 33–85 and 280–330 μCi/μg protein, respectively. For quantitative determinations of serum antibody to Tg by the antigen-binding capacity assay, the Tg's were labeled to a specific activity of approximately 0.1–0.5 μCi/μg protein.

Nitrogen Determinations.—Protein nitrogen determinations were performed by a modification of the micro-Kjeldahl technique using the Technicon autoanalyzer (Technicon Instrument Corp., Tarrytown, N. Y.) (14).

Autoradiographic Assay of Antigen-Binding Lymphocytes (ABL).—The autoradiographic method was similar to that described by Davie and Paul (15). Briefly, thymuses and spleens were removed from the mice after exsanguination and single-cell suspensions from each organ were made in minimal essential medium (MEM) or RPMI-1640 (Grand Island Biological Co., Grand Island, N. Y.) supplemented with 10% fetal calf serum (FCS) and 0.1% sodium azide. To 200 μl of medium containing 20 × 10⁶ nucleated cells was added 100 or 300 ng of [¹²⁵I]Tg; the cell suspensions were held at 4°C for 30 min. To remove unbound [¹²⁵I]Tg from the cell suspensions, each cell preparation was layered over 7 ml of FCS and centrifuged at 200g. This step was repeated three times.

Cell smears were made on precleaned slides, air-dried, fixed, and emulsed in NTB-2 (Eastman Kodak Co., Rochester, N. Y.). Exposure times varied from 14–21 days; however, in one instance the exposure time was 90 days. The slides were developed and stained with Giemsa, and in each preparation, 10–200 × 10⁶ cells were scanned for the presence of grains. A positive cell had to fulfill the following criteria: (a) the cell had to be morphologically a lymphocyte; (b)
there could be no discernible damage to the membrane or to other structures; (c) the cell could not touch other cells or cellular debris; (d) the cell had to have at least a 20-fold increase above background (~1 grain/cell) grains on its surface or distributed about its circumference.

In blocking experiments with unlabeled Tg, the cell suspensions were mixed with a 750-15,000-fold excess of unlabeled Tg diluted in MEM and the mixture incubated at 4°C for 2 h. Then, the cell suspensions were washed twice with fresh MEM before incubation with \([^{125}I]\)Tg under standard conditions. In blocking experiments with antisera, the cell suspensions (200 μl) were incubated with equal volumes of appropriately diluted antisera for 2 h at 4°C and washed twice in fresh MEM before the addition of \([^{125}I]\)Tg.

**Antisera**—Before use, all antisera were heated at 56°C for 30 min and absorbed with A/J thymocytes (10⁶ cells/ml of antiserum) in order to remove heterophile antibodies to murine cells. Normal goat and rabbit sera were obtained from animals before immunization in order to assess the background inhibitory capacity of each serum. The antimeouse Fab antiserum was prepared by repeated injections of a goat with Fab fragments from papain-digested normal mouse IgG. This antiserum detected κ, λ, and Fd determinants as ascertained by Ouchterlony analysis. A rabbit antimeouse μ-chain serum was prepared by repeatedly injecting rabbits with purified MOPC-104E IgM myeloma protein. To render the antiserum μ-chain specific, it was absorbed with neonatal mouse serum, MOPC-46 κ-chains, λ-chains of 104 E, and Fab fragments of PC-5 myeloma. The anti-IgG heavy-chain specific antiserum was prepared in rabbits by repeated injections of mouse IgG in Freund's complete adjuvant and was absorbed for γ-chain specificity with Fab fragments of PC-5 myeloma, MOPC-46 κ-chain, MOPC-315 IgA myeloma, and MOPC-104 E IgM myeloma proteins. All the antheavy-chain antisera and the anti-Fab antiserum were tested by indirect immunofluorescence and direct immunofluorescence, respectively, on ethanol-fixed mouse myeloma cells (121, W33, MOPC 315, MOPC 104E, PC-5, and 183). These studies permitted both the determination of the specificity and the relative potency of each antiserum. By this test, all the antisera were judged to be specific and to give positive fluorescence at a dilution of greater than 1:40.

