

## CELLULAR EVENTS DURING THE INDUCTION OF EXPERIMENTAL THYROIDITIS IN THE RABBIT\*

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Although experimental thyroiditis in various animals has been studied rather extensively (1-4), the immunological events at the cellular level have not been characterized. Investigations carried out at this level should provide the answer as to whether antibody to thyroglobulin is responsible for the initial damage to the thyroid gland before any significant lymphocytic infiltration occurs. Other studies (2, 5) have implicated cell-mediated activity as a contributing mechanism to the infiltration of mononuclear cells observed in the thyroid. However, this may not prove to be the case in all species, as the successful transfer of thyroiditis to rabbits and mice with sera from appropriately immunized donors is possible (6, 7). The immunoglobulin that induces thyroiditis in these species is as yet uncharacterized. Furthermore, the pathological roles of specific types of antibody to thyroglobulin are not known, as total antibody levels contain a variety of immunoglobulins with various biological capabilities (8).

Since both humoral and cellular immune activities are implicated in thyroid pathology, the present investigations were undertaken to reveal their respective importance and contribution during events leading to infiltration of mononuclear cells into the thyroid gland. Rabbits injected with an aqueous preparation of a cross-reacting (bovine) thyroglobulin were assayed daily to quantitate serum antibody to rabbit thyroglobulin, to enumerate hemolytic plaques to either bovine or rabbit thyroglobulin, and to monitor production of migration inhibition factor to both thyroglobulins.

### *Materials and Methods*

*Animals.*—New Zealand white rabbits weighing 2.5-3.0 kg were used throughout the study.

*Isolation and Purification of Thyroglobulin.*—Both bovine and rabbit thyroglobulins were isolated by differential ultracentrifugation as previously described (4) by a modification of

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Edelhoc's original method (9). Bovine and rabbit thyroid tissues were obtained from a local slaughterhouse and from Pel-Freez Biologicals, Inc., Rogers, Ark., respectively. The resulting saline (0.15 M NaCl) extracts (merthiolated 1:10,000) were kept at 4°C.

*Nitrogen Determinations.*—Protein nitrogen (N) determinations were performed by a modification of the micro-Kjeldahl technique using the Technicon AutoAnalyzer (10).

*Iodination of Proteins.*—Each thyroglobulin was passed through a column of diethylaminoethyl (DEAE)<sup>1</sup> cellulose equilibrated with 0.2 M phosphate buffer at pH 5.6 (11). This material was dialyzed against phosphate-buffered saline (pH 7.4), and 5-mg aliquots of protein were labeled with <sup>131</sup>I according to the method of McConahey and Dixon (12).

*Immunization Schedule for Rabbits.*—The schedule used to immunize the rabbits with soluble bovine thyroglobulin has already been reported (13). Briefly, rabbits received 20 mg soluble bovine thyroglobulin subcutaneously on days 1 through 4; on day 5 they received 10 mg intravenously and 10 mg subcutaneously. During days 6–19 they were either rested or assayed as indicated. Rabbits not assayed received five more subcutaneous injections of 20 mg bovine thyroglobulin on days 20–24. Animals were not immunized on the days they were to be assayed.

*Antibody Analysis.*—Antibody to rabbit thyroglobulin was quantitated by measuring the amount of <sup>131</sup>I thyroglobulin precipitated at a point near equivalence where 80% of the added thyroglobulin (antigen) was precipitated (14). The antigen/antibody ratio at equivalence with thyroglobulin is approximately 1.0, and the results are reported as micrograms ( $\mu$ g) antibody N/1.0 ml of serum.

