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STUDIES ON THYROID IMMUNITY

VII. SPLENECTOMY AND MONKEY IMMUNE THYROIDITIS: THYROIDAL FUNCTION AND THYROXINE TRANSPORT

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SUMMARY

The role of the spleen in humoral antibody formation and in the pathogenesis of immune thyroiditis was studied by splenectomizing four monkeys (*Macaca mulatta*) prior to thyroid isoimmunization. Splenectomized animals, as well as intact controls, were subjected to sequential immunizations and the course of the immune disease was followed by periodic thyroid biopsies and frequent humoral antibody measurements over a period exceeding 1 yr. Extirpation of the secondary lymphoid organ markedly inhibited agglutinating antibody response, prevented formation of complement-fixing antibodies, but had no effect on thyrocytotoxic antibodies. In animals subjected to immunization in complete adjuvant a trend towards a decrease in serum complement levels was evident at the terminal stages of the experiments. Despite the inhibitory effects on some immunological parameters, splenectomy in monkeys prior to thyroid isoimmunization did not interfere with the initiation and progression of pathological processes in the thyroid. Indeed in all splenectomized animals immunized with thyroid plus complete adjuvant, fibrotic thyroid lesions (4+) with virtual obliteration of thyroid follicles were evident, in some as early as 120 days after primary immunization; in contrast, non-fibrotic and less severe lesions were noted in the intact animal despite being repeatedly subjected to similar immunization procedures over a period of 340 days. Immunization in the absence of complete adjuvant did not induce thyroid lesions in the presence or absence of spleen. In all animals with severe thyroid lesions, thyroid function decreased as revealed by T_4 and ^{131}I-T_3 resin uptake measurements. Paper electrophoresis of serum specimens from a monkey subjected to thyroid isoimmunization in complete adjuvant (and after equilibration with ^{125}I-T_4) showed a pronounced retention of ^{125}I-T_4 radioactivity at the gamma globulin region indicating formation of antibodies to thyroxine. On the other hand, in sera with a low thyroid antibody titre as in splenectomized

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monkeys or in those animals immunized within complete adjuvant, T_4-binding antibodies were not evident. It is concluded that removal of the spleen prior to thyroid isoimmunization in monkeys, rather than inhibiting the severity of the disease may even aid and abet immunopathogenic events destructive to the thyroid.

INTRODUCTION

Rose & Witebsky (1956) first demonstrated thyroiditis in rabbits with actively induced thyroid immunity. Since that original observation, numerous investigators have employed immunological means to induce thyroiditis in a variety of subprimates to uncover intimate details that are of relevance to the pathogenesis of lymphocytic thyroiditis in man (Glynn & Holborow, 1965; Anderson, Buchanan & Goudie, 1967; Rose & Witebsky, 1968, 1971). Although such studies have contributed useful knowledge, immune thyroiditis of subprimates differs from that of the human in certain significant aspects, e.g. in man immunity develops to several components of the thyroid tissue unlike in animals. Such observations, therefore, led to a further search for an animal model which would more closely resemble the immune thyroid disease in the human. Rose and his collaborators succeeded in inducing thyroiditis in monkeys which did resemble the human disease better than the thyroiditis of subprimates (Rose *et al.*, 1965; see Rose & Kite, 1969 for exhaustive references).

In this and other laboratories, antibody binding of thyroxine has been demonstrated in the sera of several subprimates with induced (Pogoriler, van Maanen & Sellers, 1971; for other references see Premachandra, 1970) or spontaneous thyroid immunity (Nilsson, Rose & Witebsky, 1971). On the other hand, in human lymphocytic thyroiditis thyroid autoantibodies do not normally bind thyroxine save in isolated instances (Premachandra & Blumenthal, 1967). The induction of monkey thyroiditis more closely resembling that in the human was, therefore, of much interest to study thyroid hormone status and transport in thyroid immune monkey sera. Also, in order to assess the relation of the titre of humoral antibodies to the integrity of the thyroid and serum thyroxine-binding antibody activity, some monkeys were splenectomized prior to thyroid isoimmunization.

While removal of the spleen prior to thyroid isoimmunization markedly suppressed some humoral antibodies, asplenic immune animals nevertheless showed severe thyroid lesions and a decrease in thyroid function. Thyroxine-binding antibodies were demonstrated in sera with a high titre of thyroid iso- and autoantibodies.

METHODS AND MATERIALS

Seven Rhesus monkeys (*Macaca mulatta*), four females (Thor-1, Thor-2, Thor-3, Thor-4) and three males (Thor-5, Thor-6, Thor-7), ranging in age from 2–3 yr were employed in the present studies. Four animals were splenectomized 1 month before immunization.

Induction of thyroiditis

The preparation of monkey thyroid crude extracts for immunization was carried out by procedures described by Kite, Argue & Rose (1966). Briefly, normal monkey thyroid glands were minced in cold phosphate buffered saline (pH 7·2) and then subjected to homogenization for 2 min in a teflon blender. After sedimentation of the large particles, the

supernatant was extracted and mixed with equal parts of incomplete or complete adjuvant (Difco Laboratories, Detroit, Michigan). After emulsification, animals were injected (0·5 ml) intradermally between the digits of each hand and foot. The details of immunization and reimmunization schedule are also recorded in Tables 1 and 2.

Two non-splenectomized monkeys and one animal in the splenectomized series were immunized with monkey thyroid extract (isoimmunization) emulsified in incomplete adjuvant. For isoimmunization of all the remaining animals in both series complete adjuvant was used. All animals were bled prior to and at various intervals after immunization for serological and biochemical investigations. The observation period varied in individual monkeys, the maximum being 442 days.

For periodic grading of thyroiditis, biopsy specimens from monkeys were obtained under pentobarbital anaesthesia. A 2-mm fragment was fixed in buffered formalin and H & E stained sections were prepared in the manner described by Rose *et al.* (1966). In some instances 0·5 or 1-μ thick sections were made from epon blocks and stained with toluidine blue (Themann *et al.*, 1968). Autopsy was performed in some animals after the terminal observation and thyroid and other tissues were processed in the same manner.

Serological tests

Agglutinating, complement fixing and cytotoxic thyroid antibodies in thyroid immune sera were measured by techniques published previously (Kite *et al.*, 1966). Complement levels were determined by the procedure of Kabat & Mayer (1961).

Various control procedures as well as specificity tests used with all these determinations were similar to those described in detail by Kite *et al.* (1966).

Parameters of thyroidal function

^{131}I-T_3 resin uptake and serum thyroxine

For determining ^{131}I-T_3 resin uptake in the serum of monkeys, commercially available kits were employed (Abbott Laboratories, Chicago. Ill.). Serum total thyroxine (T_4) measurements were made by column chromatography at Bio-Science Laboratories (Van Nuys, Calif.).

Thyroid hormone transport

Serum thyroid hormone-protein interaction was studied by electrophoretic techniques (pH 8·6, barbital or glycine acetate buffer) as described previously (Premachandra & Blumenthal, 1967; Premachandra, Perlstein & Blumenthal, 1970). Briefly, 0·5 ml serum was equilibrated with 0·02 μg ^{125}I-T_4 (4 μg/100 ml) at 37°C for 30 min. 8 μl of the serum-radiothyroxine mixture was applied on paper and electrophoresis was carried out at 70 V for 18 hr. After the electrophoretic run the strips were dried, scanned for radioactivity and then stained. ^{125}I-T_4 (specific activity 79·5 μCi/μg) was purchased from Abbott Laboratories, Chicago, Ill., and was used soon after its receipt to eliminate possible decomposition of the material.