**Quantitation of Antibody to Mouse Tg.**—The technique employed is a modification of the antigen-binding capacity test (ABC) as described by Farr (16). To 20 μl of \([^{131}I]\)murine Tg (3.85 μg N/ml) at 0.1 μCi/μg protein was added 20 μl of antibody serially diluted in 1:2 normal mouse serum and 1 μg of \([^{125}I]\)PC5 myeloma protein (γ2ακ) or of \([^{125}I]\)mouse IgG in each tube as an internal control in order to determine the amount of mouse Ig precipitated by the coprecipitating antiserum. The tubes were mixed and incubated at 37°C for 1 h and overnight at 4°C; then, an appropriate quantity of goat antimeouse Fab (IgG) antiserum was added to each tube in order to precipitate the mouse immunoglobulins (Ig). The mixture was incubated at 37°C for 60 min and then at 0°C for 60 min, at which time a heavy precipitate formed. The precipitate was pelleted by centrifugation (in the cold) at 1000 g for 20 min and the supernate removed. The pellet was resuspended in 2 ml of cold PBS, pH 7.2, and the centrifugation process repeated. The final supernate was removed and the precipitate counted in a two channel, well-type gamma counter. For each dilution of antiserum, the percentages of Tg and of mouse Ig precipitated were calculated. A plot of the log dilution of the antiserum versus the percent Tg remaining in the supernate produced a sigmoid curve. The dilution at which 33% of the Tg was bound was determined from the semilogarithmic graph. This number allowed the calculation of the ABC value for each antiserum. Normal mouse serum served as a background control for each experiment in which less than 2% of the Tg added was bound. Between 85-100% of the mouse Ig in each antiserum dilution was precipitated by 200 μl of goat antimeouse Fab antiserum. To determine the degree to which heterologous Tg would competitively inhibit the binding of \([^{131}I]\)mouse Tg by selected antisera, the ABC assay was modified slightly. 5 μl aliquots of concentrated (40 mg protein/ml) individual heterologous Tg's were added to serial dilutions of antisera and the mixture incubated for 1 h at room temperature. Then the \([^{131}I]\)mouse Tg was added and the standard procedure was
followed as described above. As a control for dilution effect, 5 μl of FCS was added in place of heterologous Tg. Additionally, pooled immune mouse antisera were absorbed at equivalence with an equal mixture of soluble equine, human, and bovine Tg, until no further precipitation was observed, at which time 4 mg or 40 mg of an equal mixture of bovine and equine Tg were added to insure completeness of absorption. As in the above experiment, an aliquot of the antisera had a volume of either PBS or FCS added, which was equivalent to that of the added Tg. All the antisera were tested for the capacity to bind either iodinated mouse or iodinated bovine Tg, as described previously.

\textit{Antigenic Suicide of Normal Bone Marrow and Thymus.}—Syngeneic mouse Tg was iodinated with $^{125}$I at a specific activity of 280-330 μCi/μg protein as described previously (13). Normal thymocytes and bone marrow were collected from 6-8 wk old A/J female mice. The cells were suspended in MEM with 10% FCS at 5 × 10⁶ cells/ml and were incubated separately with $^{125}$I-murine Tg at a ratio of approximately 3 μg/10⁶ cells. The cell suspensions were held at 4°C for 5 h. Then, the cells (5 ml) were layered over 20 ml FCS and centrifuged at 200g for 15 min in the cold and the process was repeated twice. After the final wash, the treated cells together with untreated lymphocytes from the other cooperating cell line were suspended in MEM and injected intravenously into lethally (900 R) irradiated, thymectomized (Tx) recipients. 4-6 days after reconstitution, the animals were immunized according to the standard regimen of heterologous Tg's. In one experiment, additional mice in each group were sacrificed at a later time (day 52) to determine the incidence and extent of thyroid lesions.

\section*{RESULTS}

\textit{Detection of PFC to Thyroglobulin.}—As shown in Table I, a PFC response to bovine, equine, and human Tg's was observed in the spleens of mice injected with an equal mixture of human, bovine, and equine Tg. The responses to bovine and equine Tg were similar, whereas the response to human Tg was lower than that observed with either bovine or equine. This was a consistent finding during these studies, and for this reason, in all subsequent PFC assays, bovine Tg was selected as the antigen of choice for the PFC assay. Although not shown in Table I, D-PFC were observed only during the first 5 days after the cessation of the first series of daily Tg injections. The D-PFC response was usually 500-3,000 PFC/spleen. However, after the second and third series of Tg injections, the plaque response was almost exclusively of the 1-PFC type.

\textit{appearance of Serum Antibody and Lesions.}—Because of the excessive

\begin{table}[h]
\centering
\begin{tabular}{lcc}
\hline
Thyroglobulin tested & Indirect plaque-forming cells per: & \\
 & $10^6$ Spleen Cells$^*$ & Spleen \\
\hline
Bovine & 125 ± 11$^\dagger$ & 39864 ± 8842 \\
Equine & 82 ± 13 & 27222 ± 8040 \\
Human & 39 ± 13 & 13460 ± 5110 \\
\hline
\end{tabular}
\footnotesize{$^*$ Four mice were used in this assay and plaqued 1 day after the last Tg injection.}
\footnotesize{$^\dagger$ Arithmetic mean ± 1 standard error of mean.}
\caption{Detection of Plaque-Forming Cells in A/J Mice Immunized with a Mixture of Human, Equine, and Bovine Thyroglobulins}
\end{table}
amount of Tg required during the conjugation of antigen to erythrocytes and in consideration of the limited amount of murine Tg available, no attempt could be made to directly assay for PFC to murine Tg. Rather, a modification of the ABC was employed to measure antibody reacting with murine Tg. The data in Fig. 1 show that only after the second and third series of Tg injections do appreciable amounts of antibody reacting with syngeneic Tg appear in the serum. The dramatic drop (88%) in the serum antibody levels at days 40-45, at a time when heterologous Tg is injected, correlates well with inhibition of

![Graph showing antibody levels](image)

**Fig. 1.** The serum antibody response (○) to murine thyroglobulin measured by a modified antigen-binding capacity assay (expressed as micrograms antibody nitrogen per milliliter serum). Each point represents the mean of 6-15 mice, ±1 SE (vertical lines). The I-PFC response to bovine thyroglobulin by the spleen is included (□). The arrows indicate the number of soluble thyroglobulin injections on the indicated days.