*Hemolytic Plaque Assay.*—The hemolytic plaque assay used was a modification (15) of the Jerne-Nordin technique (16). Purified thyroglobulin was dialyzed overnight against conjugation buffer (CB) (pH 7.2) (15). The same buffer was used as the diluent for all the reactants. Goat erythrocytes (Colorado Serum Co., Denver, Colo.), washed three times with CB, were linked covalently to bovine or rabbit thyroglobulins. The procedure required that 2.5 ml of 1-methyl-3 (3-dimethylaminopropyl) carbodiimide HCl (Ott Chemical Co., Muskegon, Mich.) (100 mg/ml in CB) be added to 15 ml of CB containing both 15 mg thyroglobulin/ml and 0.5 ml washed goat erythrocytes. It was predetermined that this concentration of thyroglobulin was optimal. Goat erythrocytes (0.5 ml) were obtained from a packed suspension previously washed three times with CB. The reactants were kept in an ice bath for 1 hr, and washed twice with CB followed by two washings in a balanced salt solution (BSS) (15). The thyroglobulin conjugated red blood cells were then adjusted to a 1:12 dilution with BSS. For the actual assay, 50  $\mu$ l of the conjugate was added to tubes containing 0.4 ml agarose (0.5%) in BSS kept at 42°C. To these tubes were added 100  $\mu$ l of an appropriate lymphoid cell suspension. The contents of the tubes were mixed and poured onto microscope slides which had been precoated with 0.1% agarose. Responses to bovine and rabbit thyroglobulin are reported as the average indirect plaque-forming cells (PFC)/10<sup>6</sup> nucleated spleen or thyroid gland cells from at least five animals assayed on each particular day. The amplifying antibody was harvested from an ewe immunized with DEAE-isolated rabbit  $\gamma$ G. Any background plaques using unconjugated red blood cells were subtracted from the experimental plaques. Specificity to bovine thyroglobulin by the antibody-forming cells was confirmed when plaque formation to either bovine or rabbit thyroglobulin was completely inhibited by 75  $\mu$ g of bovine thyroglobulin protein.

*PFC from the Spleen and Thyroid Gland.*—Spleen cell suspensions were prepared individually by gently raking excised spleens from exsanguinated rabbits through a stainless-steel wire screen and washing them three times with BSS. In addition, thyroid glands were removed

<sup>1</sup> Abbreviations used in this paper: BSA, bovine serum albumin; BSS, balanced salt solution; CB, conjugation buffer; DEAE, diethylaminoethyl; MEM, Eagle's suspension medium; MEM-S, supplemental Eagle's suspension medium; MIF, migration inhibition factor; PFC, plaque-forming cells; PPD, purified protein derivative.

from each trachea, carefully trimmed of all exogenous material, and weighed. A center strip cut from each lobe of the gland was placed in Bouin's solution for histology; cell suspensions were obtained from the remaining portion (60–70%) by gently passing the cut gland through stainless steel screens. These cell suspensions were washed four times with BSS and were treated as the spleen cells had been during the plaque assay.

*Histology.*—Strips cut from the rabbit thyroids that had been fixed in Bouin's solution overnight were embedded in paraffin, sectioned, and stained with hematoxylin and eosin. The grading of thyroiditis has been established previously (7) and was determined by the degree of infiltration of mononuclear cells.