Protein measurements

The biuret technique was used to measure total serum protein. Fractionation of serum into various protein components was achieved by paper electrophoresis as described above. After the electrophoretic run the dried strips were stained with bromophenol blue and the density of the various protein bands was measured by the densicord (Photovolt Corp.).

TABLE 1. Immuno-pathological studies in non-splenectomized monkeys

Thyroid isoimmunization with Freund's complete adjuvant					Thyroid isoimmunization with Freund's incomplete adjuvant							
Monkey Thor-1					Monkey Thor-2				Monkey Thor-6			
Days	TRC titre§	Compl. levels (C'_{50})	Compl. Fix.§	Thyroid histology	Days	TRC titre§	Compl. levels (C'_{50})	Thyroid histology	Days	TRC titre§	Compl. levels (C'_{50})	Thyroid histology
0*	0	—	0		0*†	0	18		0*	0	24	
12	0	40	64		8†	0	55		7‡	0	52	
28*	8	34	0		22*†	0	34		21*†	0	18	
39	256	44	16		43†	0	27		42‡	0	19	
46	1024	55	8		75*†	4	29		74*†	8	28	
59*	—	—	—		88*†	8	38		87*‡	16	28	
60	32	58	4		95†	32	32	Normal[b]	94†	256	—	
63	—	—	4	+++[b]	121*†	256	32		120*†	512	19	
105*	512	34	0		140†	256	17		140†	256	12	
112	2048	36	0		144*	—	—		144*	—	—	
138*	2048	23	0		148†	32	—	Normal[a] (cytotoxic antibody: 1000§)	157	—	—	Normal[b]
158	2048	32	0						176*†	64	—	
173*	—	—	—						235*	—	—	
175	128	—	0						239†	8	—	Normal[b]
194	1024	30	0						263†	32	18	
249	1024	—	0	++ to +++[b]					288*†	32	27	
281	250,000	23	0						322*	—	—	
306*	512,000	30	0						371*†	16	—	
340*	—	—	—	+++[b]					417*	8	—	
351	250,000	—	4						442*†	32	—	Normal[b]

* Indicates day of immunization or reimmunization.
† Indicates negative complement fixation response on this day.
‡ Indicates anticomplementary.
a Autopsy.
b Biopsy.
++ Pronounced focal infiltration.
+++ Moderate diffused infiltration.
§ Reciprocal of serum dilution.

RESULTS

Immuno-pathologic studies in non-splenectomized monkeys

Agglutinating antibody titre

In monkey Thor-1 subjected to thyroid isoimmunization in Freund's complete adjuvant, a very low antibody titre (8*) was first detectable 4 weeks after initial antigen exposure. With further reimmunizations antibody titre fluctuated between low and moderate levels, and at 306 days an extremely high antibody titre (512,000) was evident (Table 1).

In the two monkeys Thor-2 and Thor-6 with repeated thyroid immunizations in incomplete adjuvant, a maximum antibody titre of only 512 was noted in the latter at 120 days, after which the titre began to decline. An aspect worth noting in Thor-6 was the failure of repeated reimmunizations (twelve in all) to sustain even the low antibody levels noted at 120 days.

The disparity in antibody production between complete adjuvant—thyroid and incomplete—adjuvant thyroid immunized monkeys noted above, is consistent with the established role of complete adjuvant as an inducer of intense lymphocytic proliferation.

Serum complement levels

Considerable variation in serum complement levels in individual monkeys at various intervals after immunization was noted, the range of variation being essentially similar whether complete or incomplete adjuvant was used for immunization (Table 1). However, if the mean complement levels in the 1st period (up to and including the first value after three immunizations) are compared against the mean of the remaining complement measurements after the 1st period (arbitrarily referred to as the 2nd period) significant reduction is apparent; in Thor-1, forty-six in the 1st period as against twenty-nine in the 2nd period (37%decrease). Similarly, the mean complement level in the 1st period for Thor-2 and Thor-6 was thirty-three and twenty-eight respectively, as against twenty-seven and nineteen in the 2nd period (or a mean decrease of 25·1%). While these results may not be conclusive in themselves, they do show a trend in the decrease of serum complement levels in all animals after several weeks of immunization and reimmunization, presumably indicative of immunological reactions in the tissue. It is interesting to note the more marked decrease in serum complement level in Thor-1 in comparison to Thor-2 and Thor-6, and only in the former severe thyroid lesions were noted at biopsy.

Serum complement fixing antibodies

A moderate complement-fixing antibody titre (64) was noted only once in Thor-1 at 12 days after the 1st immunization, and repeated immunizations had little effect in enhancing or even sustaining this relatively low antibody titre. There was a poor correlation of these antibodies with pathological changes in the thyroid as shown by their absence at a time when severe thyroid lesions were noted, as for example at 249 days.

In Thor-2 and Thor-6 subjected to thyroid immunization in incomplete adjuvant (rather than complete adjuvant) complement-fixing antibodies were not detected at any time during the study despite as many as ten or more reimmunizations.

Thyroid histopathology and cytotoxic antibodies

Although thyroid biopsies were not carried out in monkeys prior to immunization

* Throughout the text humoral antibody titres are expressed in terms of the highest dilution of antisera which showed a positive immune reaction.

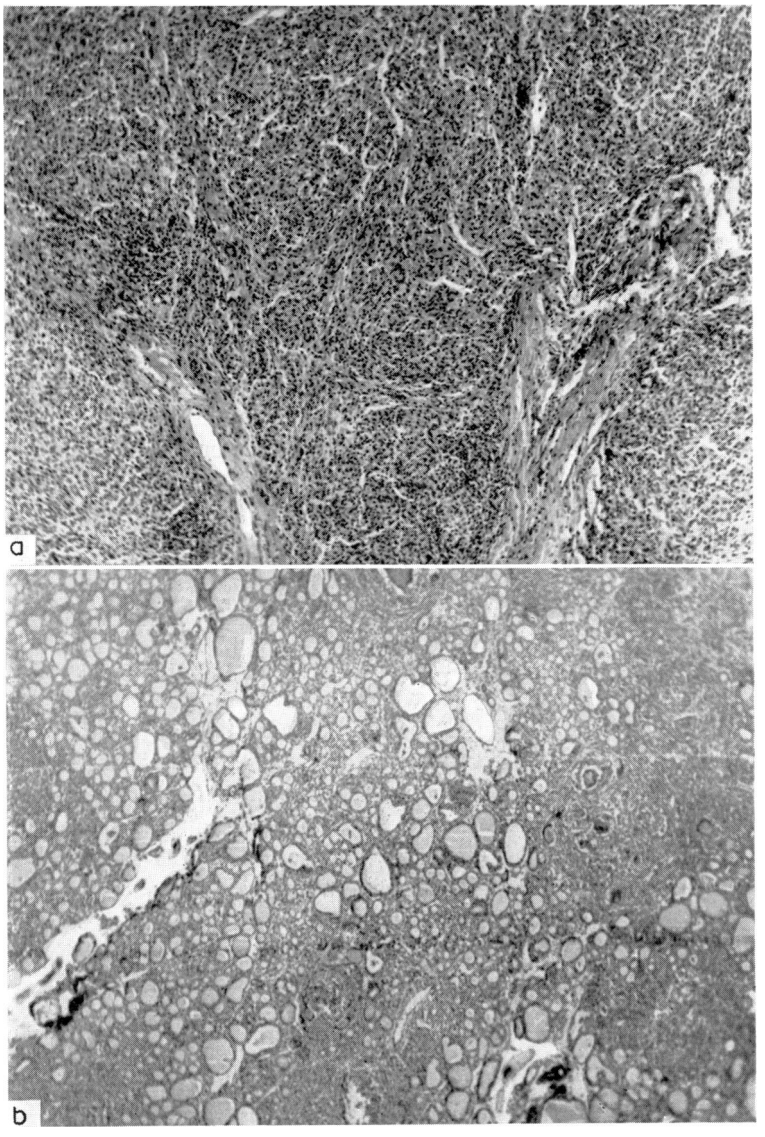

FIG. 1. Thyroid sections from splenectomized monkey Thor-5 (a) and non-splenectomized monkey Thor-1 (b). In Thor-5, a 4+ lesion was noted at autopsy 120 days after repeated thyroid isoimmunization in complete adjuvant. Most of the thyroid tissue was replaced by a dense mononuclear infiltration, and indeed no thyroid follicles are seen in the section shown in (a). Fibrotic bands appeared in some areas. (H & E stain × 36.)