The in vivo absorption of serum antibody suggests that most of the antibody produced which reacts with murine Tg, also cross-reacts with the heterologous Tg's. Furthermore, the appearance of PFC to bovine Tg precedes by 3-5 days the subsequent elevation of antibody in the sera to murine Tg. As antibody reappears in the circulation (day 48) and reaches a maximum (day 50) value, the incidence of thyroid lesions is low; however, thereafter (days 51-55) the incidence of lesions reaches a maximum of approximately 90% and the antibody to murine Tg declines.

**Histology.**—A representative lesion at day 52 is shown in Fig. 2 where the
noteworthy histopathological features of the inflammatory foci are the prevalence of neutrophiles, dissolution of colloid material, and disruption of follicular integrity. A minor mononuclear inflammatory cell reaction was also observed. The mononuclear inflammatory reaction became the prominent histological feature from day 54 through 75.

Specificity of Serum Antibody.—As previously indicated, the serum of mice immunized with a mixture of heterologous Tg's appeared to contain, in large part, antibodies cross-reacting to common determinants shared by all three of the heterologous Tg's. The administration of cross-reactive heterologous Tg's allows collaboration of T and B cells because the noncross-reactive determinants on the heterologous Tg's stimulate a specific population of T cells. At the same time, antigen-reactive B cells react with both cross-reactive determinants and heterologous-specific determinants and as such, both cross-reactive antibody and heterologous-specific antibody are produced. Thus, the specificity of antibody appearing in the serum reflects the cellular status of the host. In this respect, these experiments were designed to investigate the degree of cross-reactivity of antibody and whether any antimurine Tg-specific antibody was produced. The data in Table II demonstrate that bovine, equine, and human Tg, added individually at saturating levels, produced almost identical inhibition (73-76%) of the ABC to mouse Tg. As the level of soluble inhibitor decreased, the degree of inhibition declined correspondingly. As a control for dilution and specificity, an excess of non-Tg proteins and FCS was added to an aliquot of pooled immune serum. The FCS produced no detectable inhibition of the ABC value. An additional aliquot of the antiserum was absorbed at equivalence with an equal mixture of equine, bovine and human Tg's, and an excess unlabeled Tg (4 mg or 40 mg) added to insure completeness of absorption. This absorbed antiserum contained no antibodies capable of binding iodinated bovine Tg, but retained a small but significant population of antibodies specific for murine Tg when a mixture of 4 mg of the excess unlabeled Tg's were added. At 40 mg excess of bovine and equine Tg, no antibody to murine Tg remained. An aliquot of appropriately diluted unabsorbed antiserum served as a control to which the absorbed antiserum was compared. These data show that all the antibody produced that reacts with murine Tg also cross-reacts with the heterologous antigens.

Thymus and Bone-Marrow Synergism in Production of Antibody and of Thyroid Lesions.—As shown in Table III, lethally irradiated and Tx mice reconstituted with normal bone marrow, and injected with heterologous Tg's did not produce antibody (PFC) and did not have discernible lesions. Conversely, lethally irradiated Tx mice reconstituted with normal bone marrow or “B” spleen cells and normal thymocytes were able to generate both I-PFC

Fig. 2. Sections of thyroid removed at day 52. Hematoxylin and eosin. 3+ reaction. Fig. 2 A X 96. Fig. 2 B, X 440.
and thyroid lesions, after heterologous Tg injections. Similarly, recipients of normal spleen cells produced PFC and lesions. By the nature of the experimental protocol, the possibility that recipients reconstituted with only thymocytes could produce lesions was untestable, since such mice died from hematopoietic stem cell failure 7-10 days postirradiation. Thus, these T-cell reconstituted mice do not survive the 50-60 days normally required for lesion production.

Effect of Prior Exposure of B Cells to Tg as Measured by Synergistic Response with T Cells.—The experiment described in Table IV was designed to answer the following two questions: (a) can B cells be stimulated by repeated exposure to antigen, in the absence of T cells, to produce antibody; and (b) what are the effects of repeated Tg injections on the synergistic capacity of such B cells? Two groups of animals were Tx, irradiated, and reconstituted with $40 \times 10^6$ normal bone marrow/recipient. One group received the standard heterologous Tg injection regimen, and the other received saline. 2 wk after the last Tg injection, the mice were sacrificed and the B spleen cells of the donors plus
TABLE III

Thymus and Bone-Marrow Cell Collaboration in Antibody Plaque-Forming Response and Production of Lesions in Experimental Autoimmune Thyroiditis

| No. of Animals | Tx, lethally irradiated* recipient mice reconstituted with: | PFC per: | Lesions |
|----------------|-------------------------------------------------------------|-----------|---------|
|                |                                                             | 10^6 Spleen cells | Spleen |         |
| 8              | 40 × 10^6 normal bone-marrow cells‡ and 100 × 10^6 normal thymocytes | 134 ± 33** | 34,769 ± 6,668 | + ||
| 11             | 60 × 10^6 “B” spleen cells§ and 100 × 10^6 normal thymocytes | 78 ± 13 | 26,799 ± 4,992 | + ||
| 6              | 40 × 10^6 normal spleen cells | 70 ± 27 | 9,992 ± 3,504 | + ||
| 11             | 40 × 10^6 normal bone-marrow cells‡ | 0 | 0 | - ¶ |

* 850 R, whole body irradiation.
‡ Normal bone marrow was treated with AKR anti-C₃H₀ serum plus C.
§ “B” spleen cells were derived from spleens of animals, thymectomized, irradiated (850 R), reconstituted 6 wk before with normal bone marrow treated with AKR anti-C₃H₀ serum plus C.
|| The incidence of lesions ranged from 30 to 90% of the animals in each group.
¶ None of the animals reconstituted with normal bone marrow showed any evidence of lesions at anytime during the course of experimentation.
** Arithmetic mean ± 1 SE of the mean.