*Migration Inhibition Factor (MIF) Production.*—The technique used generally followed the modification established by David et al. (17) of the original method of George and Vaughn (18). Rabbits immunized identically to those described above were assayed for production of MIF when either bovine or rabbit thyroglobulin was added to the test system. Exudate cells from normal (nonimmune) rabbits were used as control cells. To obtain a population of exudate cells, the rabbits were injected intraperitoneally with 60 ml sterile Bayol F. After 4 days, the rabbits were exsanguinated, the thyroid gland was removed for histology, and 200 ml of Eagle's suspension medium (MEM) (Microbiological Associates, Inc., Bethesda, Md.) supplemented with glutamine (200 mM), penicillin (100 units/ml), and streptomycin (100  $\mu\text{g}/\text{ml}$ ) was injected into the peritoneal cavity. After gentle kneading of the abdomen, the skin was reflected and a small aperture was cut into the abdominal muscles so that a sterile siliconized plastic tube, drilled out at the tip, could pass into the cavity. This procedure allowed withdrawal of the exudate cells using a 10 ml pipette. The cells were washed three times (300 g) with MEM and adjusted to  $30 \times 10^6/\text{ml}$  with 10% heat-inactivated (56°C, 60 min) normal rabbit serum in supplemented MEM (MEM-S). Differential counts on the harvested cells revealed that 75–85% were macrophages. The cells were then placed in sterile, acid-washed, capillary tubes (Dade Supplies, Miami, Fla.), and centrifuged at 60 g for 3 min. The tubes were cut at the cell-fluid interface and placed in Mackness-type chambers. The antigen was diluted with MEM-S and 200  $\mu\text{g}/\text{ml}$  of either bovine or rabbit thyroglobulin was added to the chamber. Control chambers received 200  $\mu\text{g}/\text{ml}$  bovine serum albumin (BSA) and 25  $\mu\text{g}/\text{ml}$  purified protein derivative (PPD) (a gift from the Ministry of Agriculture, Waybridge, England) or MEM-S. The migration of cells was taken as an average of four fan patterns per antigen after 24 hr incubation at 37°C. The area of migration of the fan was photographed, projected on a screen, and traced on high grade bond paper. The fan was then cut out, weighed, and per cent migration inhibition was calculated as:

$$\% \text{ Migration inhibition} = 100 - \frac{\text{Average weight of migration pattern with antigen}}{\text{Average weight of migration pattern in MEM-S}} \times 100.$$

In our hands, statistical analysis revealed that any inhibition of migration that was less than 20% was insignificant.

## RESULTS

*Profile of the Daily Appearance of PFC to Bovine and Rabbit Thyroglobulin from the Spleen and Thyroid Glands.*—The appearance of PFC in the spleens and thyroid glands of immunized animals is presented in Fig. 1 *a* and *b*. At all times during the assays, PFC from the spleen to bovine thyroglobulin were considerably greater than PFC to rabbit thyroglobulin. Two peaks of PFC to bovine thyroglobulin occurred in the spleen (Fig. 1 *a*). One appeared on day 8 with 412 PFC/ $10^6$  nucleated cells, and the other appeared on day 25 with 6019 PFC/ $10^6$  cells. The PFC to rabbit thyroglobulin (Fig. 1 *a*) from the spleen

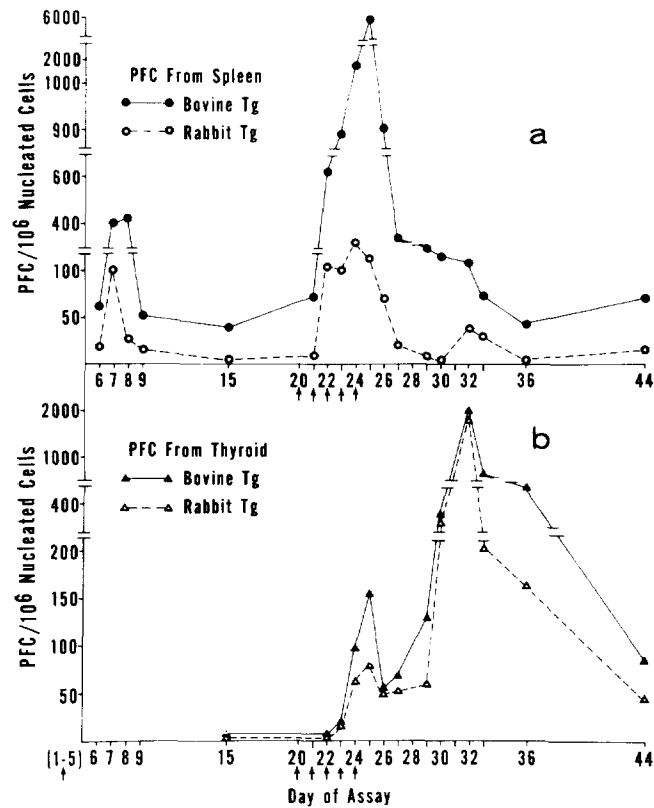


FIG. 1. The PFC response to bovine and rabbit thyroglobulin (Tg) by the spleen (panel *a*) and the thyroid gland (panel *b*). The arrows indicate the number of injections performed at the indicated days.

peaked at day 7 with 94 PFC/10<sup>6</sup> cells and at day 24 with 176 PFC/10<sup>6</sup> cells. Both these peaks occurred 1 day before the observed peaks to bovine thyroglobulin. A third minor peak containing significant numbers of spleen PFC (36 PFC/10<sup>6</sup> cells) to rabbit thyroglobulin occurred at day 32.

