TRANSFER OF EXPERIMENTAL AUTOIMMUNE THYROIDITIS BY SERUM FROM THYROIDECTOMIZED DONORS*

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(Received for publication 24 March 1969)

The development of experimental thyroiditis with production of autoimmune antibodies may be considered a termination of the natural unresponsive state to native thyroglobulin (1–5). As yet, the exact pathogenetic mechanisms involved are poorly understood, and there has been much speculation as to the role of circulating antibody. The lack of correlation between antibody level and severity of thyroiditis in rabbits and guinea pigs has been previously reported (6, 7). Furthermore, the course of thyroiditis in guinea pigs was more closely correlated with delayed hypersensitivity against thyroid tissue than serum antithyroid antibody levels (6, 8, 9). Experimental thyroiditis has been transferred by spleen and lymph node cells to rabbits (10) and guinea pigs (11). However, the pathogenetic role of one or more specific types of antibody cannot be ruled out, as the total antibody levels reflect a wide variety of immunoglobulins with different and opposing functions (12).

In the present experiments various studies were initiated to define further the role of humoral antibodies. In order to determine optimal conditions for serum transfer, the temporal sequence in the production of thyroid lesions and autoimmune antibodies in rabbits following an injection of homologous thyroglobulin in complete adjuvant was studied. Serum transfers from thyroidectomized and nonthyroidectomized donor animals were performed in various ways. So that recipients would receive antibody in the same manner in which it appeared in immunized rabbits, the immunized donor rabbits were bled periodically and the sera were injected sequentially into the recipients. Similarly, purified antithyroglobulin was isolated from pooled sera and transferred in a sequential fashion to recipient animals. Passive transfer experiments were

* This is publication 323 from the Department of Experimental Pathology of Scripps Clinic and Research Foundation. This work was supported by United States Public Health Service grant AI 07007-03 and Atomic Energy Commission contract AT (04-3)-410.

‡ Supported by a fellowship from the Arthritis Foundation.

§ Supported by United States Public Health Service research career award 5-K6-GM-6936

also performed with heterologous guinea pig antithyroglobulin antibodies in rabbits rendered unresponsive to guinea pig γG-globulin.

Materials and Methods

Animals.—New Zealand white rabbits weighing 1.8–2.5 kg were used in the present experiments. Hartley strain guinea pigs weighing 350–500 g and adult sheep were used for the production of antisera. In most of the experiments, animals were fed Purina chow and water containing KI to prevent reincorporation of liberated radioactive iodine during in vivo radioisotope tracer studies.

Total thyroidectomy was performed on rabbits with intravenous pentobarbital anesthesia.

Isolation and Purification of Thyroglobulin.—Rabbit thyroglobulin was isolated and purified by differential ultracentrifugation as previously described (1), using a modification of the method described by Edelhoch (13). Rabbit thyroid tissue was excised from fresh, unfrozen tracheas of New Zealand white rabbits obtained from Pel-Freez, Rogers, Ark.

Protein Antigens.—Guinea pig γG-globulin and rabbit γG-globulin were isolated and purified by DEAE-cellulose chromatography as previously described (14). Crystalline bovine serum albumin (BSA), lot D-71209, was obtained from Armour Pharmaceutical Co., Kankakee, Ill.

Nitrogen Determination.—Protein nitrogen determinations were performed by a modification of the micro-Kjeldahl technique using the Technicon AutoAnalyzer (15).

In Vitro Iodination of Proteins.—5 mg aliquots of protein were labeled with ^{131}I or ^{125}I according to the method of McConahey and Dixon (16). After extensive dialysis following the labeling procedure, all preparations were checked for trichloroacetic acid-precipitable radioactivity.

Antisera.—Specific antiserum to rabbit γ-globulin was prepared in sheep by repeated immunization with rabbit γG-globulin in incomplete Freund's adjuvant. The sheep γG-globulin fraction of anti-rabbit γ-globulin was isolated and labeled with fluorescein isothiocyanate (FITC), according to the method of Wood et al. (17). To remove nonspecific background staining, the fluoresceinated antibody was reisolated by DEAE-cellulose chromatography (17).

Rabbit β_{1C}-globulin was isolated from fresh serum according to the method of Mardiney and Müller-Eberhard (18). The β_{1C}-globulin–zymosan complex was incorporated into incomplete Freund's adjuvant and injected into guinea pigs, which were tested every 2 wk for antibody to β_{1C}-globulin by immunoelectrophoresis and were exsanguinated at the time of its appearance. The sera of guinea pigs showing antibody response to other serum proteins were discarded. The guinea pig γG-globulin fraction was isolated by DEAE-cellulose chromatography with 0.02 M phosphate buffer at pH 8.0. The guinea pig γG-antibody to rabbit β_{1C} was conjugated with FITC according to the method described for sheep anti-rabbit γG-globulin.

Guinea pig anti-rabbit thyroglobulin was prepared as previously described (3).

Sheep anti-rabbit globulin was prepared by repeated injections of rabbit globulin incorporated in incomplete Freund's adjuvant. The rabbit globulin fraction was isolated by 50% ammonium sulfate fractionation. The sheep antisera contained antibody to rabbit γM-immunoglobulin, as evidenced by radioimmunoelectrophoresis when reacted against rabbit sera known to contain γM-immunoglobulin antibodies to keyhole limpet hemocyanin.[1]

Preparation and Staining of Sections for Fluorescent Studies.—The technique employed for immunofluorescent studies was similar to that described originally by Coons and Kaplan (19).

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[1] Spiegelberg, H. L. Personal communication.

The tissue was snap-frozen in a bath of dry ice and butanol. The frozen tissue was sectioned at 5 μ in a cryostat. After the thyroid sections were washed in phosphate-buffered saline, the sections were stained with either FITC sheep anti-rabbit γ-globulin or FITC guinea pig anti-rabbit β_{1C} complement for 40 min. The sections were then washed twice and mounted in glycerol-saline.

Immunoelectrophoresis and Radioimmunoelectrophoresis.—Immunoelectrophoretic analyses were made with the Agafor 1 apparatus (National Instrument Laboratories, Washington, D. C.), using the technique described by Scheidegger (20). Electrophoresis was performed in 1.5% Ionagar No. 2 (Consolidated Laboratories, Inc., Chicago, Ill.). Radioimmunoelectrophoretic studies were performed according to a method described by Goodman et al. (21).

Injection and Bleeding of Rabbits.—Rabbits were injected with adjuvant containing varying amounts of thyroglobulin and mycobacteria (H37 Ra, Difco Laboratories, Detroit, Mich.). As previously described (5), the complete Freund's adjuvant was composed of 9 parts Bayol F (Humble Oil Co., Houston, Tex.) to 1 part Arlacel 83 (McKesson and Robbins, Los Angeles, Calif.) and 10 parts 0.15 M NaCl containing the thyroglobulin. The animals were bled at various times from either the veins or the arteries of the ear.

Histology.—Thyroid tissue was removed from rabbits either by a hemithyroid biopsy or at autopsy and fixed in Bouin's solution. The tissue was embedded in paraffin, sectioned, and stained with hematoxylin and eosin. The grading of thyroiditis was determined by the degree of inflammatory cell infiltration. The lesions were graded ± if two or four foci the size of one follicle of infiltration were present in a longitudinal section of both lobes. A 1+ lesion contained at least five to nine foci the size of one follicle in a longitudinal section of both thyroid lobes. A 2+ lesion contained 10–20 foci which occupied areas the size of several follicles in one lobe. Lesions were graded 3+ if numerous foci were present in each section which occupied relatively large areas. A 4+ lesion showed inflammatory infiltration of 90% or more of the longitudinal sections of the thyroid lobe.