In monkey Thor-1 subjected to repeated thyroid isoimmunization in Freund's complete adjuvant a 2+ to 3+ lesion was seen in a biopsy specimen 63 days after antigen exposure. Fairly uniform dense inflammatory infiltration may be seen. Note also the absence of fibrotic changes. Although thyroid follicular architecture was damaged by mononuclear cells, some follicles still remained intact. (H & E stain × 36.) Even at the final biopsy examination 340 days after several immunizations and reimmunizations, some follicles, though irregular, were still left, and morphological lesions as seen in Thor-5 (a) were not revealed.

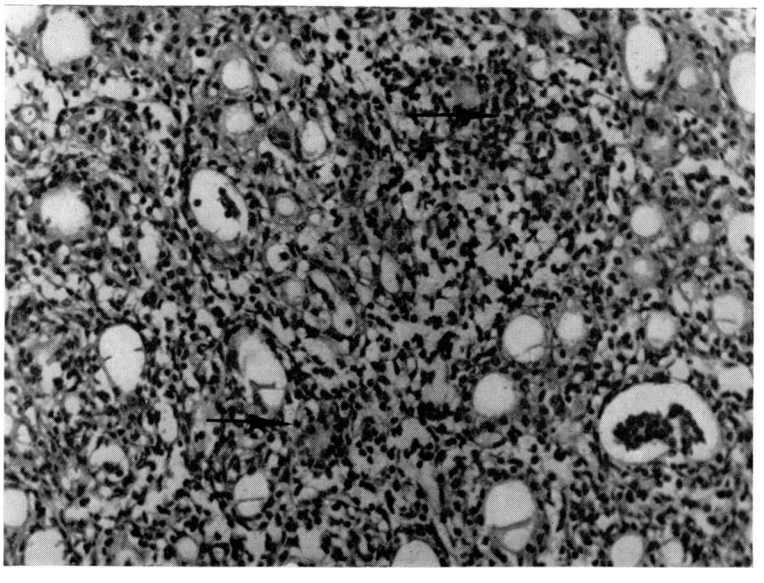

FIG. 2. H & E stained thyroid biopsy section from the monkey Thor-1, 249 days after repeated thyroid isoimmunization in Freund's complete adjuvant. (× 54.) Lesion 2+ to 3+. Severe infiltration may be noted which in some areas completely obscures the follicles. In other areas, the vast accumulation of leucocytes appeared to suggest formation of small lymph follicle-like nodules (arrows). Vacuolization of the colloid in several follicles and macrophagic infiltration may also be noted.

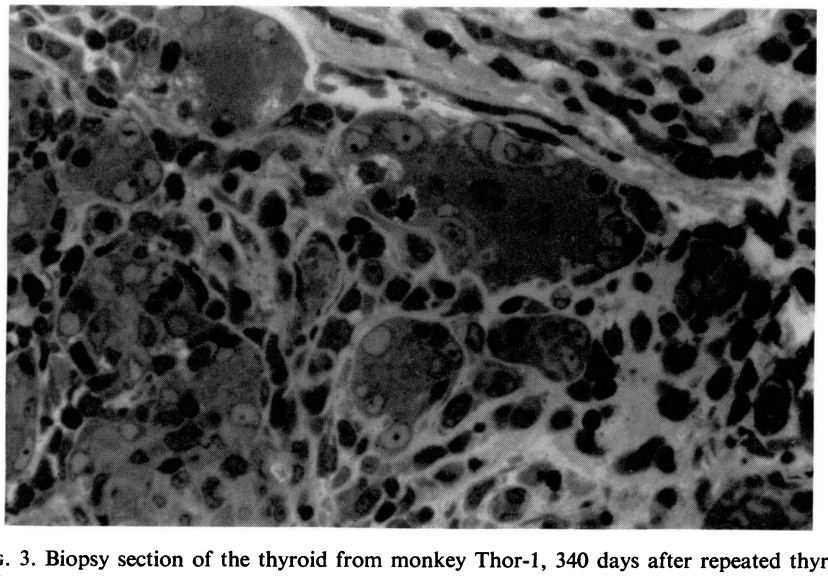

FIG. 3. Biopsy section of the thyroid from monkey Thor-1, 340 days after repeated thyroid isoimmunization (1-μ thick section. Epon embedding, toluidine stained). Follicles are of irregular shape, and vacuolization in some of them may also be noted. Many infiltrative mononuclear cells (lymphocytes and plasma cells) are also seen in the lumen and surrounding follicles. (× 360.)

TABLE 2. Immuno-pathological studies in splenectomized monkeys

Thyroid isoimmunization with complete adjuvant												
Monkey Thor-7				Monkey Thor-4				Monkey Thor-5				
Days	TRC titre§	Compl. levels (C'_{50})	Thyroid Histology	Days	TRC titre§	Compl. levels (C'_{50})	Thyroid Histology	Days	TRC titre§	Compl. levels (C'_{50})	Thyroid Histology	Cytotox.§
0*†	0	46		0*†	0	60		0*†	0	—		10
7†	0	41		7†	0	45		29*†	8	—		100
33*†	0	24		33*†	0	44		41†	8	58		100
53†	0	17		53†	0	23		60*†	32	58		100
68*	—	—		68*	—	—		74†	32	35		0
70†	0	—		70†	0	—	+[b]	103*†	16	—	++[b]	0
89†	4	—		89†	32	—		120†	32	32	++++[a]	0
148*	—	—		148*	—	—						
152†	0	—	++[b]	176†	32	33						
176†	2	25		201*†	4	44						
201*†	2	31		235*	—	—						
235*	—	—		253†	32	—	+++[b]					
284*†	16	—		258†	32	—	++++[a]					
292†	128	—	++++[a]									

Table 2. (*continued*)

Thyroid isoimmunization with Freund's incomplete adjuvant

Monkey Thor-3					
Days	TRC titre§	Compl. levels (C'_{50})	Compl. Fix.§	Thyroid histology	Cytotox.§
0*	0	—	0		0
29*	4	—	0		0
41	4	22	0		100
60*	4	40	0		0
74*	4	37	0		0
103	2	—	0	Normal[b]	0
123	0	—	0		0
133*	0	—	—		—
165*	0	37	0		0
200	0	35	0	Normal[a]	0

* Indicates day of immunization or reimmunization.
† Indicates negative complement fixation response on this day.
[a] Autopsy.
[b] Biopsy.
\+ Small areas of infiltration and interstitial reaction.
\+ + Pronounced focal infiltration.
\+ + + Moderate diffused infiltration.
\+ + + + Severe lymphocytic infiltration and fibrosis.
§ Reciprocal of serum dilution.