The first indication that antibody-forming cells could be found in the thyroid was at day 15 with 6 and 9 PFC/10<sup>6</sup> cells to rabbit and bovine thyroglobulin, respectively (Fig. 1*b*). The PFC in the thyroid next appeared at day 22 followed by a peak at day 25 against bovine thyroglobulin of 156 PFC/10<sup>6</sup> cells. PFC (74/10<sup>6</sup> cells) to rabbit thyroglobulin now peaked on the same day as did the bovine thyroglobulin PFC response. In addition, the two peaks from the thyroid gland against both thyroglobulins on day 25 occurred the day after the last injection of bovine thyroglobulin, and the difference between the PFC to both thyroglobulins was not as large as the difference which was noted in the spleen PFC response. The peak response on day 25 was followed

by a slight decrease after which numbers of PFC to both thyroglobulins increased. At day 32 the major peaks occurred in the thyroid and consisted of 1810 PFC/10<sup>6</sup> cells and 2001 PFC/10<sup>6</sup> cells to rabbit and bovine thyroglobulin, respectively. The similarity in number of PFC to the homologous and heterologous thyroglobulins was in sharp contrast to the marked difference in the number of PFC to these thyroglobulins observed during the peak response in the spleen. The peak of PFC to both thyroglobulins occurring in the thyroid gland was 8 days after the last injection of bovine thyroglobulin and 7 days after the peak of spleen PFC to bovine thyroglobulin. Cell preparations obtained from kidney or liver tissues contained no PFC to either thyroglobulin.

*Relationship Between PFC in the Spleen and Thyroid Gland to Rabbit Thyroglobulin and the Degree of Infiltration of Mononuclear Cells*—The degree of infiltration of mononuclear cells and PFC to rabbit thyroglobulin obtained both from the spleen and thyroid gland are presented in Fig. 2. The spleen PFC response to rabbit thyroglobulin demonstrated three peaks which occurred at days 7, 24, and 32. The PFC response from the thyroid gland also is plotted where the PFC peak response at day 32 and the rise of infiltration of mono-

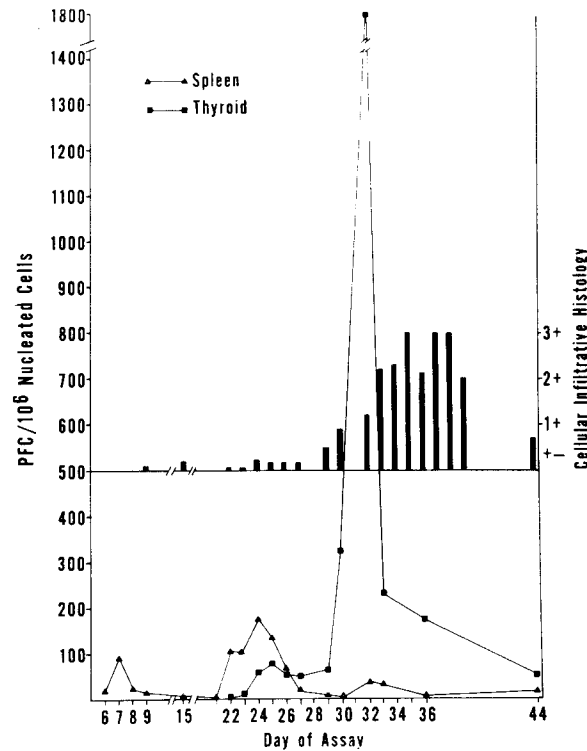


FIG. 2. The PFC response to rabbit thyroglobulin of cells from the spleen  $\Delta$ — $\Delta$  and the thyroid gland  $\blacksquare$ — $\blacksquare$ . The degree of infiltration of mononuclear cells is shown by solid bars.