Antibody Analyses.—Antibody to rabbit thyroglobulin was measured by quantitative precipitation (22) and hemagglutination (23) techniques. The quantitative precipitin test employed measured the amount of ^{131}I-thyroglobulin precipitated at a point near equivalence where 80% of the added antigen was precipitated. The antigen/antibody ratio at equivalence with thyroglobulin is approximately 1.0. In the hemagglutination test, a 2.5% suspension of tannic acid-treated sheep erythrocytes was sensitized with 0.5 mg of thyroglobulin/ml. Test sera were first heated at 56°C for 20 min and then absorbed with an equal volume of washed, packed sheep erythrocytes.

Density Gradient Ultracentrifugation.—Sucrose density gradient centrifugation was performed according to a procedure previously described (3). A 10–37% linear sucrose gradient in 0.15 M NaCl buffered at pH 7.0 with 0.01 M phosphate was prepared. The serum sample was diluted 1:1 with 0.15 M NaCl, and 0.2 ml samples were placed on top of the gradient and centrifuged at 35,000 rpm in the SW 39 rotor for 18 hr in a Spinco model L preparatory ultracentrifuge. 24 fractions of approximately 0.2 ml were collected by a pin puncture of the bottom of the tube.

Test for Passive Cutaneous Anaphylaxis.—Passive cutaneous anaphylaxis in rabbits for homocytotropic antibody was performed according to the technique of Zvaifler and Becker (24).

Purification of Homologous Anti-rabbit Thyroglobulin Antibodies.—Purified anti-rabbit thyroglobulin antibodies were isolated with the use of a bromoacetyl cellulose immunoadsorbent, according to the method described by Robbins et al. (25). The physical adsorption of

thyroglobulin to bromoacetyl cellulose was done in 0.15 M sodium phosphate citrate buffer at pH 4.8. The covalent binding of thyroglobulin to bromoacetyl cellulose was performed at pH 8.9.

Induction of Unresponsiveness to Guinea Pig γG-Globulin in Adult Rabibts.—^{131}I-Labeled guinea pig γG-globulin was first heat-aggregated at 63°C for 15 min. The heat-aggregated guinea pig γG-globulin was allowed to settle at 0°C for 8–12 hr. The supernatant solution, containing 10 mg protein/ml of guinea pig γG-globulin, was ultracentrifuged at 105,000 *g* for 90 min in an SW 39 rotor. After ultracentrifugation, the upper 3 ml of the tubes were carefully aspirated into 1 ml disposable syringes, and 5 mg of ^{131}I-protein was injected within 10 min intravenously into rabbits. Rabbits which did not show an immune elimination of the guinea pig γG-globulin within 16 days were rechallenged 1 month after the first injection with 5 mg ^{131}I-labeled guinea pig γG-globulin. Rabbits which did not show an immune elimination by day 16 as monitored by daily counting of whole-body radioactivity (3) were considered unresponsive.

RESULTS

Effect of Varying Amounts of Rabbit Thyroglobulin and Mycobacteria in Complete Adjuvant on the Severity of Thyroiditis in Rabbits.—These experiments were designed to establish the optimal concentration of antigen and mycobacteria in the adjuvant which would consistently induce severe thyroid lesions in rabbits.

Adult rabbits were injected with varying doses of rabbit thyroglobulin incorporated in 1.0 ml of complete Freund's adjuvant containing 10 mg mycobacteria. Five groups of six animals each were injected with 0.1, 1.0, 2.0, 5.0, and 10.0 mg of rabbit thyroglobulin, respectively. A total of 1.0 ml of the antigen in adjuvant was injected into all four footpads of each animal. All animals were bled and killed at 1 month, and their thyroid glands were removed for histology. Sera obtained at this time were analyzed for precipitating antibody to rabbit thyroglobulin.

The animals receiving the 5.0 and 10.0 mg doses of rabbit thyroglobulin showed greater degrees of thyroid inflammation than the animals receiving smaller doses (Fig. 1). The animals receiving 0.1 and 1.0 mg doses of rabbit thyroglobulin showed lower grades of thyroiditis and lower levels of precipitating antibody than animals receiving the 2, 5, and 10 mg doses of rabbit thyroglobulin. In general, the animal groups receiving 2, 5, and 10 mg doses of rabbit thyroglobulin showed no difference in the amount of precipitating antibody to homologous thyroglobulin. There was no correlation between the precipitating antibody level to rabbit thyroglobulin and the severity of thyroiditis.

In another experiment, adult rabbits were injected with varying doses of mycobacteria incorporated in 1.0 ml of complete Freund's adjuvant containing 10.0 mg of rabbit thyroglobulin. Five groups of six animals each were injected with 0, 0.1, 1.0, 5.0, and 10.0 mg of mycobacteria, respectively. A total of 1.0 ml of the adjuvant was injected into all four footpads. All animals were bled and killed at 1 month, and their thyroid glands were removed for histology. Sera were analyzed for precipitating antibody to rabbit thyroglobulin.

It can be seen from Fig. 2 that the combination of 10.0 mg of mycobacteria with 10.0 mg of rabbit thyroglobulin induced 2+ or greater thyroiditis in all the animals. Most of the animals receiving either 1, 5, or 10 mg of mycobacteria showed measurable precipitating antibody to rabbit thyroglobulin. Rabbits receiving the 5 or 10 mg dose of mycobacteria showed the highest levels of precipitating antibody to rabbit thyroglobulin.

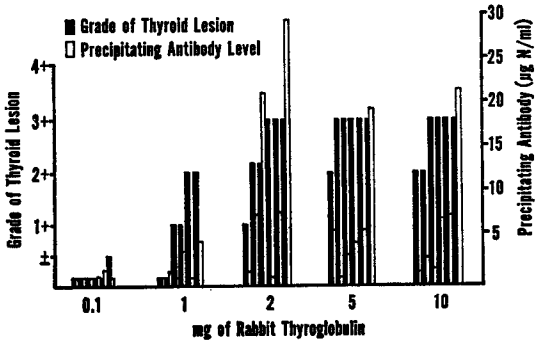

FIG. 1. Effect of varying amounts of rabbit thyroglobulin in complete adjuvant on severity of thyroiditis in rabbits.

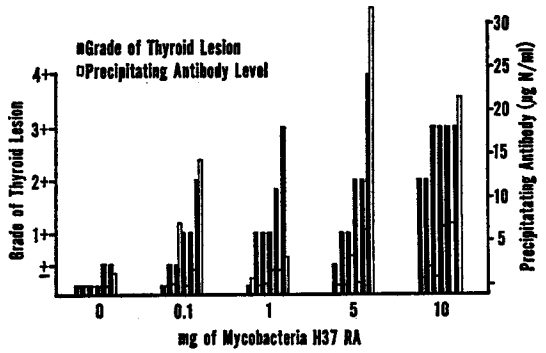

FIG. 2. Effect of varying amounts of mycobacteria on the severity of rabbit thyroiditis induced with 10 mg rabbit thyroglobulin in adjuvant.