TABLE IV

Effect of Prior Exposure of B Cells to Antigen as Measured by Synergistic Response with T Cells

| No. of Animals | Tx, lethally irradiated* recipient mice reconstituted with: | Indirect PFC to: | Bovine Tg per spleen | HoRBC per spleen |
|----------------|-------------------------------------------------------------|------------------|----------------------|-----------------|
|                |                                                             |                   |                      |                 |
| 5              | 30 × 10^6 “B” spleen cells‡ and 90 × 10^6 normal thymocytes | 49,446 ± 10,881¶ | 113,116 ± 36,900¶ |
| 5              | 30 × 10^6 “B” spleen cells‡ | 0 | 0 |
| 5              | 30 × 10^6 “B” spleen cells§ and 90 × 10^6 normal thymocytes | 25,299 ± 13,621¶ | 54,283 ± 16,137¶ |
| 5              | 30 × 10^6 “B” spleen cells§ | 0 | 0 |

* 850 R, whole body irradiation.
‡ “B” spleen cells derived from donors previously Tx, lethally irradiated (850 R), reconstituted with 40 × 10^6 bone marrow cells treated with AKR anti-C₃H₀ serum plus C, and challenged with the standard regimen of Tg before transfer.
§ “B” spleen cells derived from donors previously Tx, lethally irradiated (850 R), reconstituted with 40 × 10^6 normal bone-marrow cells treated with AKR anti-C₃H₀ serum plus C.
¶ Arithmetic mean ± 1 SE of the mean.
¶ Not significantly different (P > 0.5).
normal thymocytes were transferred to intermediate groups of Tx, irradiated recipients. All groups were challenged with heterologous Tg and, in addition, were immunized appropriately with horse red blood cells (HoRBC) to produce maximal PFC response simultaneously with that of the Tg PFC. The spleens of animals reconstituted only with B cells, whether previously exposed to heterologous Tg in the donor or not, did not contain I-PFC to bovine Tg or to the unrelated antigen, HoRBC. Conversely, the two groups of mice reconstituted with B spleen cells plus thymocytes produced I-PFC to both bovine Tg and HoRBC. However, statistical analysis showed that the responses of both groups to bovine Tg or to HoRBC were not significantly different. This suggests that prior exposure of B cells to antigens (Tg’s) in the absence of T cells, does not influence their capacity to synergize with T cells in a second host.

**Autoradiographic Detection of [125I]Tg-Binding Lymphocytes.**—The frequency of cells from nonprimed mice that bind [125I]syngeneic Tg is shown in Table V. In a thymocyte preparation incubated with the [125I]murine Tg and exposed for 6 wk, the frequency of positive cells was less than 1/10⁶ cells. On the contrary, an aliquot of the same thymocyte suspension incubated with an equal mixture of [125I]heterologous Tg’s contained 30 positive cells/10⁵ cells, when exposed for 6 wk. Higher concentrations of iodinated antigen (500 ng) and longer exposure times (6 wk) were employed in order to increase the probability of finding cells that bind syngeneic Tg. The number of ABL in the spleen of normal nonprimed A/J mice that bind [125I]mouse Tg was 68/10⁵ lymphocytes counted, while the frequency of such cells to bovine or human Tg was 38 and 130/10⁵ lymphocytes, respectively.

**Inhibition of [125I]Tg-Binding Lymphocytes.**—To study the nature of the Ig receptors on lymphoid cells that bind [125I]syngeneic Tg, spleen cells from nonprimed mice were pretreated with various antisera as shown in Table VI. Incubation of spleen cells with normal goat or rabbit sera did not diminish the

| Amount of [125I]Tg | Source of normal lymphoid cells | Iodinated Tg       | Antigen-binding lymphocytes per 10⁵ cells |
|-------------------|---------------------------------|-------------------|----------------------------------------|
| 500 ng            | Thymus                          | Mouse             | <1                                     |
| 500 ng            | Thymus                          | Heterologous mixture | 30                                    |
| 100 ng            | Spleen                          | Mouse             | 68                                     |
| 100 ng            | Spleen                          | Human             | 130                                    |
| 100 ng            | Spleen                          | Bovine            | 38                                     |

TABLE V
Frequency of Antigen-Binding Lymphocytes to Tg in Spleen and Thymus of Normal A/J Mice
Inhibition of Antigen-Binding Lymphocytes in Spleens of Normal A/J Mice by Antiglobulin Sera and Unlabeled Antigen

| Amount of [125I]murine Tg | Inhibitor* | Antigen-binding lymphocytes per 10⁸ cells | Inhibition of [125I]murine Tg binding |
|---------------------------|-----------|----------------------------------------|-----------------------------------|
| 100 ng                    | MEM       | 67                                     | 0                                 |
| 100 1:10 normal rabbit serum | 66       | 0                                      | 0                                 |
| 100 1:10 normal goat serum  | 80       | 0                                      | 0                                 |
| 100 1:10 goat anti-Fab (IgG) | 5        | 94                                     | 0                                 |
| 100 1:10 rabbit anti-μ-chain | 9        | 86                                     | 0                                 |
| 100 1:10 rabbit anti-γ-chain | 70       | 0                                      | 0                                 |
| 100 750 X unlabeled murine Tg | 50      | 25                                     | 0                                 |
| 100 15,000 X unlabeled murine Tg | 13      | 80                                     | 0                                 |