nuclear cells causing the lesions within the gland are clearly seen. The dramatic increase occurring between days 30 and 32 of from 336 PFC/10<sup>6</sup> to 1810 PFC/10<sup>6</sup>, respectively, is of particular interest. There is an obvious association between the appearance of the PFC to rabbit thyroglobulin and the increasing intensity of the infiltration. The peak of antibody-forming cells at day 32 to rabbit thyroglobulin preceded by 1 day the maximum infiltration of mononuclear cells. There was a significant infiltration on day 32 (> 1+), a substantial increase (> 2+) by day 33, which remained at this level for a number of days. By day 44 the infiltration diminished significantly (< 1+), tissue repair was initiated, and resolution of the damaged gland occurring.

*Relationship Between Antibody to Rabbit Thyroglobulin and Infiltration of Mononuclear Cells.*—Precipitating antibody to rabbit thyroglobulin reached the first peak of 2.3  $\mu$ g antibody N/ml of serum at day 8 (Fig. 3). Upon resumption of the immunization schedule at day 20, antibody to rabbit thyroglobulin steadily increased up to a maximum of 20.1  $\mu$ g antibody N/ml at day 31. Thereafter, a pronounced decrease occurred and by the 33rd day the level of antibody N was 2–3  $\mu$ g/ml. Included in Fig. 3 is the degree of infiltration of mononuclear cells. Decreased precipitating antibody to rabbit thyroglobulin by day 33 to 2.4  $\mu$ g N/ml correlated with increased pathology.

*Relationship of the Production of MIF and Infiltration of Mononuclear Cells.*—This series of experiments was designed to show the relationship between cell-mediated immunity to both rabbit and bovine thyroglobulin and the degree of infiltration of mononuclear cells. As Fig. 4 illustrates, the first significant

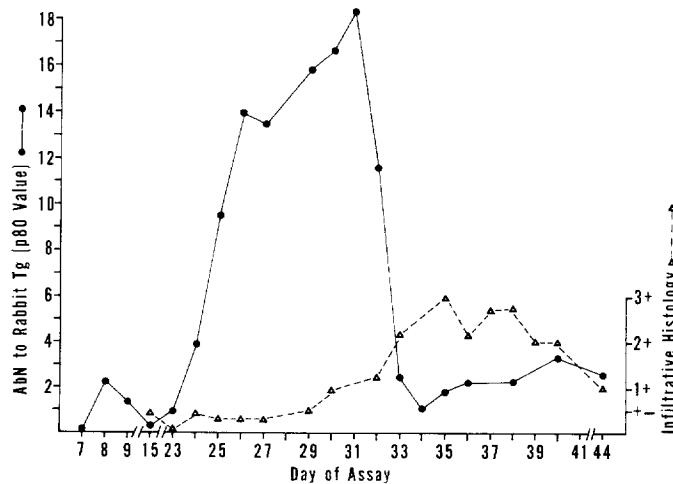


FIG. 3. The cross-reactive antibody (in  $\mu$ g) response (●) to rabbit thyroglobulin as measured by a quantitative precipitin assay. The degree of infiltration of mononuclear cells ( $\Delta$ ) is included to show the inverse relationship between the antibody and infiltration. AbN = antibody N.