Study of Temporal Sequence in the Development of Thyroiditis and Antibody following Injection of Homologous Thyroglobulin in Complete Adjuvant.—This experiment was designed to determine when the earliest thyroid lesions and antithyroglobulin antibody would appear, and the time required for establishment of uniformly severe thyroiditis in rabbits following an injection of 10.0 mg of rabbit thyroglobulin in complete adjuvant containing 10.0 mg mycobacteria.

Adult rabbits were divided into nine groups, with 6–10 animals in each group. Various groups of rabbits were bled and killed on days 5, 7, 9, 11, 13, 14, 16, 28, and 56 after injection. The thyroid glands were removed for histology, and the sera were analyzed for precipitating antibody to rabbit thyroglobulin.

It can be seen from Fig. 3 that early thyroid lesions were observed on day 5. Small amounts of precipitating antibody to rabbit thyroglobulin were observed in six of eight animals on day 7. Significant thyroiditis was seen in five of eight animals on day 9, and by day 14 a majority of the rabbits had severe thyroid lesions which were not significantly different from the thyroid lesions of animals observed at 1 and 2 months postimmunization. In general, the animals killed on days 14, 16, 28, and 56 showed the highest levels of precipitating antibody

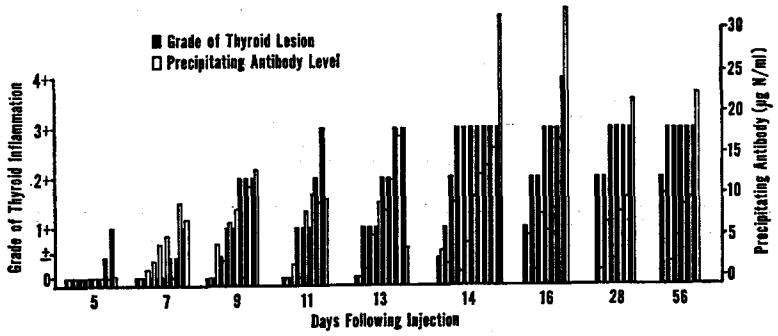

FIG. 3. Temporal sequence in the development of thyroiditis in rabbits following a single injection of 10 mg of rabbit thyroglobulin in complete adjuvant containing 10 mg of mycobacteria.

to rabbit thyroglobulin. Again, no significant correlation between precipitating antibody to rabbit thyroglobulin and severity of thyroid lesions was observed.

Study of Antibody Produced in Nonthyroidectomized and Thyroidectomized Rabbits Injected with Rabbit Thyroglobulin in Complete Adjuvant.—This experiment was performed to detect possible quantitative differences in antibody production of nonthyroidectomized and thyroidectomized rabbits injected with 10.0 mg rabbit thyroglobulin in complete adjuvant with 10.0 mg of mycobacteria.

A group of 12 rabbits was immunized 1 month after total thyroidectomy. The animals were bled on days 5, 6, 7, 9, 11, 14, 29, 31, and 56. On day 56 the animals were killed, and none showed evidence of residual thyroid gland tissue. Individual sera were analyzed for (*a*) precipitating antibody, (*b*) 19S and 7S antibodies by density gradient ultracentrifugation and radioimmunoelectrophoresis, and (*c*) homologous anaphylactic antibody. A second group of 12 normal, nonthyroidectomized rabbits of similar age was immunized and studied in the same fashion as above.

The animals were killed at day 56, and the thyroid glands of all 12 thyroidectomized animals showed a 3+ thyroiditis. The average precipitating antibody levels in thyroidectomized and nonthyroidectomized rabbits on various days following immunization are shown in Fig. 4. The average levels of precipitating antibody to rabbit thyroglobulin were higher in thyroidectomized rabbits. In both groups of rabbits, the peak precipitating antibody level was observed on day 14, and the levels observed on days 28 and 56 were similar. Significant

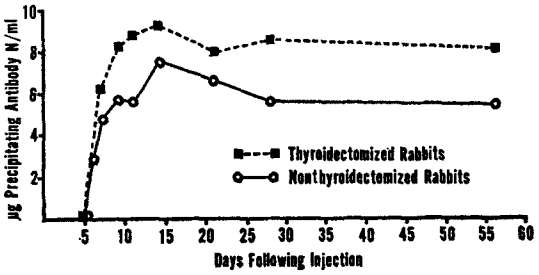

FIG. 4. Average antibody levels in nonthyroidectomized and thyroidectomized rabbits injected with rabbit thyroglobulin in complete Freund's adjuvant.

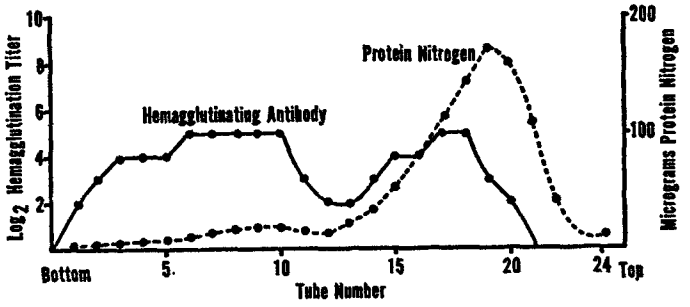

FIG. 5. Density gradient of day 6 serum obtained from a representative rabbit injected with rabbit thyroglobulin in complete adjuvant.

levels of either precipitating or hemagglutinating antibody were not observed on day 5. However, 19S hemagglutinating antibody was detected on days 6, 7, 9, and 11 by density gradient ultracentrifugation (Fig. 5) in three representative animals demonstrating precipitating antibody to rabbit thyroglobulin. Radioimmunoelectrophoretic studies showed the presence of 7S rabbit γG but not γM antithyroglobulin antibodies in sera obtained on the various days. Repeated experiments with sera obtained on different days from thyroidectomized and nonthyroidectomized animals were negative for homocytotropic antibody.

Study of the Development of Delayed and Immediate (Arthus) Hypersensitivity during Induction of Experimental Thyroiditis in Rabbits following a Single Injection of Rabbit Thyroglobulin in Complete Freund's Adjuvant.—

Adult rabbits were injected in the footpads with 10.0 mg rabbit thyroglobulin in complete Freund's adjuvant with 10.0 mg mycobacteria. On days 3, 5, 8, 12, and 20, histological and capillary permeability studies were performed. For the histological studies, different groups of two animals were studied on each day. 100 μg, 20 μg, 2 μg, and 0.2 μg doses of rabbit thyroglobulin were injected intradermally 2.5 cm apart in a horizontal row. Three such horizontal rows of intradermal injections with the different doses of thyroglobulin were injected into the same animal. At 3, 24, and 48 hr after intradermal injections the animals were anesthesized with pentobarbital; a biopsy with removal of a row of skin lesions was performed. The skin wound was closed with metallic clips. The skin biopsies containing the injection sites were fixed in Bouin's solution for histology.