* Spleen cells were pretreated with the various inhibitors, washed twice with fresh medium, and exposed to the [125I]murine Tg.

capacity of lymphocytes to bind [125I]syngeneic Tg. However, pretreatment of cells with goat anti-Fab (IgG) or rabbit anti-μ antisera inhibited the binding by 93 and 87%, respectively. The rabbit anti-IgG heavy-chain antisera did not inhibit the appearance of ABL to [125I]murine Tg. Unlabeled murine Tg inhibited the binding of iodinated murine Tg by 25 and 80% when spleen cells were pretreated with a 750- and 15,000-fold excess of unlabeled murine Tg and subsequently washed.

Frequency of ABL in Spleens of Primed A/J and Normal Nude Mice.— If [125I]syngeneic Tg binds only to B lymphocytes, then it would follow that normal nude (nu/nu) spleens should contain [125I]murine Tg-binding B lymphocytes with a frequency comparable to that of normal A/J mice. Secondly, prolonged immunization with a heterologous Tg should produce an increased pool of ABL to the injected Tg and to a lesser extent for other cross-reacting Tg's. To test these hypotheses, the frequencies of ABL in the spleens of homozygous athymic nude and primed A/J mice were examined as indicated in Table VII. In the nude mouse, the frequency of ABL for [125I]murine and [125I]human Tg was 128 and 140/10⁸ cells counted. Mice previously immunized only with human Tg by a standard regimen of injections were used to investigate the frequency of ABL to human, murine, and bovine Tg. A fourfold enhanced binding over normal (Table V) was observed with the three test anti.
TABLE VII

Frequency of Antigen-Binding Lymphocytes in Spleens of Primed A/J and Nude Mice

| [125I]Test antigen | Source of spleen cell | Inhibitor | Antigen-binding lymphocytes per 10^6 cells | Inhibition of [125I] Tg binding |
|--------------------|----------------------|-----------|------------------------------------------|-------------------------------|
| 100 murine         | Normal nude          | None      | 128                                      | —                             |
| 100 human          | Normal nude          | None      | 140                                      | —                             |
| 100 human          | A/J mice immunized* 8 mo before sacrifice | None      | 470                                      | —                             |
| 100 murine         | A/J mice immunized* 8 mo before sacrifice | None      | 220                                      | —                             |
| 100 bovine         | A/J mice immunized* 8 mo before sacrifice | None      | 130                                      | —                             |
| 100 human          | A/J mice immunized* 8 mo before sacrifice | 1:10 normal goat serum | 410                                      | 13                            |
| 100 human          | A/J mice immunized* 8 mo before sacrifice | 1:10 goat anti-Fab (IgG) | 143                                      | 65                            |

* Injected only with human Tg by standard immunization regimen.

gens. The binding of [125I]human Tg in the primed spleen was inhibitable, in large part, by antimouse Fab sera, but not by normal sera.

Antigen Suicide of Normal Bone Marrow Cells by [125I]Syngeneic Tg.—As previously indicated, it is believed that the ABL observed with [125I]syngeneic Tg represent, in part, immunocompetent B cells. Therefore, such cells should be rendered immunoincompetent or suicided by heavily iodinated syngeneic Tg. Since normal thymocytes did not bind [125I]syngeneic Tg to any significant degree, the specific function of this cell-type should not be affected by the incubation in [125I]syngeneic Tg. The data in Table VIII clearly show that normal bone-marrow cells were susceptible to the lethal effects of heavily iodinated syngeneic Tg. In two experiments the degree of suppression of the I-PFC response to bovine Tg was 80% and 88%. Of great importance was the observation that 56% of such animals had no thyroid lesions at a time (day 52) when 100% of the animals reconstituted with normal thymocytes and bone marrow had lesions. Not only was the incidence diminished in the bone-marrow suicidal group, but the severity of the lesions was less than in the control group in which 85% of the animals had moderate inflammatory involvement (grade 1-2+) as compared to 37% in the bone-marrow suicidal group. Equally as important was the finding in both experiments that normal thymocytes were
TABLE VIII
Antigen Suicide of Normal Bone-Marrow Cell by [125-I]-Syngeneic Tg

| Exp. no. | T cells | B cells | no. animals per group for PFC assay* | I-PFC per spleen to bovine Tg | Incidence of thyroid lesions: Grade |
|----------|---------|---------|--------------------------------------|-------------------------------|---------------------------------|
|          |         |         |                                      |                               | 0 | 1+ | 2+ | 3+ | 4+ |
| 1        | 100 × 10^6 normal thymocytes | 25 × 10^6 bone marrow and [125-I]-mouse Tg | 7 | 10,460 ± 2,691† | ND§ |
| 1        | 100 × 10^6 normal thymocytes | 25 × 10^6 normal bone marrow | 6 | 81,903 ± 15,446 | ND |
| 2        | 100 × 10^6 normal thymocytes | 25 × 10^6 normal bone marrow and [125-I]-mouse Tg | 2 | 50,187 ± 19,387 | ND |
| 2        | 100 × 10^6 normal thymocytes | 25 × 10^6 normal bone marrow | 9 | 3,385 ± 534 | ¼+ ½† ½† ½† ½† |
| 2        | 100 × 10^6 normal thymocytes | 25 × 10^6 normal bone marrow | 16 | 28,037 ± 3,980 | ½† ½† ½† ½† ½† |
| 2        | 100 × 10^6 normal thymocytes and [125-I]-mouse Tg | 25 × 10^6 normal bone marrow | 8 | 20,693 ± 2,422 | ½† ½† ½† ½† ½† |