(exception, see Fig. 5), examination of thyroid sections from several normal monkeys as also those immunized with incomplete adjuvant, did not reveal inflammatory infiltrates or other interstitial changes. On the other hand, in monkey Thor-1, 63 days after primary thyroid immunization in complete adjuvant (total of three immunizations) the biopsy examination showed severe changes in the architecture of the thyroid (Fig. 1b). There was a fairly uniform dense infiltration of inflammatory cells. Despite the assault on the integrity of the parenchyma by invading mononuclear cells, some thyroid follicles still remained intact and no fibrotic changes were noted. Histological examination of the thyroid biopsy specimen at 249 days after initial immunization (followed by reimmunizations) showed focal as well as diffused areas of leucocytic and macrophagic infiltration which obscured thyroid follicles in some areas (Fig. 2). The pronounced lymphocytic infiltration in some areas formed small lymph follicle-like nodules. Vacuolization of the colloid was noted in several follicles. The final biopsy examination at 340 days showed irregular follicles with varying degrees of vacuolization (Fig. 3). As noted in the examination of earlier biopsy specimens, there was extensive infiltration of lymphocytes and plasma cells in the thyroid parenchyma.

In contrast, in Thor-2 and Thor-6 which were subjected to isoimmunization with incomplete adjuvant (i.e. lacking in acid-fast bacilli) biopsy and autopsy of the thyroid revealed normal architecture. These observations along with the normal histology of the thyroid noted in monkey Thor-3 (Table 2) would argue against the possibility that the thyroid lesions in Thor-1 (and in other monkeys to be described subsequently) were induced as a result of surgical embarrassment effected by frequent thyroid biopsies rather than due to autoimmune reactions. Despite the normal histological appearance of the gland in monkey Thor-2, its serum contained a very high titre of cytotoxic thyroid antibodies—a paradoxical finding. Some of the possible reasons for this apparent discrepancy are amplified later in the discussion.

Immunopathological studies in splenectomized monkeys

Agglutinating antibody titre

In all splenectomized monkeys whether immunized with the thyroid in complete or incomplete adjuvant, only very low levels of TRC antibodies were detected despite repeated antigenic stimulation (maximum TRC titre, 128).

These results, in comparison to those noted with non-splenectomized monkeys reviewed previously, show that removal of the spleen inhibits formation of circulating thyroid antibodies. The effect of splenectomy on humoral antibody formation is rendered more dramatic, however, when the comparison is made between splenectomized and non-splenectomized animals immunized with complete adjuvant (cf. titre of 512,000 in Thor-1 against 128 in Thor-7, Tables 1 and 2).

Serum complement levels

Variation in serum complement levels in splenectomized monkeys was essentially similar to that noted in non-splenectomized animals. As with the latter, the initial complement levels (first two observations) in monkeys Thor-7, Thor-4 and Thor-5 soon after or a few weeks after immunization were higher than those noted at the terminal period of observation. In all these animals immunological thyroid lesions were revealed by biopsy or autopsy. In Thor-3 which was immunized with incomplete adjuvant, the mean of the

terminal complement levels (i.e. at 165 and 200 days, Table 2) was higher than the mean of the initial levels (from 41 to 74 days); no thyroid lesions were revealed in this animal at autopsy.

Although both in splenectomized and non-splenectomized monkeys some semblance of a consistent trend in the direction of change in serum complement levels in relation to the immunological disease of the thyroid seems apparent, no firm conclusions are possible based on changes in serum complement alone for various reasons.

Complement-fixing antibodies

Removal of the spleen, apparently, totally inhibited the formation of complement-fixing antibodies in all monkeys whether or not they were subjected to thyroid isoimmunization in complete or incomplete adjuvant.

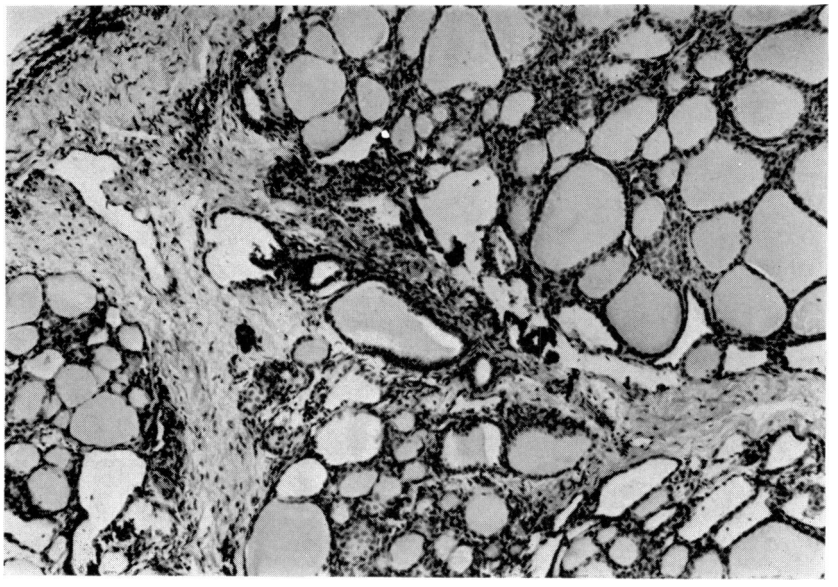

FIG. 4. Thyroid biopsy section from splenectomized monkey Thor-4, 70 days after repeated thyroid isoimmunization in Freund's complete adjuvant. Lesion 1+. The bulk of the tissue seems well preserved. Several distinct foci of interstitial reactions accompanied by a decrease of the colloid in some follicles can be noted. (H & E stain × 54.)

Thyroid histopathology and cytotoxic antibodies

Thyroid biopsy specimens of all monkeys subjected to thyroid immunization in complete adjuvant showed moderate to severe lesions. In Thor-7, a 2+ reaction involving focal mononuclear infiltration of the thyroid was noted after three reimmunizations, at 152 days. Several distinct foci of interstitial reaction in the process of replacing thyroid follicles were noted. The absence of spleen in no way appeared to inhibit the initiation or the progression of the thyroid lesion as exemplified by morphological alterations in Thor-4 as early as 70 days after initial immunization (followed by booster immunizations on days 33 and 68, Table 2, Fig. 4). The lesion involved discrete areas of moderate to extensive infiltration of

parenchyma followed by a variable decrease of the colloid. After five reimmunizations severe lesions (3+) were noted at 253 days, and 5 days later fibrotic bands were evident (4+lesion, Table 2). A similar 4+ thyroid lesion was also observed in Thor-5 after three booster immunizations and as early as 120 days (Table 2, Fig. 1a). Practically, the entire parenchyma was replaced by a dense mononuclear infiltration with virtually no visible thyroid follicles. Fibrosis was apparent in some areas.

In summary, in all splenectomized monkeys immunized with thyroid in complete adjuvant severe 4+ lesions (with fibrotic changes and virtual obliteration of thyroid follicles) were seen, in contrast to the less severe lesions (3+) noted in nonsplenectomized monkey Thor-1 despite its being subjected to a more intense antigenic stimulation in complete adjuvant.