On day 3 both rabbits showed a few polymorphonuclear cell infiltrations in the dermis at 24 and 48 hr in sites injected with 100 and 20 μg rabbit thyroglobulin. No significant accumulation of mononuclear cells was seen. On day 5 polymorphonuclear cell infiltration was observed at 24 hr in the skin of both rabbits injected with 100, 20, and 2 μg of thyroglobulin. One rabbit showed necrotizing vasculitis with polymorphonuclear cell infiltration at 48 hr in a skin site injected with 100 μg of thyroglobulin. No significant mononuclear cell infiltration was observed. On days 8, 12, and 20, extensive polymorphonuclear cell infiltration around the vessels, characteristic of an immediate hypersensitivity reaction, was observed at 3, 24, and 48 hr in skin sites injected with the varying doses of rabbit thyroglobulin.

Similarly, capillary permeability studies were done on groups of two animals on days 3, 5, 8, 12, and 20, with intradermal injections of the varying doses of thyroglobulin given to the same animal 48, 24, 6, and 2 hr prior to sacrifice. The different doses were injected 2.5 cm apart along the same horizontal skin row as described above. 20 min prior to sacrifice each animal was injected with 20 μc of ^{131}I-BSA intravenously. Immediately after injection of ^{131}I-BSA, 2.0 μg of histamine (base) was injected in duplicate skin sites for positive controls. After sacrifice the skin was removed, flattened, and frozen. Injection sites were punched out from frozen skin with a 1.0 cm diameter punch and hammer. The punch was washed after each skin biopsy. The ^{131}I radioactivity in each biopsy specimen was measured in a well-type scintillation counter containing a NaI crystal. Normal, uninjected skin was punched out to indicate background.

On days 3 and 5 no significant increase in ^{131}I radioactivity was observed in the skin biopsies removed at varying times. On day 8, increased radioactivity (greater than twice background) was observed in skin sites injected with 100 and 200 μg of rabbit thyroglobulin 24 and 48 hr prior to sacrifice. On day 12 significant increases in ^{131}I radioactivity were observed in skin biopsies injected with 100, 20, and 2 μg 48, 24, 6, and 2 hr prior to sacrifice.

Passive Transfer of Serum Containing Early Antibody from Thyroidectomized Donors Injected with Homologous Thyroglobulin in Complete Adjuvant.—Since

the critical period of development of thyroid lesions and antithyroglobulin antibodies in rabbits injected with homologous thyroglobulin in complete adjuvant was completed during the first 2 wk following injection, experiments were designed to transfer sera containing early antibody in a sequential manner.

Donor animals were completely thyroidectomized and maintained with 50 μg of L-thyroxine injected subcutaneously once a week. 1 month after the operation the donor animals were each injected with 10 mg rabbit thyroglobulin in complete Freund's adjuvant containing 10

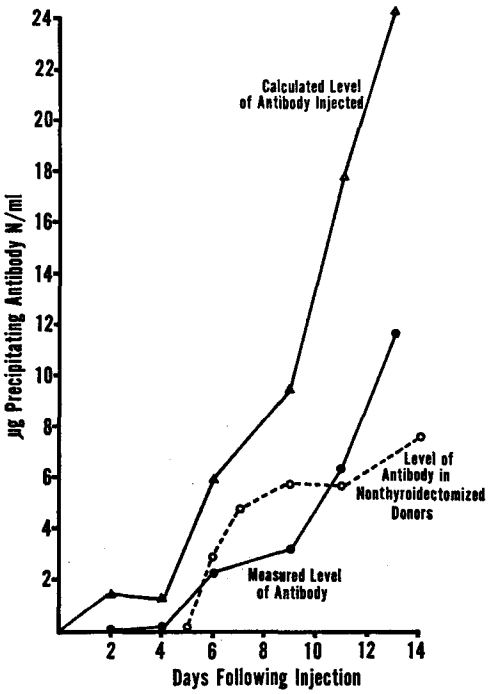

FIG. 6. Experiment A. Passive transfer of serum containing early antithyroglobulin antibody from thyroidectomized donors.

mg mycobacteria. 30–40 ml of blood was taken from each donor on days 4, 6, 8, 10, 13, and 15 after injection. Donor sera of various days were pooled separately and sequentially injected into normal recipients on days 0, 2, 4, 6, 9, and 11. The recipients were bled just prior to each injection of the various donor sera to measure circulating anti-rabbit thyroglobulin precipitating antibody. The recipient animals were sacrificed on day 13. Thyroid glands were removed for histological and fluorescent antibody studies. Samples of donor pooled sera obtained on various days also were analyzed for precipitating antibody levels to rabbit thyroglobulin.

In experiment A, on day 0, nine 1.6–1.8 kg recipient rabbits were first bled and 35 ml of blood was removed. A total of 65–90 ml of donor sera was then given by intravenous and subcutaneous routes in divided doses on each of the

various days. In Fig. 6, the average measured levels of antibody in the 9 recipient rabbits were compared with the average antibody levels in a group of 12 rabbits immunized with rabbit thyroglobulin in complete Freund's adjuvant (Fig. 4). It can be seen that on days 6, 7, and 9 the antibody levels in the passively transferred recipients were less than those observed in actively immunized rabbits. Four of the nine recipient rabbits demonstrated 1+ thyroiditis. One of the animals showed minimal thyroid lesions graded as ±. Typical focal thyroid lesions are shown in Fig. 11. The inflammatory lesions showed disruption of thyroid follicles and basement membrane with accumulation of lymphocytes and histiocytes. Fluorescent antibody studies of the

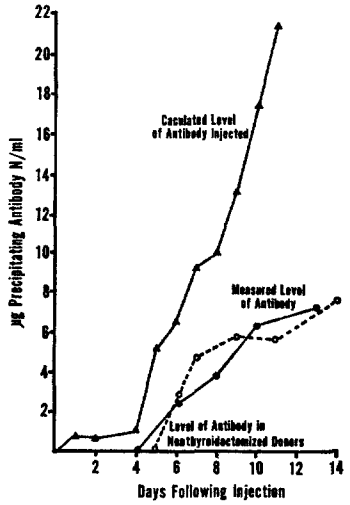

FIG. 7. Experiment B. Passive transfer of serum containing early antithyroglobulin antibody from thyroidectomized donors.

recipient thyroid glands showed focal fixation of rabbit γ-globulin and β_{1C} complement in thyroid follicles (Fig. 12). The focal fixation of rabbit γ-globulin and β_{1C} complement was noted only in the glands showing inflammatory lesions. Normal rabbit thyroid glands showed no evidence of specific fluorescent staining when reacted with the conjugated, fluoresceinated antisera as above. The antibody levels in the recipient sera were quantitated and compared with the total antibody in the sera injected and calculated to be present after assuming a half-life of rabbit γG-globulin in rabbits to be 5.6 days (14) and the extravascular equilibration of rabbit γG-globulin to be about 50% (26).

In experiment B, whole serum was obtained from thyroidectomized donors as in experiment A. Recipients were given over twice the quantity of pooled donor sera as was given in experiment A. 160–200 ml pooled donor sera ob-

tained on each of various days was transferred sequentially in divided doses to four 2.0 kg recipient rabbits over a 2-day period, instead of 1 day as in experiment A. The measured precipitating antibody level to rabbit thyroglobulin in recipient animals varied from 2.3 to 2.4 and from 3.4 to 4.5 μg N/ml on days 6 and 8, respectively. The average passively transferred antibody levels between days 6 and 9 were lower than the average antibody levels in a group of rabbits injected with thyroglobulin in complete adjuvant (Fig. 7).