* PFC assay done on day 45.
† Mean ±SE of mean.
§ ND, not done.

DISCUSSION

The data presented in this paper have demonstrated the cellular requirements for murine autoimmune thyroiditis induced by immunization with heterologous Tg's. The production of autoantibody to murine Tg and the induction of lesions required the presence of both specific T and B cells. Animals that were Tx, irradiated, reconstituted with normal bone marrow, and injected with a mixture of soluble heterologous Tg's did not produce any D-PFC or I-PFC to heterologous Tg. These same animals showed no histological evidence of thyroiditis. By contrast, mice that were T x, irradiated, reconstituted with normal thymocytes and bone marrow or spleen cells were able to produce both D-PFC and I-PFC and thyroid lesions.

Secondly, the absence of significant numbers of [125-I]syngeneic Tg ABL in the thymus of normal mice would be compatible with the hypothesis that un-
responsiveness to some self-antigens resides exclusively in the thymus. However, the hypothesis also assumes that the B-cell compartment is immunocompetent and as such may recognize cross-reacting and self-antigens (\textsuperscript{125}I syngeneic Tg). Further, the T-cell pool must be capable of recognizing cross-reacting Tg's via noncross-reacting determinants. The ABL data fully support this hypothesis. No significant number of thymic ABL reacting with \textsuperscript{125}I syngeneic Tg were demonstrable while under similar conditions, significant numbers (30/10^6 lymphocytes) of ABL were demonstrable for the cross-reacting heterologous Tg's. Normal A/J spleen cell preparations contained comparable quantities of ABL for syngeneic and the two heterologous Tg's tested (human and bovine). As expected, an excess of cold syngeneic or heterologous Tg inhibited the binding of \textsuperscript{125}I syngeneic Tg. In general, the inhibition data are in agreement with those reported by Byrt and Ada (17) who found that pretreatment of normal spleen cells with a 10,000-fold excess of noniodinated flagellin reduced substantially the numbers of flagellin ABL.

In the homozygous athymic nude mouse, comparable numbers of ABL were observed with \textsuperscript{125}I human Tg and with \textsuperscript{125}I murine Tg. The cells binding the iodinated antigens in the spleens of these athymic mice are primarily, if not exclusively, B-derived cells (18). This type of experimental approach does not constitute formal proof that the ABL's found with \textsuperscript{125}I syngeneic Tg in an intact normal mouse are exclusively B lymphocytes although by inference it must be considered strong supportive evidence for it. Further evidence implicating B cells as the ABL for self-antigens may be found in a recent report of Bankhurst et al. (19). They demonstrated that peripheral blood lymphocytes from normal human patients subjected to double-layer antihuman Ig-coated bead column which removes lymphocytes bearing large amounts of surface Ig, effectively abolished or drastically reduced the number of autoradiographic positive cells to \textsuperscript{125}I homologous Tg.

Along these same lines, there was an increase in the number of ABL for both syngeneic Tg and heterologous Tg's when mice were immunized with only human Tg 8 mo before sacrifice. The lymphocytes binding \textsuperscript{125}I syngeneic Tg were most probably a pure B population; however, the source of those cells binding the \textsuperscript{125}I heterologous Tg may well have been mixture of B and T cells. Ample evidence exists for the presence of T cells functioning as ABL (20–22). Whether the ABL which reacted with \textsuperscript{125}I syngeneic Tg are B memory cells or precursors of PFC and whether the immunoglobulin surface receptors on these cells are \(\mu\) or \(\gamma\)-chains remains to be investigated.

Thirdly, radioactively labeled syngeneic Tg incubated with normal bone-marrow cells under conditions favoring radiation damage abrogated the capacity of these cells to collaborate with normal thymocytes when adoptively transferred to a Tx-irradiated host. Upon challenge with cross-reacting Tg's, such animals exhibited a markedly decreased antibody response (I-PFC) and subsequently had a decreased incidence of thyroid lesions. When normal
thymocytes were incubated with $[^{125}\text{I}]{\text{syngeneic Tg}}$ and injected with normal bone-marrow cells into a Tx-irradiated host, a response comparable to that of the control group was observed upon challenge with the cross-reacting Tg's. This experiment established directly the fact that the B-cell pool in normal mice recognizes the self-antigens and therefore is immunocompetent while the T-cell pool does not recognize self-antigens and therefore may be considered unresponsive.