The virtual destruction of the thyroid in Thor-5 was not accompanied by cytotoxic antibodies in serum. Rather, the moderate cytotoxic antibody titre noted from the 29th to

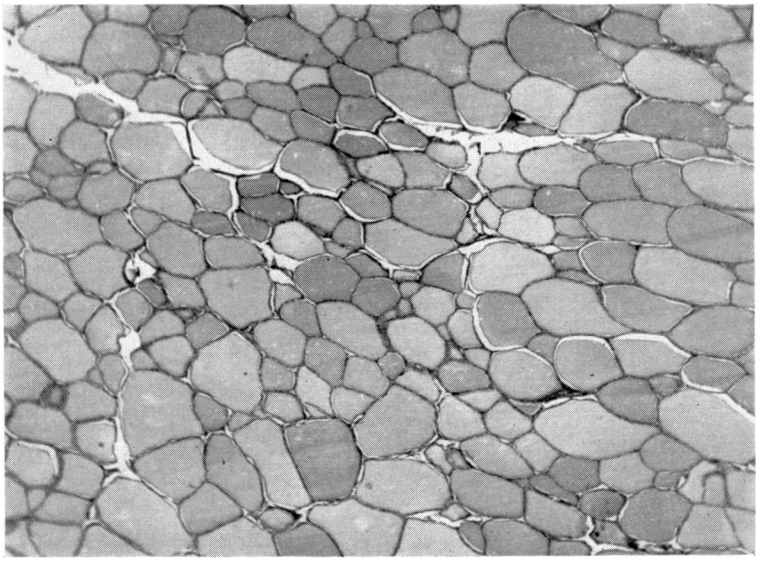

FIG. 5. Biopsy section of the thyroid from monkey Thor-3 prior to isoimmunization. Follicles are of normal size, contain colloid and are lined by flattened epithelium. Note the absence of interstitial reaction. (H & E stain × 54.)

60th day was reduced to undetectable levels at the terminal observation (Table 2). Cytotoxic tests were always carried out with a known thyrocytotoxic antiserum as a control to be certain of the susceptibility of thyroid cells used in these studies.

In contrast to these observations, no lesions were noted either in biopsy or the autopsy thyroid specimens of Thor-3 subjected to thyroid isoimmunization without the use of complete adjuvant. The preimmunization thyroid histology (Fig. 5) was unaltered. Nevertheless, in one instance, i.e. 41 days after initial immunization, a moderate cytotoxic antibody titre (100) was evident (Table 2).

Thyroxine transport and thyroidal function

The *in vitro* parameters of thyroid activity were determined in pre- and post-immunization

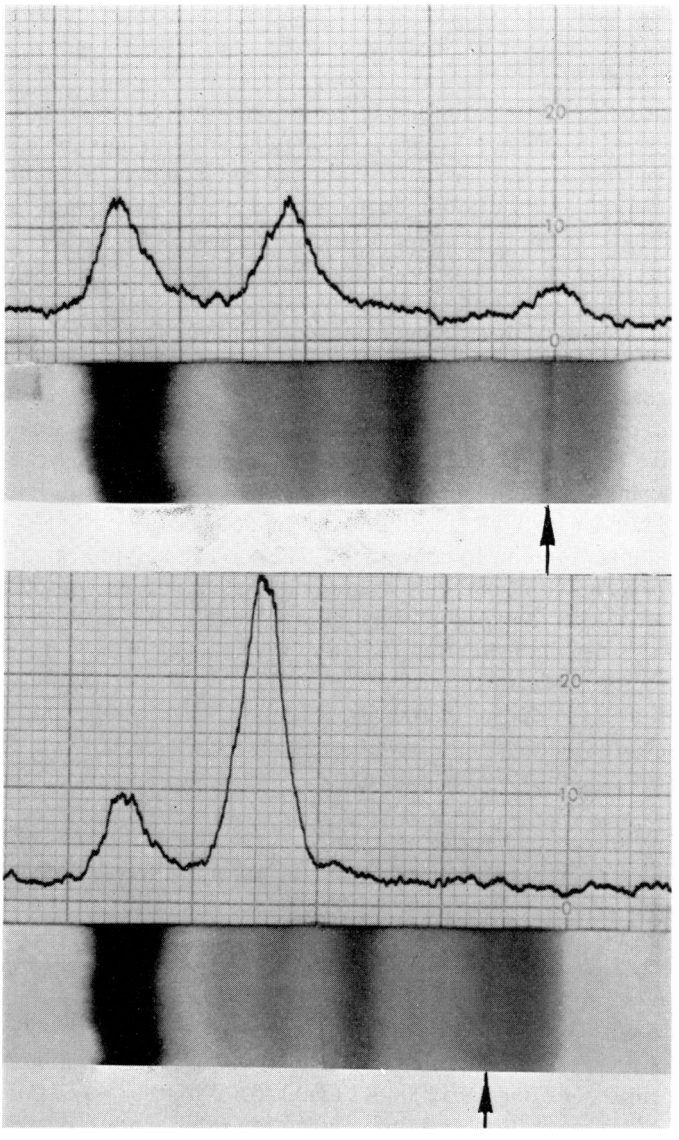

FIG. 6. Paper electrophoretograms showing distribution of radiothyroxine (^{125}I-T_4) in the serum of a non-splenectomized (top) and a splenectomized (bottom) monkey subjected to thyroid isoimmunization in Freund's complete adjuvant. Electrophoresis was carried out in barbital buffer at pH 8·6. The paper strips are aligned with respective radioactivity scans. The arrows indicate origin. The distribution of radiothyroxine in the preimmunization serum sample was similar to that shown in the splenectomized animal. Bulk of the added ^{125}I-T_4 was bound by TBG (first radioactivity peak to the left of the origin) and the remainder by albumin. Approximately 4 weeks after immunization, ^{125}I-T_4 distribution in the serum of a non-splenectomized monkey showed an additional radioactivity peak at the gamma globulin region (at and to the right of the arrow) which indicated formation of thyroxine-binding antibodies.

sera of some animals and are recorded in Table 3. In all these tests each animal served as its own control.

Thyroid hormone transport

The paper electrophoretic distribution of radiothyroxine in the serum of non-splenectomized monkey Thor-1 subjected to thyroid isoimmunization in complete adjuvant differed substantially from that of splenectomized animals subjected to a similar immunization procedure. When preimmunization monkey sera were enriched with labelled thyroxine *in vitro* (4 μg/100 ml) 75–90% of radioactivity was bound by TBG and the remainder by albumin (barbital buffer pH 8·6). ^{125}I-T_4 distribution pattern in the serum of monkeys is similar to that seen in normal human serum as reported previously (Premachandra, 1970). However, in the serum of non-splenectomized monkey Thor-1,4 weeks after thyroid immunization in complete adjuvant, there was a small but significant electrophoretic retention of thyroxine radioactivity at the gamma globulin region in addition to the conventional binding of ^{125}I-T_4 at TBG and albumin (Fig. 6). Gamma globulin bound radioactivity (indicative of antibody binding of thyroxine) represented 16·4% of ^{125}I-T_4 added *in vitro*. With increase in antibody titre, ^{125}I-T_4 binding at gamma globulin increased and in the serum of Thor-1 with a TRC antibody titre of 250,000, 57% of ^{125}I-T_4 added *in vitro* was bound by gamma globulin. In comparison to Thor-1 it is interesting that in sera with a low antibody titre as noted in monkeys either immunized without complete adjuvant or in those subjected to splenectomy, no T_4-binding antibodies were detected as determined by radioelectrophoresis despite formation of severe thyroid lesions in some of these animals. These results, therefore, indicate the lack of correlation between T_4-binding thyroid antibody activity in serum and the degree of the thyroid lesion, i.e. the antigenic determinants involved in eliciting antibodies which cause tissue damage are probably different from those involved in circulating antibody formation.