Of the four recipient animals, two showed definite thyroid lesions. One rabbit had 1+ thyroiditis, and the animal showing the highest level of passively transferred antibody had 2+ thyroiditis. The thyroid gland of the animal with 2+ thyroiditis showed marked vacuolation and loss of follicular colloid with accumulation of many histiocytes (Fig. 13). The calculated level of injected antibody was much higher than in experiment A, since larger amounts of donor sera were transferred. The thyroid glands of both the rabbits with thyroid lesions showed definite focal fixation of rabbit γ-globulin and rabbit β_{1C} complement by immunofluorescent antibody studies. Of the rabbits showing no definite histological lesions, one animal showed minimal focal fixation of rabbit γ-globulin in the thyroid gland surrounding and partially within colloid follicles.

A control experiment consisted of immunizing adult rabbits with complete Freund's adjuvant containing 10.0 mg mycobacteria but no thyroglobulin. Serum was collected from many such donors on days 4, 6, 8, 10, 13, and 15 following injection. Donor sera of various days were pooled separately and injected sequentially into six normal recipients on days 0, 2, 4, 6, 9, and 11 as in experiment A. The recipient animals were sacrificed on day 13. The thyroid glands were removed and showed no thyroid lesions upon histological examination. As an additional control, four nonthyroidectomized rabbits were injected with 10.0 mg rabbit thyroglobulin in complete adjuvant and bled (30–40 ml) on various days in a fashion similar to the donor animals in this experiment. The thyroid glands were examined on day 15, and all showed 2+ to 3+ thyroiditis.

Passive Transfer of Serum Globulin Fraction from Nonthyroidectomized Donors Injected with Homologous Thyroglobulin in Complete Adjuvant.—

Nonthyroidectomized adult rabbits were used as donors. Each rabbit was injected with 10.0 mg rabbit thyroglobulin in complete adjuvant containing 10.0 mg mycobacteria. Serum was collected on days 4, 6, 8, 10, 13, and 15 after injection. Donor sera of various days were pooled separately, and the globulin fraction was obtained by 50% ammonium sulfate fractionation. The globulin fraction of the donor sera obtained on the various days was sequentially injected subcutaneously into five 2.0 kg recipient rabbits on days 0, 2, 4, 6, 9, and 11. The recipient rabbits were bled periodically just prior to injection of donor sera for antibody analyses. The recipients were killed on day 13, and their thyroid glands were removed for histological examination.

The calculated and measured passively transferred antibody levels are shown in Fig. 8. The antibody levels were calculated in a manner similar to that in the previous experiments. It can be seen that the average precipitating antibody level to rabbit thyroglobulin in the recipient animals was much less than that observed in experiments A and B when sera from thyroidectomized donors were transferred. None of the five recipient rabbits showed any evidence of thyroid lesions. The average measured precipitating antibody level to homologous thyroglobulin was much less than the calculated level of injected antibody, assuming a half-life of rabbit γG-globulin of 5.6 days (14). The

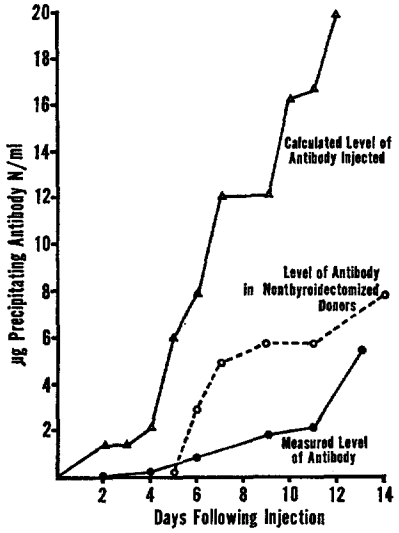

FIG. 8. Passive transfer of serum globulin fraction from nonthyroidectomized donors injected with rabbit thyroglobulin in complete Freund's adjuvant.

difficulty in obtaining a high level of passively transferred antibody is complicated by a marked increase in catabolism of γG-globulin when large amounts of serum proteins are infused into the animal (27).

Passive Transfer of Serum Containing Late Antibody to Rabbit Thyroglobulin from Thyroidectomized Donors Injected with Homologous Thyroglobulin in Complete Adjuvant.—

Donor animals were completely thyroidectomized and were maintained and immunized with rabbit thyroglobulin in complete Freund's adjuvant as in the above experiments. The donor animals were bled on days 56, 58, 60, 62, 65, and 66 after immunization. The donor sera of the various days were pooled separately and injected sequentially into four 2.0 kg normal recipient rabbits on days 0, 2, 4, 6, 9, and 11. 150–170 ml of pooled donor sera obtained on

each of various days were transferred sequentially over a 2 day period and injected intravenously and subcutaneously. Donor sera were quantitated for precipitating antibody to rabbit thyroglobulin. The animals were bled periodically prior to injection of various pooled donor sera for antibody analyses. The recipients were bled and killed on day 13, and the thyroid glands were removed for histology.

None of the recipient animals showed any evidence of thyroid lesions. The average measured precipitating antibody in the recipient rabbits on days 2, 4, 6, 9, 11, and 13 was more than twice that observed in a group of nonthyroidectomized rabbits injected with rabbit thyroglobulin in complete Freund's adjuvant (Fig. 9).

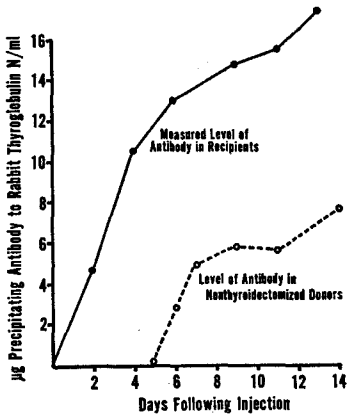

FIG. 9. Passive transfer of serum containing late antibody to rabbit thyroglobulin from thyroidectomized donors.

It has been previously reported that passively transferred heterologous antibodies to thyroglobulin can cause early thyroid lesions which may be seen in 1–5 days (28, 29). In the present experiment, sera containing *late* antibody to rabbit thyroglobulin were obtained from thyroidectomized donors as above and pooled.

The globulin fraction was obtained by fractionation with 50% ammonium sulfate. The precipitating antibody of the donor globulin fraction was quantitated and injected intravenously and intraperitoneally into five recipient rabbits on day 0. On day 1 the recipient rabbits were anesthesized and bled, and one lobe of the thyroid was removed for histological examination. On day 5 the recipients were bled, and the remaining thyroid lobe was removed for histological examination. The sera were analyzed for precipitating antibody to rabbit thyroglobulin.

None of the recipient rabbits showed any evidence of thyroid lesions on day 1 or day 5. The measured levels of antibody to rabbit thyroglobulin are shown in Table I. The precipitating antibody level on day 1 varied from 10.2 to 19.8

μg N/ml, which was higher than the peak average antibody level observed in a group of nonthyroidectomized rabbits injected with rabbit thyroglobulin in complete Freund's adjuvant.

Passive Transfer of Purified Antibody to Rabbit Thyroglobulin Obtained from Thyroidectomized Donors Injected with Homologous Thyroglobulin in Complete Adjuvant.—In this experiment sera containing early antibody to rabbit thyroglobulin were collected on various days from thyroidectomized donors as in experiments A and B.