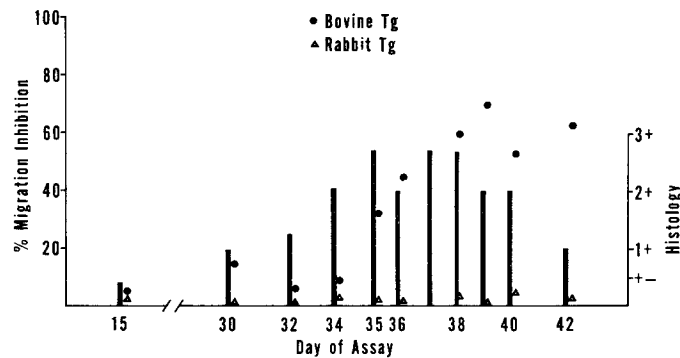


FIG. 4. The per cent migration inhibition to bovine (●) and rabbit (△) thyroglobulin and its relationship to the plateau of maximal infiltration of mononuclear cells.

production of MIF occurred at day 35 corresponding to a migration inhibition of 36%. The production of MIF occurred only after the addition of 200  $\mu\text{g}/\text{ml}$  bovine thyroglobulin, as 200  $\mu\text{g}/\text{ml}$  of rabbit thyroglobulin did not produce MIF at any time during the entire series of experiments. As seen in Fig. 4, the maximal infiltration of mononuclear cells first appeared on day 33, 2 days before the initial production of MIF. In addition, MIF was still capable of being produced as reflected by its appearance at day 42 despite the observed reduction in the degree of infiltration of mononuclear cells. The migration of macrophages from normal, nonimmune rabbits in 200  $\mu\text{g}$  bovine thyroglobulin resulted in large fan patterns identical to those shown by immune cells in only MEM-S. Immune peritoneal cell populations were never observed to display migration inhibition when BSA or PPD was added as a nonspecific control.

#### DISCUSSION

By modifying the Jerne hemolytic plaque assay for PFC to thyroglobulin, the cellular kinetics of the immune response in rabbits injected with aqueous preparations of bovine thyroglobulin could be studied. Thus, sequential examination of the appearance of PFC to thyroglobulin could be quantitated before the appearance of thyroid lesions. The reported data support the contention that thyroiditis in the rabbit is mediated and perpetuated by circulating antibody. PFC to both bovine and rabbit thyroglobulin appeared in the spleens of rabbits after injection of bovine thyroglobulin with the major peak of PFC to bovine thyroglobulin being 20 times greater than that to rabbit thyroglobulin. Previously similar injections of bovine thyroglobulin resulted in the production of antibody which cross-reacted with rabbit thyroglobulin (19), but all of the antibody to rabbit thyroglobulin could be absorbed with bovine thyroglobulin.

Events from the present studies were very similar to those involved in the termination of unresponsiveness to BSA in rabbits after the injection of heter-

ologous (cross-reacting) albumins (20, 21). In this latter report it was suggested that the termination of the unresponsive state to BSA resulted from unresponsiveness of the thymus but not the bone marrow-derived cells. Thus, determinants unrelated to BSA could stimulate specific thymus-derived cells which then could stimulate the normal complement of bone marrow-derived cells to differentiate and make some antibody reactive to both BSA and the cross-reacting albumin. Thyroiditis and production of antibody to rabbit thyroglobulin can be explained similarly. Since relatively large concentrations of "tolerogen" are required to maintain unresponsiveness of bone marrow cells in comparison to thymus cells (22), it is unlikely that a natural unresponsive state to autologous thyroglobulin is present in bone marrow-derived cells.

Of special interest is the presence of PFC to thyroglobulin in the thyroid itself after immunization with bovine thyroglobulin. The major peak of PFC cells from the gland appeared approximately 7 days after the major peak of cells from the spleen. As in the spleen, PFC to both bovine and rabbit thyroglobulin were present in cells from the thyroid. However, unlike the spleen, there was little or no difference in the number of PFC from the thyroid to bovine and rabbit thyroglobulin. It appears as if the antibody-damaged thyroid gland acts as an immunoabsorbant. Thus, locally exposed thyroglobulin removes from the circulation migrating memory cells which are reactive to rabbit thyroglobulin determinants. It is difficult to explain why a 7-day period is required before these cells produce antibody; however, similar observations have been made with another system<sup>2</sup> which is presently being investigated. The above results are compatible with the appearance of plasma cells in thyroid lesions of rabbits (23).