^{131}I-T_3 resin uptake

The interpretation of this *in vitro* parameter in relation to thyroid function is well known. The resin uptake of ^{131}I-T_3 is related to the degree of saturation of T_4-binding sites in serum by endogenous thyroid hormones. Thus, in hyperthyroidism the saturation of T_4-binding proteins is high and hence little ^{131}I-T_3 is abstracted by the serum resulting, therefore, in a high resin uptake and the converse situation would prevail in hypothyroidism. The ^{131}I-T_3 resin uptake in non-immune animals varied between 25–49%. In comparison to these observations, values rather suggestive of hyperthyroidism were noted in preimmunization samples of Thor-5 and Thor-3 (Table 3). With the loss of thyroid parenchyma in consequence of immunization and reimmunization (and hence a decrease in circulating T_4), ^{131}I-T_3 uptake by TBG sites increased greatly and hence there was a drop in ^{131}I-T_3 resin uptake. This decline was precipitous in Thor-5 (71%) and Thor-3 (56%, despite lack of apparent morphological alterations), and less dramatic in Thor-4 and Thor-1.

Thyroid hormone transport investigations reviewed previously showed that thyroxine was bound by gamma globulin in Thor-1. In other words, additional T_4-binding sites were provided by thyroid antibody molecules. These observations then suggest that the decrease in ^{131}I-T_3 resin uptake in Thor-1 may have resulted not only from the loss of thyroid tissue but probably also due to the peripheral binding of thyroxine by the receptors on antibody molecules. If this should be the case one would have expected a much lower ^{131}I-T_3 uptake

TABLE 3. Thyroxine transport and thyroidal function in monkey immune thyroiditis

Treatment	γ-Globulin (antibody) binding of T_4 (% of total)		^{131}I-T_3 resin uptake‡ (%)		Serum total T_4† (μg/100 ml)		Thyroid lesions*
	Pre-immun.	Post-immun.*	Pre-immun.	Post-immun.*	Pre-immun.	Post-immun.*	
Splenectomized monkeys							
Thyroid isoimmunization with:							
Freund's complete adjuvant							
Thor-4	0	0	35·5	28·1	4·8	3·0	++++
Thor-5	0	0	64·0	18·3	4·1	2·9	++++
Freund's incomplete adjuvant							
Thor-3	0	0	64·2	28·1	—	—	neg.
Non-splenectomized monkeys							
Thyroid isoimmunization with:							
Freund's complete adjuvant							
Thor-1	0	57	36·9	28·8	2·3	0·3	+++

* Terminal observations.
† Normal variation 1·7–4·5 μg/100 ml.
‡ Normal variation 25–49%.
+++ Moderate diffused infiltration.
++++ Severe lymphocytic infiltration accompanied by fibrosis.

in Thor-1 than the data indicates. While a rational explanation of this apparent discrepancy is not clear at this time, it would be well to recognize the limitations inherent in these *in vitro* techniques, especially when used in sera of animals suffering from immunological diseases; in this connection, the disparity in reduction in post-immunization ^{131}I-T_3 uptake between Thor-4 and Thor-5 and with similar thyroid lesions, is also worth noting. These latter observations once again point out the hazard of a direct extrapolation of morphologic data to dynamic function as measured by various *in vitro* parameters.

Serum total thyroxine

The normal range in total thyroxine (T_4) in the sera of non-immunized animals was 1·7–4·5 μg/100 ml. Decreases in circulating hormone levels over preimmunization values were noted in all animals which developed severe thyroid lesions. The decrease in T_4 in Thor-1 was from 2·3 to 0·3 μg/100 ml or a reduction of 87% after immunization. Similarly, the decreases in T_4 over preimmunization values in Thor-4 and Thor-5 were 38% and 29% respectively (Table 3). Obviously, the decreases in circulating thyroxine levels after immunization resulted from the loss of substantial amounts of thyroid parenchyma. Whether or not this loss of tissue can be entirely attributed to autoimmune reactions in the gland may be questioned in view of the serial thyroid biopsies carried out in these animals. No doubt one would expect compensatory replacement of the tissue via pituitary—thyroid axis but knowledge of such responses in animals with actively induced immunological thyroid disease is not available. It is also possible that biochemical abnormalities in consequence of immunologically induced thyroid disease may have been partly responsible for lowering of T_4 levels in these animals.

DISCUSSION

Although the effects of splenectomy on humoral antibody formation to a variety of antigenic stimuli have been studied by various workers (*vide infra*), the present thyroid histopathological studies in asplenic monkeys constitute the first detailed observations reflecting the role of spleen in cellular immune response to thyroid isoimmunization. The results and conclusions from our studies may be of added significance in that the qualitative nature of thyroid immune response, anatomy and relative spleen size in monkeys are comparable to that of man. The investigations clearly indicate that splenectomy in monkeys (2–3 yr) does not interfere with the animal's capacity to manifest a delayed-type hypersensitivity to thyroid antigens. The severity of the lesion in splenectomized monkey Thor-5 after four immunizations, at 120 days (Table 2, Fig. 1a), was not matched by commensurate pathological changes in the thyroid of non-splenectomized monkey Thor-1 having twice the number of immunizations, as noted at various times during the course of the disease up to 340 days (Table 1, Figs 1b, 2 and 3); these observations raise the possibility that some absorption of tissue damaging cellular and/or humoral immune factors may occur in the spleen. As noted previously (Andrada, Rose & Kite, 1968) the morphologic changes occurring in the thyroid glands of immunized animals were specific and similar lesions were not noted in other tissues examined post-mortem [some of the similarities and the pathologic significance of monkey thyroid lesions to the human disease have been elaborated previously (Rose *et al.*, 1966; Andrada *et al.*, 1968)]. All these results would suggest that removal of the spleen, one of the secondary lymphoid organs, does not interfere with the expression of

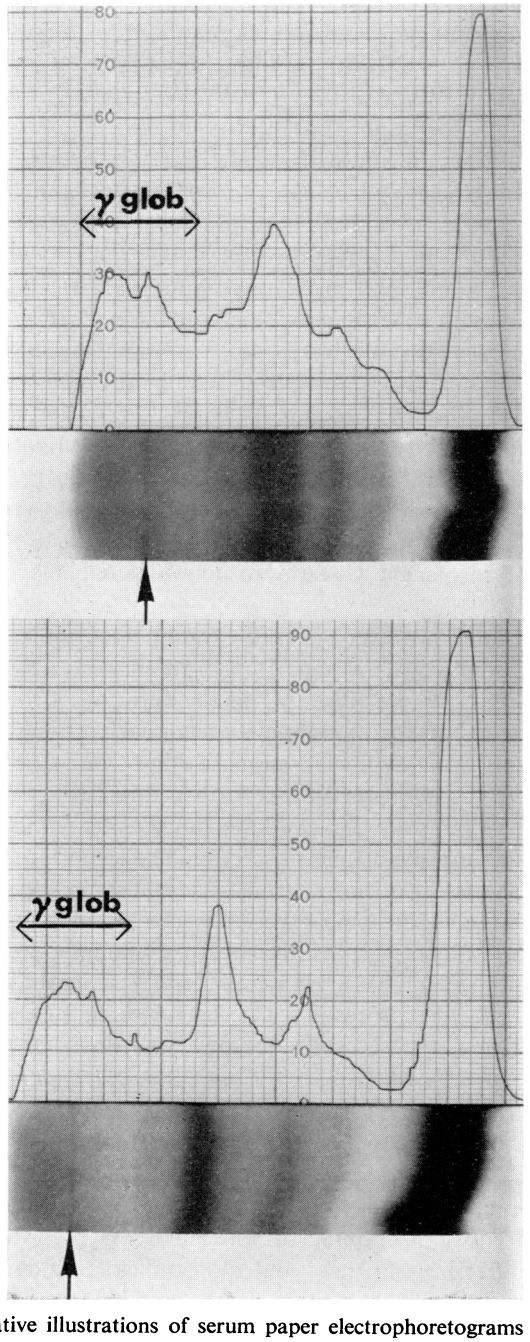

FIG. 7. Representative illustrations of serum paper electrophoretograms of splenectomized (top) and non-splenectomized (bottom) monkeys subjected to thyroid isoimmunization. Electrophoresis was carried out in barbital buffer at pH 8·6. The arrow represents origin. Densitometric scans showing the concentration of various protein bands are aligned against each stained paper strip. The lack of a decrease in gamma globulin concentration in the splenectomized monkey is readily apparent.