The donor sera of various days were pooled separately, and purified thyroglobulin antibody was isolated from each donor pool by use of a bromoacetyl cellulose immunoadsorbent. The purified antibody to rabbit thyroglobulin of the donor sera pools was then injected sequentially

TABLE I

Passive Transfer of Globulin Fraction Containing Late Antibody to Rabbit Thyroglobulin from Thyroidectomized Donors

Recipient rabbit	Measured precipitating antibody level to rabbit thyroglobulin	
	Day 1	Day 5
	μgN/ml	
1	19.8	11.4
2	10.2	4.7
3	18.2	10.9
4	11.0	6.0
5	10.6	5.4
Average	14.0	7.7

(intravenously) into two normal recipients on days 0, 2, 4, 6, 9, and 11 as above. The amount of precipitating antibody injected was measured, as well as the level of antibody in the recipients just prior to each injection of purified antibody. The recipient rabbits were sacrificed on day 13. The thyroid glands of both rabbits showed 1+ thyroiditis.

The thyroid lesions in the recipients showed an accumulation of lymphocytes and histiocytes with disruption of the follicular architecture (Fig. 14). In Fig. 10 is shown the average measured antibody level in the recipient rabbits, which was much lower than the calculated level of antibody injected or the observed average antibody level in normal rabbits immunized with rabbit thyroglobulin in complete adjuvant. Six control recipient rabbits received injections of similar quantities of nonantibody rabbit γ-globulin intravenously on the various days, as outlined above. None of these animals showed any evidence of thyroiditis.

Injection of Guinea Pig Anti-rabbit Thyroglobulin in Rabbits Previously Rendered Unresponsive to Guinea Pig γG-Globulin.—

Adult rabbits were first rendered unresponsive to guinea pig γG-globulin according to a technique described above. The animals were then divided into two groups with five rabbits

in each group. The experimental group was injected with the globulin fraction obtained by 50% ammonium sulfate fractionation of guinea pig anti-rabbit thyroglobulin thrice weekly for 8 wk. A total of 8.0 ml was given in divided doses the first week, and 1.0 ml/injection was given thereafter. The protein content of the globulin fraction was 45 mg/ml and contained 1.15 mg N/ml of precipitating anti-rabbit thyroglobulin. The total quantity of antibody injected was 33.4 mg antibody N.

The control group received injections of normal guinea pig globulin, fractionated in a similar manner, containing protein concentrations varying from 40 to 50 mg/ml. On the sixth week of injections, the animals were challenged with 10 mg ^{131}I-labeled guinea pig γG-globulin to test for persistence of unresponsiveness to guinea pig γG-globulin.

All of the animals remained unresponsive, since they did not show an immune elimination of the ^{131}I-labeled guinea pig γG-globulin. Upon termination of the experiment, the animals were killed and their thyroid glands were removed for histological examination. No significant lesions were observed in the thyroid glands of any of the rabbits.

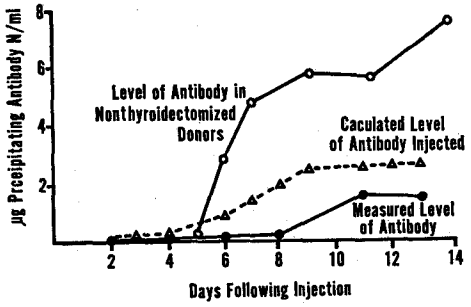

FIG. 10. Passive transfer of purified antibody to rabbit thyroglobulin from thyroidectomized donors.

DISCUSSION

The events leading to thyroiditis occur over a relatively short period of time following injection of rabbits with homologous thyroglobulin in adjuvant. Detectable thyroid lesions were observed in rabbits as early as the fifth day following injection, and by day 14 the majority of animals had severe thyroid lesions which were not significantly different from thyroid lesions observed at 1 and 2 months postimmunization. The peak level of precipitating antibody to rabbit thyroglobulin was observed on day 14. As previously shown in guinea pigs by McMaster and Lerner (30), the severity of the thyroid lesions in rabbits of the present experiments was influenced by the amount of thyroglobulin and mycobacteria incorporated into the adjuvant. Lesions of maximal severity were observed following injection of adjuvant containing 10 mg of mycobacteria and 2–10 mg of thyroglobulin.

Since the critical events involved in the production of thyroiditis in the rabbit occur over a relatively short period of time, it was practical to attempt

passive transfer of thyroiditis with serum taken at various times from immunized donors and injected sequentially into normal rabbits. The results of these experiments strongly suggest that humoral antibody may play a role in experimental autoimmune thyroiditis in the rabbit. Successful transfer of thyroiditis occurred with serum containing early antithyroglobulin antibodies obtained from thyroidectomized donor animals. Thyroid lesions were observed in recipients when pooled sera obtained on various days from thyroidectomized donors and containing early antibodies were transferred in a sequential manner. Immunofluorescent studies of recipient thyroid glands showed focal fixation of rabbit γ-globulin and β_{1C} complement in thyroid follicles. No thyroid lesions were observed in control recipients receiving sera from donors injected with complete Freund's adjuvant without homologous thyroglobulin. Similarly, no thyroid lesions were seen in recipient animals injected sequentially with early antithyroglobulin obtained from nonthyroidectomized donors. The latter observation suggests that the pathogenetic antibodies may have been removed from the circulation by the target organ and is in agreement with previous studies with experimental glomerulonephritis, in which transfer of glomerulonephritis with serum from sheep immunized with kidney antigens occurred only when serum was obtained from the donor after complete nephrectomy (31). The removal of circulating antibody by thyroglobulin either in the target organ or by release into the circulation might also have been responsible for the higher levels of precipitating antithyroglobulin in thyroidectomized rabbits than in nonthyroidectomized rabbits.

The antibodies responsible for the serum transfer of thyroiditis are produced relatively early following immunization of the host. Whether two or more types of immunoglobulins are involved which react in sequence to produce thyroid lesions cannot be definitely discerned from the present experiments. In any event, late antibody produced 2 months after immunization does not appear to be pathogenetic. When large amounts of either homologous or heterologous antibody to thyroglobulin obtained relatively late after immunization were transferred, no lesions developed in the recipients. It is unlikely that 7S γ_1 homocytotropic antibody is involved, since it could not be detected in the donors' sera after immunization. Homocytotropic antibody recently has been associated with experimental thyroiditis in guinea pigs, in that some guinea pigs immunized with thyroglobulin in complete Freund's adjuvant had thyroid lesions and only the homocytotropic antibody was found in their sera (32). In the present studies only 7S γ_2 antibody could be detected in the donors' sera by autoradioimmunoelectrophoresis. However, low levels of 19S hemagglutinating antibody were observed in serum taken 6 days after immunization. What role, if any, 19S antibody plays in experimental autoimmune thyroiditis is currently being investigated.

Since large volumes of serum were transferred in the present studies, the

possibility that adjuvant containing thyroglobulin was transferred with the serum must be considered. The presence of adjuvant in the serum might lead to active immunization of the recipients. However, the ability of purified antibody to cause thyroiditis following transfer probably rules out this possibility. When antibodies isolated and purified on an immunoadsorbent were transferred sequentially to recipient rabbits, significant thyroid lesions resulted. It is unlikely that any adjuvant present in serum would be selectively concentrated by this procedure.