There is a direct correlation between the appearance of PFC in the thyroid gland and the appearance of thyroid lesions. Significant lesions do not appear before PFC are observed in the gland, and the more severe lesions are found shortly after the peak of PFC. It is possible that the initial lesions releasing thyroglobulin locally permit absorption of memory cells and that release of antibody directly within the gland from these cells causes more severe damage. It should be emphasized at this point that the test system is mainly detecting what appears to be IgG PFC. However, in rabbits immunized with homologous thyroglobulin incorporated into complete Freund's adjuvant, IgG antibody formed late in the response does not appear to be involved in the initiation of the lesion (7). It may be that a different class or subclass of antibody initiates subtle injury which is then enhanced by the IgG antibody produced locally in the gland.

Levels of precipitating antibody to rabbit thyroglobulin also correlate with the appearance of lesions. Again, antibody precedes the occurrence of lesions, and as the lesions begin to appear there is a dramatic disappearance of antibody from the serum at a rate much greater than that which can be accounted for by

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<sup>2</sup> Romball, C. G., and W. O. Weigle. Manuscript in preparation.



the 5.8 day half-life of IgG in the rabbit (24). Apparently, the antibody is removed by thyroglobulin either exposed in the gland or released into the body fluids. This latter observation may explain the failure of others to find a correlation between the presence of circulating antibody and degree of thyroid lesions (2). That antibody plays a role has previously been shown by passive transfer of thyroiditis with serum to rabbits (7) and to mice (6).

Further evidence for the role of antibody is the absence of a cell-mediated response to rabbit thyroglobulin. The failure of rabbit thyroglobulin to elicit MIF activity from peritoneal cells of immunized rabbits suggests that the thymus-derived cells have not been stimulated to determinants on bovine thyroglobulin that are shared with rabbit thyroglobulin. These results also suggest that the rabbit is unresponsive to homologous thyroglobulin at the level of the thymus-derived cell and support the contention that the induction of autoantibody and thyroiditis after immunization with bovine thyroglobulin is the result of responsive bone marrow-derived cells but unresponsive thymus cells. In any event, the above hypothesis may not apply for all species. A cell-mediated component has been adequately demonstrated for thyroiditis in guinea pigs (2, 25). Possibly guinea pig thymus-derived cells are not completely unresponsive to their own thyroglobulin because a sufficient concentration of thyroglobulin is not maintained in the circulation. The half-life of homologous thyroglobulin in the guinea pig is, at the most, only a few hours,<sup>3</sup> whereas the half-life of rabbit thyroglobulin in the rabbit is 2.3 days (26). The spontaneous thyroiditis observed in chickens (obese strain) is apparently also mediated by antibody, since bursectomy but not thymectomy prevents the disease (27).

#### SUMMARY

With a modification of the Jerne plaque technique to enumerate plaque-forming cells (PFC) to bovine and rabbit thyroglobulin, the cellular kinetics of the antibody response were followed during two 5-day series of injections of an aqueous preparation of bovine thyroglobulin. The results support the suggestion that thyroiditis in the rabbit is mediated by antibody. The peak PFC appear in the spleen at the end of the second series of injections and are considerably greater for bovine than for rabbit thyroglobulin. PFC also appear in the thyroid gland; however, the numbers of PFC for bovine and rabbit thyroglobulin were similar, and they did not reach a peak until 7 days after the peak PFC in the spleen. There was an excellent correlation between the appearance of PFC in the thyroid gland and the appearance of thyroid lesions. The disappearance of antibody to rabbit thyroglobulin from the serum also correlated with the appearance of lesions. Migration inhibition factor (MIF) activity was not produced at any time throughout the study when rabbit thyroglobulin was added to peritoneal exudates of immunized rabbits containing circulating

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<sup>3</sup> Nakamura, R. M. 1967. Personal communication.

antibody to rabbit thyroglobulin. MIF activity was observed when bovine thyroglobulin was added to similar cells in the later stages of the study after lesions were present.

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