cellular immunity. Analogous observations in transplantation immunology such as failure of splenectomy to prevent allograft rejection (Krohn, 1953; Rapaport & Dausset, 1968), the delay in allograft rejection effected by intralymphatic ^{198}Au irradiation but not by splenectomy alone (Wheeler, White & Calne, 1965), etc. are well known.

Dissemination of the immune response to soluble antigens may be effected not only by the rapid distribution of the immunogen in the body, but also by interlymphoid organ communicating sensitized lymphocytes which carry information on antibody synthesis (Nilsson, 1971). Evidently, in asplenic thyroid immune animals immunocompetent lymphocytes were sufficiently inhabited in the peripheral lymph nodes and other extra-splenic lymphoid tissues and, on antigenic stimulation proliferated and elicited cellular immune responses (probably in cooperation with other macrophagic cells) even better than that noted in the non-splenectomized animal also subjected to similar immunization procedures. The ability of immunocompetent lymphocytes of nonsplenic lymphoid organs to undergo proliferation on contact with the antigen (Baney, Vazquez & Dixon, 1962) and to compensate for splenic contribution in humoral antibody formation (Rosenquist & Wolfe, 1962) is known. Consistent with these observations is the lack of significant differences in serum gamma globulin concentration between splenectomized and non-splenectomized thyroid immune monkeys (Fig. 7). These results corroborate the findings of Azar, Naujoks & Williams (1963) and Saslaw *et al.* (1959), and also suggest a store of immunocompetent lymphocytes in extra-splenic lymphoid tissues.

Removal of the spleen did, however, markedly inhibit TRC agglutinating antibody titre, the effect being more dramatic when comparisons were made between normal and asplenic monkeys immunized with complete adjuvant. As the duration of the experiments exceeded over a year, and in view of repeated immunizations in each animal, the lack of formation of high antibody levels in splenectomized animals cannot be attributed to post-operative stress. The credibility of this interpretation is further strengthened by the observations of low humoral antibody response in all monkeys immunized without complete adjuvant, whether or not they were subjected to splenectomy. The inhibitory effects of splenectomy on humoral antibody formation to intradermally administered thyroid antigens as noted in this communication are, with some exceptions (Bednarik & Cajthamlova, 1971), in general agreement with results of various investigators who have studied immune response to intravenously administered soluble or particulate antigens in asplenic animals (Luckhardt & Becht, 1911; Motohashi, 1922; Rowley, 1950; Taliaferro & Taliaferro, 1950; Rosenquist & Wolfe, 1962; Campbell & La Via, 1967; De Carvalho, Borel & Miescher, 1967) and in man (Rowley, 1950a).* On the other hand, our results are at apparent variance from those of Bednarik & Cajthamlova (1970) who reported that spleen plays no role in antibody formation to non-intravenously administered antigens; in their studies, intact and splenectomized rabbits were subjected to depot immunization (administration of antigen in complete adjuvant by subcutaneous and intramuscular routes) against human IgG and other antigens, and no differences in immune response in these two groups were noted. The interpretation of the differences in these and our studies is rendered difficult in view of

* In contrast, microbic antigens regardless of the route of their administration (subcutaneous, aerosol or intravenous), appear to elicit strong and effective immune responses in the absence of spleen in animals (Grimm, Theiel & Tischer, 1965; Fitch & Winebright, 1962; Saslaw & Carlisle, 1964; 1964a) and in man (Saslaw *et al.*, 1959). The efficacy of subcutaneous route of immunization in splenectomized rats has also been shown recently by an *in vitro* assay utilizing leucocytic pneumococcal phagocytosis (Biggar *et al.*, 1972).

differences in species, antigen and techniques of immunization and quantification of immune response.

While the long delayed and very low agglutinating antibody formation followed by a virtual failure to mount an accelerated antibody response to sequential sensitizations in splenectomized monkeys are also consistent with the conclusions of Campbell & La Via (1967) despite differences in species, antigens and their mode of administration, our results differ from those of Pierce (1967). In his investigations increased primary (20 days after immunization) and secondary immune responses (8–12 days after sensitization) over that of intact animals were noted in splenectomized rats sensitized with bovine gamma globulin alone or with endotoxin (*Salmonella typhosa*). In our studies primary antibody response in asplenic monkeys was measured at 7 or 29 days after the first immunizing dose, whereas the secondary immune response was determined at 12 or 20 days (Table 2) after sensitization. It is possible that in view of these differences in the time of measurement of immune response, apart from a number of differences in procedural details, the secondary immune responses noted in splenectomized animals in Pierce's investigations were not apparent in our studies.

Removal of the spleen inhibited formation of complement-fixing antibodies. These results in monkeys are at apparent variance with those of De Carvalho *et al.* (1967) who reported formation of complement-fixing antibodies in splenectomized rats 35 days after intravenous immunization of the antigen (heat aggregated gamma globulin). The variation in results noted in the two studies may, apart from differences in species, immune systems and other factors, also be due to events associated with increasing complexities in immune response attendant on the increasing order in phylogeny.

Although removal of the spleen depressed or prevented agglutinating and complement-fixing antibody formation, cytotoxic immune response was not affected by splenectomy. The relation of thyrocytotoxic antibodies to the pathology of the thyroid has been a puzzling one. Paradoxical observations in either direction were noted in the present studies, i.e. thyroid morphology was normal in the presence of a very high cytotoxic antibody titre and conversely, animals showing a severe thyroid lesion showed no cytotoxic antibody. In regard to the former, the *in vitro* method of determining cytotoxicity involved exposure of thyroid cells to trypsin. The enzymatic treatment may have altered membranal integrity and facilitated cytotoxicity. Mere exposure of thyroid cells, without some membranal change, to the action of cytotoxic antibodies is of no avail, as also noted by others (Forbes *et al.*, 1962; Irvine, 1962). [The implication of these observations *in vivo* is the prerequisite of a surface injury to the follicular cell by cytopathologic reactions (e.g. emperipolesis) or other immunopathogenic events before cytotoxic antibody can gain access to its intracellular antigen.] Another consideration would be whether or not cytotoxic antibodies in sufficient concentration gained access to the thyroid. Furthermore, tests for cytotoxic antibodies were made with thyroid cells from young monkeys (2 yr). These 'young' cells have been shown to be more sensitive than the thyroid cells of older (3–5 yr) monkeys (Kite *et al.*, 1966).