It was difficult to maintain levels of antithyroglobulin in recipient animals comparable to the levels in the donor serum transferred very early after immunization. The level of antibody in the serum of the recipient always was considerably lower than that calculated from both the amount of antibody transferred and the plasma volume of the recipient. It is most likely that the lower levels of antibody resulted in large part from an increase in the rate of catabolism of plasma proteins (27). However, this would not be the case when purified antibody was injected, since the total amount of protein injected would not significantly increase the plasma level. The differences between the observed and calculated levels of antibody in the recipients injected with purified antibody probably resulted from technical difficulties in quantitating precipitating antibodies at the low concentrations present in the sera of these recipients.

The ability to produce thyroiditis by passive transfer of serum raises the question of what role, if any, is played by cellular hypersensitivity. Certainly the severity and incidence of thyroiditis were considerably less in rabbits receiving serum than in rabbits actively immunized. However, this difference may be explained by the lower levels of circulating antibody in recipients than in actively immunized rabbits. The lack of correlation between the level of antibody to thyroglobulin observed in both the present study with rabbits and previous studies with guinea pigs (6, 7), and the close relationship in the guinea pig between the course of thyroiditis and cellular sensitivity (6, 8, 9), further suggest that factors other than circulating antibody are involved. However, the total antibody in the serum consists of a wide variety of immunoglobulins, and the presence of one or more pathogenetic antibodies may be obscured when a correlation is made between the severity of thyroid lesions and total serum antibody. Furthermore, the course of thyroiditis in rabbits is not as closely correlated with cellular hypersensitivity as it has been shown to be in guinea pigs. In the present experiment, skin reactivity to homologous thyroglobulin, characteristic of cellular sensitivity, was not observed in rabbits injected with homologous thyroglobulin in complete Freund's adjuvant. Different amounts of thyroglobulin were injected intradermally at different intervals of time on various days after a single injection of rabbit thyroglobulin in complete Freund's adjuvant. No significant infiltration of mononuclear cells or increased capillary permeability characteristic of cellular hypersensitivity was observed on days 3,

5, 8, 12, or 20 following injection of the adjuvant. Perivascular polymorphonuclear infiltration, indicative of an Arthus reaction, was observed 24 hr after intradermal injection of thyroglobulin as early as the fifth day after the adjuvant injection. Rose and coworkers (6) have reported that cellular hypersensitivity to thyroid extracts in rabbits was not significant until after 1½–2 months after immunization. Furthermore, these animals required 1–2 months to develop significant thyroid lesions. The difference in their findings from those of the present study may be explained by their use of thyroid extracts as well as by differences in amounts of thyroglobulin and mycobacteria in the adjuvant. In any event, detectable cellular hypersensitivity is not evident in the rabbit early after injection of thyroglobulin in complete adjuvant at a time when severe thyroiditis is developing. The spontaneous development of autoimmune thyroiditis in chickens also appears to be dependent to a large extent on circulating antibody, in that bursectomy markedly interferes with the development of lesions (33). The observation of Brown, Glynn, and Holborow (34), working with autoimmune orchitis in guinea pigs, suggests that both cellular hypersensitivity and circulating antibody are involved in some of the autoimmune diseases. The development of autoimmune thyroiditis in the rabbit and chicken may be more dependent on circulating antibody and less dependent on cellular sensitivity than the development of thyroiditis in the guinea pig.

Heterologous antisera obtained late in the course of immunization were ineffective in producing thyroid lesions. Rabbits rendered unresponsive to guinea pig γG-globulin and periodically injected with guinea pig anti-rabbit thyroglobulin showed no thyroid lesions. Previous reports have demonstrated eosinophilic infiltration of the thyroid, which developed over 1 day in guinea pigs following passive transfer of heterologous rabbit antisera to guinea pig thyroglobulin (28, 29). Thyroiditis was observed in rats with injections of rabbit anti-rat thyroid serum only after pretreatment of recipients with Freund's adjuvant or low doses of radioiodine (35); however, the experiments could not be repeated with the homologous antibody (autoantibody) (12). It is not known whether injection of heterologous antiserum obtained early in the course of immunization would result in thyroiditis.

SUMMARY

When rabbits were injected with 10.0 mg rabbit thyroglobulin in complete Freund's adjuvant, the earliest thyroid lesions were seen on day 5 and uniformly severe thyroid lesions were seen by day 14; these observations were not significantly different from the thyroid lesions observed at 1 and 2 months post-immunization. Pooled sera were obtained from immunized, thyroidectomized, and nonthyroidectomized donors on various days and transferred to normal recipient rabbits in different experiments. Successful transfer of thyroid lesions was seen when serum containing early antithyroglobulin antibody obtained

from thyroidectomized donor animals at various times after immunization was injected into normal recipients in a sequential manner. Immunofluorescent studies of recipient thyroid glands showed focal fixation of rabbit γ-globulin and β_{1C} complement in thyroid follicles. When purified antibody to rabbit thyroglobulin obtained from thyroidectomized donor sera was transferred sequentially as above, significant thyroid lesions were seen in recipient rabbits. In contrast, no thyroid lesions were seen in recipient animals injected with rabbit sera containing late antithyroglobulin antibody from thyroidectomized donors or hyperimmune sera from guinea pigs.

No thyroid lesions were seen in recipient animals injected either with sera from donors given complete adjuvant without thyroglobulin or with globulin fraction of pooled sera containing early antithyroglobulin antibody obtained on various days from nonthyroidectomized donors. Similarly, rabbits rendered unresponsive to guinea pig γG-globulin and periodically injected with guinea pig anti-rabbit thyroglobulin showed no thyroid lesions.

The authors acknowledge the capable technical assistance of Mrs. Judith Bolls and Miss Carole Romball.

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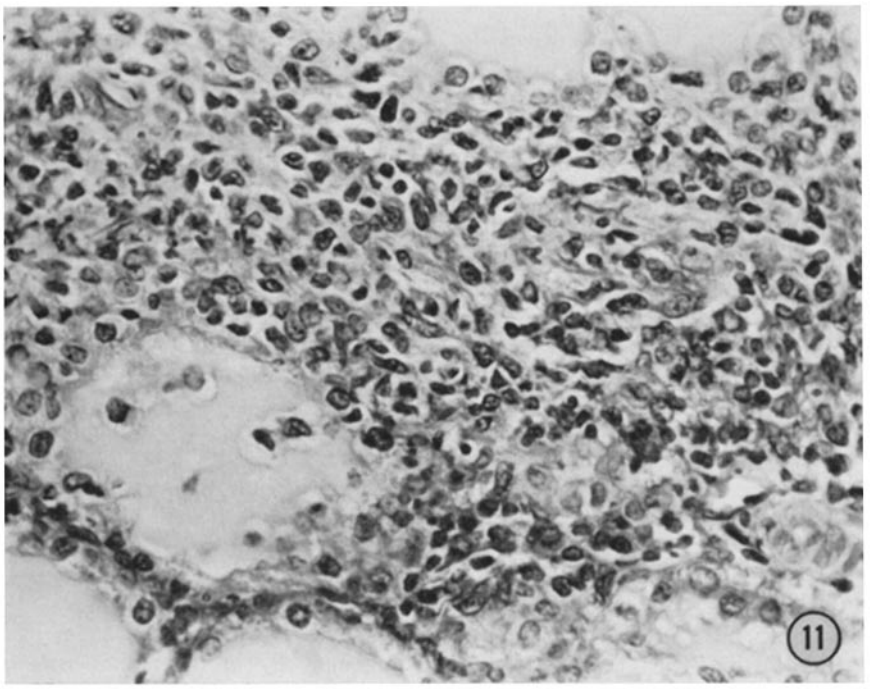