The problem of antigen suicide of normal bone marrow versus suicide of normal B-derived lymphocytes is still a matter of controversy. Unanue (23) was able to demonstrate antigen suicide of normal bone marrow with heavily iodinated KLH; on the other hand, Basten et al. (24) were unable to suicide bone-marrow cells but did suicide B-derived cells. Yet it is clear from the work of Chiller et al. (3) that bone marrow does contain antigen-sensitive cells since they are capable of being rendered unresponsive with deaggregated human gamma globulin. This type of data suggests that normal bone marrow does contain antigen-specific immunocompetent cells. One reason why Unanue (23) and we were able to suicide normal bone marrow may be that substantially larger amounts and more intensely iodinated antigen were employed.

The interrelationship between thyroid lesions and antibody was dramatically clarified with the suicide of normal bone marrow. When antibody is decreased dramatically, the incidence and severity of lesions decreased. Further support for the role of antibody may be ascertained from the temporal relationship between the elevation (day 48–50) of serum antibody reacting with mouse Tg and the subsequent appearance of inflammatory cells in the thyroid on day 52. The histopathological nature of these lesions was in part neutrophilic in character; this type of lesion has often been associated with acute inflammatory reactions mediated by antigen, antibody, and the complement system (25, 26). Clinton and Weigle (10) reported that rabbits immunized with soluble bovine Tg exhibited a similar kinetic interrelationship between the appearance of splenic PFC and serum antibody to Tg. In their rabbit model, they found that coincidental with the appearance of thyroid lesions, I-PFC to rabbit Tg reached a peak in the thyroid. As they suggested, the thyroid may act as a specific immunoabsorbent for circulating B memory cells and as such, an accumulation of sensitized lymphocytes specific for rabbit Tg would be expected in the thyroid at this time. That specific antibody to Tg passively transfers thyroiditis has been clearly demonstrated for rabbits (27) and mice (28). On the other hand, the degree of thyroiditis in guinea pigs has been correlated best with the level of cellular immunity to Tg (29, 30).

The immunoglobulin receptor on spleen cells that binds $[^{125}\text{I}]{\text{syngeneic Tg}}$ was composed of $\mu$-chains and light chains, since antisera specific for mouse $\mu$-chain and for mouse $\kappa$, $\lambda$, and Fd (IgG) determinants significantly blocked the binding of $[^{125}\text{I}]{\text{syngeneic Tg}}$. The binding site did not appear to be influenced by anti-IgG-specific antisera. It has been reported that both anti-$\mu$
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and -γ (20) or only anti-μ (31–33) heavy-chain serum significantly inhibit the binding of iodinated antigens in normal spleen cell populations. Perhaps, in the immunocompetent B-cell pool, the receptors on cells specific for self-antigens are class restricted to μ-chains only.

SUMMARY

The data presented in this paper support the hypothesis that unresponsiveness to autologous thyroglobulin (Tg) exists in the T cells and responsiveness exists in the B cells. Such a conclusion is based on the results of antigen-binding studies where few, if any, thymocytes recognized syngeneic Tg. Comparable numbers of antigen-binding lymphocytes for syngeneic Tg were found in the spleens of normal intact mice and of nude mice. The latter fact suggested that B cells exist which recognize self-constituents. From antigen-suicide experiments, a clearer picture of the susceptibility of B cells to iodinated self-antigen and of the obligatory role of antibody in the induction of lesions was developed. Only bone marrow cells (B cells) were affected by [125I]syngeneic Tg, in which case the incidence of lesions was diminished. From adoptive transfer experiments, the results demonstrate that unresponsiveness may be terminated by immunization with a mixture of heterologous (cross-reacting) Tg's. In this situation T cells are required since a B-cell reconstituted host failed to make antibody (plaque-forming cells) and to develop lesions. T cells in this form of an unresponsive state may recognize determinants on the heterologous Tg unrelated to autologous Tg and as such stimulate the normal complement of B cells to produce antibody that both reacts with autologous and heterologous Tg.

We gratefully acknowledge the skilled technical assistance of Ms. Kay Miller, Ms. Karen Prescott, Ms. Patricia Wright, and Mr. Gerry Sanford.

REFERENCES

1. Claman, H. N., E. A. Chaperon, and R. F. Triplett. 1966. Thymus-marrow cell combinations. Synergism in antibody production. Proc. Soc. Exp. Biol. Med. 122:1167.
2. Miller, J. F. A. P., and G. F. Mitchell. 1967. The thymus and the precursors of antigen reactive cells. Nature (Lond.). 216:659.
3. Chiller, J. M., G. S. Habicht, and W. O. Weigle. 1970. Cellular sites of immunological unresponsiveness. Proc. Natl. Acad. Sci. U. S. A. 65:551.
4. Benjamin, D. C., and W. O. Weigle. 1970. The termination of immunological unresponsiveness to bovine serum albumin in rabbits. I. Quantitative and qualitative response to cross-reacting albumins. J. Exp. Med. 132:66.
5. Weigle, W. O. 1965. The induction of autoimmunity in rabbits following injection of heterologous or altered homologous thyroglobulin. J. Exp. Med. 121:289.
6. Wick, G. J., J. H. Kite, R. K. Cole, and E. Witebsky. 1970. Spontaneous thyroiditis in the obese strain of chickens. III. The effect of bursectomy on the development of the disease. J. Immunol. 104:45.
7. Nakamura, R. M., and W. O. Weigle. 1968. Experimental thyroiditis in complement intact and deficient mice following injections of heterologous thyroglobulins without adjuvant. *Proc. Soc. Exp. Biol. Med.* **129**:412.