The converse situation, i.e. a full-blown lesion and no cytotoxic antibodies, is reminiscent of the results of Kite *et al.* (1965) who reported that not all human thyroid cells are destroyed by a known human cytotoxic thyroiditis serum. By analogy, therefore, it may be postulated that not all monkey thyroid cells are destroyed by a known cytotoxic monkey thyroid immune serum. It is also possible that antibodies may have lost their cytotoxic potency by their previous confrontation with cytotoxic antigens. Consistent with this suggestion are the

investigations of Nakamura & Weigle (1969) who induced thyroiditis by passive transfer of thyroid immune serum from thyroidectomized donor rabbits; the removal of the target gland presumably facilitated the survival of cytotoxic antibodies in circulation. The rapid loss of susceptibility of thyroid cells to cytotoxic antibodies has also been stressed by Kite *et al.* (1966) and may also be a factor in the apparent discrepancies noted. Finally, very simply, the animal may not have produced cytotoxic antibody at all.

In the sera of three monkeys (Thor-2, Thor-3 and Thor-5), tests for complement-fixing antibodies were negative whereas these same sera showed moderate or a high titre of cytotoxic antibodies. These results, therefore, suggest the non-identity of complement fixing and cytotoxic antibodies, at least in some monkeys, a finding in agreement with the results of Kite *et al.* (1966). Irvine (1962) suggested that this discrepancy may be due to the higher sensitivity provided by the cytotoxic tests, and in the human various groups of investigators have suggested that complement fixing and cytotoxic properties are functions of the same antibody (Forbes *et al.*, 1962; Irvine, 1962; Halberg, 1964). However, both Kite *et al.* (1965) and Goudie & McCallum (1962) have described complement-fixing antibodies in sera containing no thyrocytotoxic antibodies. These observations taken together with the differences in sedimentation characteristics of cytotoxic and complement-fixing antibodies plus the insensitivity of the former to mercaptoethanol (in contrast to marked sensitivity of complement-fixing antibodies, Kite *et al.*, 1966) would seem to suggest that thyrocytotoxic and gross complement-fixing activities may not be synonymous, at least in some thyroid immune sera.

Although a trend in the decrease of serum complement levels at terminal periods of observation appeared to bear some relationship to the severity of the lesions of the thyroid as also noted previously in the human (Andrada *et al.*, 1965), and some reports suggest an apparent correlation of decrease of serum complement levels with the cytotoxic thyroid antibody titre (Rose & Kite, 1969), the interpretation of serum complement levels, nevertheless, is beset with various difficulties and complications. In this connection, among other things, the various glomerular lesions in thyroid immunized animals (Premachandra, 1967) are worthy of note; if such severe lesions were present in immunized monkeys they might have seriously compromised the integrity of the glomerular capillaries thereby clouding the interpretation of the decrease in serum complement levels to antigen–antibody reaction in the tissue. Since the primary site of antigen–antibody reaction was in the thyroid, changes in complement levels in this gland would have more accurately reflected an immunological tissue lesion, although it is unlikely that much complement exists in the thyroid. It is for some of these reasons also that a relationship would not be expected between serum complement levels and complement-fixing antibodies in thyroid immunized animals, as also noted in these investigations.

The marked inhibitory effects of splenectomy on agglutinating and complement-fixing thyroid antibodies in the presence of a full-blown thyroid lesion (Table 2), again raises the much debated question of the relative importance of humoral and cellular immune responses in inducing pathological changes in the thyroid. Present observations complement the investigations of others who have induced thyroiditis by adoptive (passive) transfer of sensitized lymph node cells (Felix-Davies & Waksman, 1961; McMaster & Lerner, 1967; Nakamura & Weigle, 1967; Twarog & Rose, 1970), as well as studies of Shimazaki (1968) which indicate a direct action of sensitized lymphocytes on thyroid cells, and thus provide another argument in favour of supremacy of cellular immune reactions in initiating thyroid

lesions (Flax, 1971). Nevertheless, none of these experiments unequivocally prove that humoral factors may not also play a role in cellular immune reactions. In splenectomized animals for instance, there is always the possibility that humoral factors in undetectable amounts may have caused thyroid damage; or thyroid cell membrane may have been damaged by immunogenic or other pathologic events facilitating cytotoxic action of humoral factors (cytotoxic antibody, lymphotoxins, etc.). Furthermore, various other investigations illustrate the importance of humoral antibodies in evoking thyroid tissue damage, e.g. adoptive transfer of immune thyroid disease has been accomplished by serum from sensitized donors (Nakamura & Weigle, 1969; Vladutiu & Rose, 1971). Bursectomy in obese strain chickens has been reported to interfere with development of spontaneous thyroiditis (Cole, Kite & Witebsky, 1968) implicating a major role for circulating antibodies in the disease. Finally, convincing thyrocytotoxic effects *in vitro* have been obtained only with anti-thyroid serum rather than sensitized leucocytes. Because of the difficulty in specifically incriminating either humoral or cellular immunity, there is a growing sentiment that both types of immune responses may be important in tissue damage (Brown, Glynn & Holborow, 1967; Roitt & Doniach, 1967) although the relative importance of these effector mechanisms may vary at different stages of the disease as well as in species (Ringertz *et al.*, 1971). The role of humoral and cellular factors in inducing thyroid damage has been reviewed recently (Rose & Witebsky, 1968a; Rose & Kite, 1969; Rose *et al.*, 1971).

Although none of the asplenic thyroid immunized monkeys showed serum antibody binding of thyroxine (in contrast to the immune-T_4 interaction in the intact animal immunized with complete adjuvant), this would not suggest that spleen is the exclusive site of formation of T_4-binding antibodies. Rather, the more pertinent point is the lack of T_4-binding antibodies in all animals (whether or not splenectomized) immunized with incomplete adjuvant. In these animals, the titre of thyroid antibodies was very low and the absence of T_4-binding antibodies in such sera would indicate that poor immune responses are not attended by antibody formation to all of the antigenic determinants in the thyroid. Such observations appear to differ from those noted in other primates (baboons) subjected to thyroid heteroimmunization where a small but significant T_4-immune interaction was noted in the presence of a low titre of agglutinating thyroid antibodies (Premachandra, 1970). These differences may be due to a more intense scrutiny and processing of the antigen afforded in heteroimmunization, thereby facilitating formation of antibodies to more internally located small antigenic fragments. An equally plausible suggestion for the failure to detect T_4-binding antibodies in sera with a low antibody titre is that the techniques used were not sensitive enough to detect the low order of immune-T_4 interaction. While this is possible, it is rather unlikely in view of the great sensitivity of radioelectrophoretic techniques. Finally, a 4+ thyroid lesion in the absence of humoral antibodies is a manifestation of severe cellular immunity and the transmigration of cell-bound antibodies to the site of immunological reaction. Whether or not such immunocompetent cells (plasma cells) would contain antibodies to T_4 determinants is not known.

In passing, it may be of relevance to note that cognizance of the stage of thyroid lesion and antibody binding of thyroxine may resolve apparent discrepancies in the analysis of thyroid function in thyroid immunized animals (Premachandra, 1971). Invariably, thyroxine-binding antibodies in thyroid immunized animals contribute to spurious increases in plasma PBI or total thyroxine (Beall, Chopra & Solomon, 1971; Beall & Solomon, 1968; McKenzie & Haibach, 1967; Premachandra, Berns & Blumenthal, 1965). The knowledge of immuno-

globulin interaction of thyroxine in the sera of thyroid immune animals has also been helpful in differentiating (experimentally induced) true LATS response from the artifactual increase in organic iodine (Florsheim, Williams & Schonbaum, 1970; Solomon, Beall and Chopra, 1970).

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