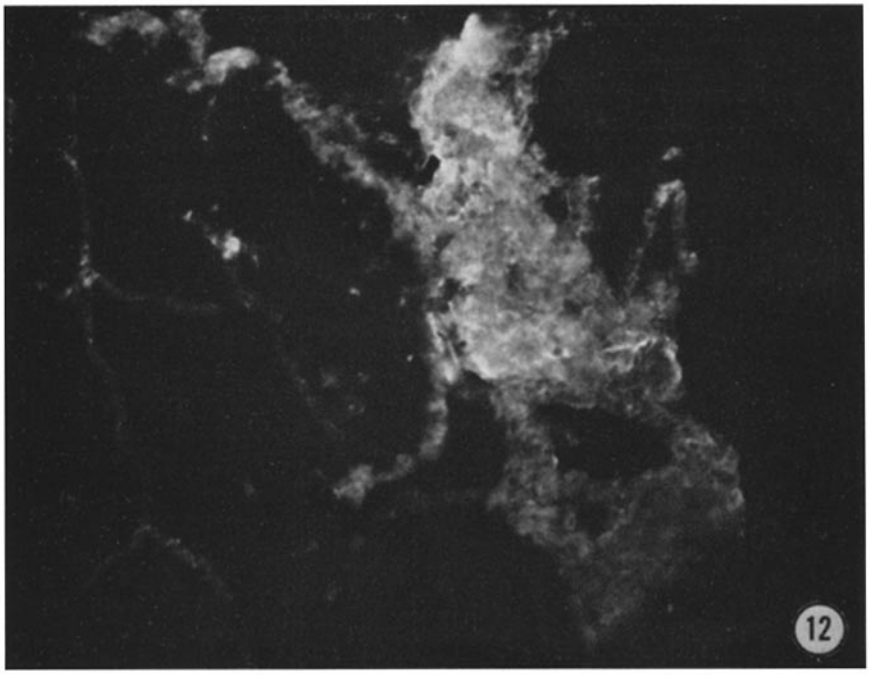

FIG. 11. Experiment A. Photomicrograph of typical thyroid lesions in recipient animals injected with early sera from thyroidectomized donors. There is disruption of thyroid follicles and basement membrane, with accumulation of lymphocytes and histiocytes. Hematoxylin and eosin, × 440.

FIG. 12. Photomicrograph of thyroid gland of recipient animal, showing lesions after injection of early sera from thyroidectomized donors. The thyroid glands were stained with FITC-conjugated sheep anti-rabbit γ-globulin. A similar pattern was observed when sections were stained with FITC-conjugated guinea pig anti-rabbit β_{1C}-globulin. Focal fixation of rabbit γ-globulin and rabbit β_{C1}-globulin was observed in thyroid follicles and follicular lining epithelial cells. × 480.

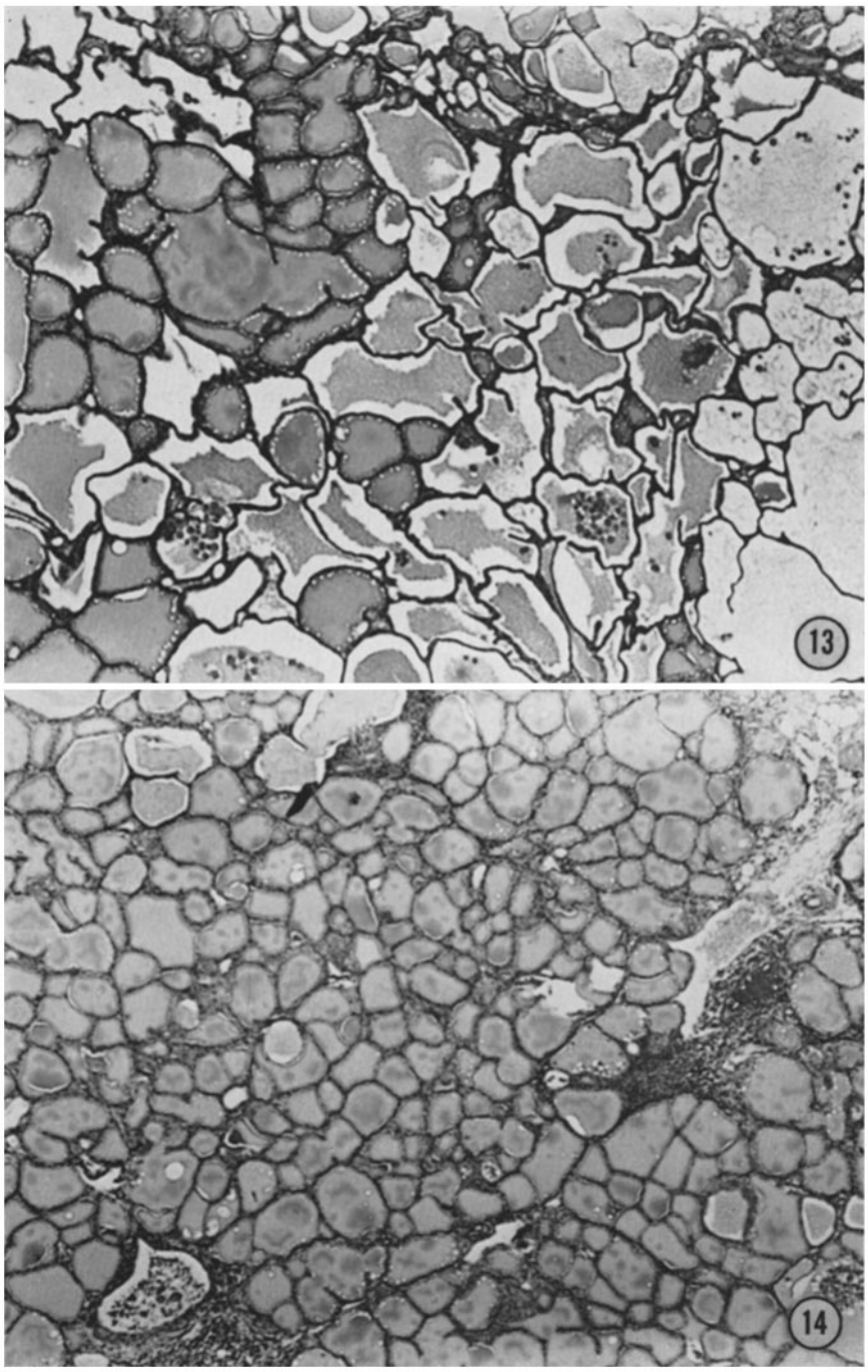

FIG. 13. Experiment B. Photomicrograph of thyroid gland of recipient animal following injection of early sera from thyroidectomized donors. The thyroid gland showed marked depletion of follicular colloid with atrophy of follicular epithelial cells and accumulation of many histiocytes. Hematoxylin and eosin, × 44.

FIG. 14. Photomicrograph of recipient animal, showing thyroid lesions after passive transfer of purified antibody. There is disruption of follicular architecture with accumulation of ymphocytes and histiocytes. Hematoxylin and eosin, × 56.