8. McLaughlin, J. F., P. Y. Paterson, R. S. Hartz, and S. H. Embury. 1972. Rheumatic carditis: *in vitro* responses of peripheral blood leukocytes to heart and streptococcal antigen *Arthritis Rheum.* **15**:600.

9. Rose, N. R., and W. A. Stylos. 1969. Splitting of human thyroglobulin. I. Reduction and alkylation. *Clin. Exp. Immunol.* **5**:129.

10. Clinton, B. A., and W. O. Weigle. 1972. Cellular events during the induction of experimental thyroiditis in the rabbit. *J. Exp. Med.* **136**:1605.

11. Golub, E. S., R. I. Mishell, W. O. Weigle, and R. W. Dutton. 1968. A modification of the hemolytic plaque assay for use with protein antigens. *J. Immunol.* **100**:133.

12. Jerne, N. K., and E. A. Nordén. 1963. Plaque formation in agar by single antibody producing cells. *Science (Wash. D. C.)*. **140**:405.

13. McConahey, P., and F. J. Dixon. 1966. A method for trace iodination of proteins for immunologic studies. *Int. Arch. Allergy Appl. Immunol.* **29**:185.

14. Ferrari, A. 1960. Nitrogen determination by a continuous digestion and analysis system. *Ann. N. Y. Acad. Sci.* **87**:792.

15. Davie, J. M., and W. E. Paul. 1971. Receptors of immunocompetent cells. II. Specificity and nature of receptors on dinitrophenylated guinea pig albumin 125I-binding lymphocytes of normal guinea pig. *J. Exp. Med.* **134**:495.

16. Farr, R. S. 1958. A quantitative immunochemical measure of the primary interaction between 125I-BSA and antibody. *J. Infect. Dis.* **103**:239.

17. Byrt, P., and G. L. Ada. 1969. An *in vitro* reaction between labelled flagellin or haemocyanin and lymphocyte-like cells from normal animals. *Immunology.* **17**:503.

18. Dwyer, J. M., S. Mason, N. L. Warner, and I. R. MacKay. 1971. Antigen binding lymphocytes in congenitally athymic (nude) mice. *Nat. New Biol.* **234**:252.

19. Bankhurst, A. D., G. Torrigiani, and A. C. Allison. 1973. Lymphocytes binding human thyroglobulin in healthy people and its relevance to tolerance for auto-antigens. *Lancet.* **1**:226.

20. Modabber, F., S. Morikawa, and A. H. Coons. 1970. Antigen-binding cells in normal mouse thymus. *Science (Wash. D. C.)*. **170**:1102.

21. Lawrence, D. A., H. L. Spiegelberg, and W. O. Weigle. 1973. 2,4-Dinitrophenyl receptors on mouse thymus and spleen cells. *J. Exp. Med.* **137**:470.

22. Roelants, G. E. 1972. Quantification of antigen specific T and B lymphocytes in mouse spleens. *Nat. New Biol.* **236**:252.

23. Unanue, E. R. 1971. Antigen binding cells. II. Effect of highly radioactive antigen on the immunologic function of bone marrow cells. *J. Immunol.* **107**:1663.

24. Basten, A., J. F. A. P. Miller, N. L. Warner, and J. Pye. 1971. Specific inactivation of thymus-derived (T) and non-thymus-derived (B) lymphocytes by 125I-labelled antigen. *Nat. New Biol.* **221**:104.

25. Cochrane, C. G., E. R. Unanue, and F. J. Dixon. 1965. A role of polymorphonuclear leukocytes and complement in nephrotoxic nephritis. *J. Exp. Med.* **122**:99.

26. Henson, P. M. 1972. Pathologic mechanisms in neutrophil-mediated injury. *Am. J. Pathol.* **68**:593.
27. Nakamura, R. M., and W. O. Weigle. 1969. Transfer of experimental autoimmune thyroiditis by serum from thyroidectomized donors. *J. Exp. Med.* 130:263.

28. Vladutiu, A. O., and N. R. Rose. 1971. Transfer of experimental autoimmune thyroiditis of the mouse by serum. *J. Immunol.* 106:1139.

29. McMaster, P. R., E. M. Lerner, and E. D. Exum. 1961. The relationship of delayed hypersensitivity and circulating antibody to experimental allergic thyroiditis in inbred guinea pigs. *J. Exp. Med.* 113:511.

30. Romagnani, S., M. Ricci, A. Passaleva, and G. Biliotti. 1970. Cell-mediated immune responses to heterologous and homologous thyroglobulin in guinea pigs immunized with heterologous thyroid extracts. *Immunology.* 19:599.

31. Hämmerling, G. J., T. Masuda, and H. O. McDevitt. 1973. Genetic control of the immune response. Frequency and characteristics of antigen-binding cells in high and low responder mice. *J. Exp. Med.* 137:1180.

32. Dwyer, J. M., and I. R. MacKay. 1972. Validation of autoradiography for recognition of antigen binding lymphocytes in blood and lymphoid tissues. *Clin. Exp. Immunol.* 10:581.

33. Warner, N. L., P. Byrt, and G. L. Ada. 1970. Blocking of the lymphocyte antigen receptor site with anti-immunoglobulin sera in vitro. *Nature (Lond.)* 226:942.