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Localization of Thyroid and Spinal Cord Autoantibodies by Fluorescent Antibody Technic.* (23855)

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Recently production of autoantibodies against constituent(s) of the thyroid gland, presumably thyroglobulin, has been reported from this laboratory(1-6). Highly specific autoantibodies have been observed in several experimental animal species, such as rabbits, dogs, and guinea pigs, as well as in human beings suffering from certain types of chronic thyroiditis. Concurrently, characteristic changes in thyroid gland were frequently found. Demonstration of paralysis by immunization of experimental animals with brain substance has created considerable interest(7-9). In many instances brain-specific circulating antibodies were found in serum of these animals by both complement fixation and precipitation tests. However, antibody formation against brain substance including that of antibody-producing animal has been noted in absence of observable paralysis. On the other hand, brain lesions have been observed repeatedly in absence of detectable circulating antibodies. Therefore, relationship of circulating antibodies to what is frequently referred to as *allergic encephalomyelitis* is still a moot question. The fluorescent antibody technic of Coons and Kaplan(10) seemed to offer an interesting approach to the study of the possible direct combination of circulating antibodies and tissue sections. This technic might shed some new light on specific localization of tissue-specific antibodies. Simultaneous studies were therefore undertaken of these 2 autoantibody systems with Coons technic since they seem to constitute perfect controls for each other.

Methods. Sections of quick frozen tissues were cut 6 to 8μ thick with standard microtome in a cryostat according to method of Coons and Kaplan. The air-dried sections were fixed in formalin or in formalin followed by acetone. Formalin fixation was carried out by immersion in 10% formalin in pH 7.2 buffered saline solution at room temperature. Thyroid sections could be fixed in formalin for 2 to 10 minutes. Formalin fixation followed by 10 min. acetone fixation also gave satisfactory results. However, 2-minute formalin fixation was used in most experiments. Brain and spinal cord sections, on the other hand, were fixed in formalin for only 2 minutes because they lost their capacity to stain by fluorescent antibody technic when fixed in formalin for longer than 2 minutes or when 2 minute formalin fixation was followed by acetone fixation. Rabbit antisera to homologous brain or spinal cord antigens were prepared by intracutaneous injection of 20% aqueous suspensions in equal volume of Freund adjuvant. Aqueous suspensions were prepared by homogenizing fresh brain or spinal cord in Servall omnimixer for 30 seconds at maximum speed and centrifuging 20 seconds. As source of thyroid autoantibodies anti-rabbit thyroid rabbit sera were used from the same series as in previous studies(3,4,5). Complement fixation tests for thyroid autoantibodies and spinal cord autoantibodies as well as tanned cell hemagglutination tests with experimental thyroiditis sera were carried out as described previously(1,11). Evaluations of histologic

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changes of the thyroid gland were made as described previously(4). Observations for paralysis of rabbits immunized with homologous spinal cord or brain extracts were made daily. Fluorescent antibody staining was carried out by a two-step reaction; *i.e.*, sections of rabbit brain or spinal cord and rabbit thyroid were treated 30 to 60 min. with undiluted, active rabbit sera or serum dilutions, washed with buffered saline for 10 min., and stained with fluorescein isocyanate conjugated goat antiserum to rabbit immune globulin for 15 to 30 min., washed again and mounted. Goat antiserum was obtained from goats immunized with washed immune precipitates formed by reaction of goat serum and rabbit antisera to goat globulin. Goat antiserum was fractionated by precipitation with 50% saturated ammonium sulfate and the globulin fraction conjugated with fluorescein isocyanate† according to the method of Coons and Kaplan(10). The conjugated goat antisera to rabbit immune globulin were dialyzed against pH 7.2 buffered saline and adsorbed once or twice with acetone powders of rabbit or human livers as indicated by observed staining reactions. Some experiments were also carried out on rabbit and human thyroids by one-step staining reactions, *i.e.* rabbit and human thyroiditis sera were fractionated by 50% ammonium sulfate precipitation and the resulting globulin fraction was directly conjugated with fluorescein isocyanate. These conjugates were dialyzed and adsorbed with an ion exchange resin.‡ A few 2-step staining reactions were also carried out with human thyroid sections using human thyroiditis sera and rabbit antisera to human gamma globulin conjugated with fluorescein isocyanate. Observations of preparations were carried out with standard Reichert microscope using Reichert ultra-high intensity light source. Ultraviolet light was supplied by Osram 200 mercury vapor light. A UV pass filter transmitting light at 365 mμ was inserted between light source and specimen. The use of this filter made it possible to visualize autofluorescence of the fixed rabbit tissue as a blue color which contrasted clearly with the green color of fluorescein-labeled conjugates. A dark field condenser was used throughout. Fluorescent antibody staining reactions were regarded as negative when the intensity of reaction was no higher than that obtained with normal rabbit sera constantly used as controls. The colored photomicrographs of this publication were kindly made by Dr. Jacinto Vazquez of the University of Pittsburgh School of Medicine. The methods employed in making these photomicrographs have been described by Vazquez and Dixon(12).

† We are indebted to Dr. Coons for synthesis of the fluorescein isocyanate. The fluorescein amine from which the isocyanate was prepared was donated by Sterling Winthrop Corp.

‡ Dowex 2-X, 200 mesh used according to method of Coons (unpublished).

TABLE I. Summary of Serologic Reactions of Rabbit Spinal Cord Autoantibodies with Homologous Antigens and Observations on Degree of Paralysis.

	Serologic reactions		
Rabbit serum*	Fluorescent antibody reactions	Complement fixation titers†	Degree of paralysis
59	Positive	320	++++
13	Questionable‡	80	none
14	Negative	40	++++
64	"	40	none

* All sera from rabbits immunized with rabbit spinal cord suspension with adjuvants.

† Greatest antiserum dilution giving complete inhibition of hemolysis using a 1:5 dilution of 20% rabbit brain.

‡ Usually weak positive reaction.

Results. Results of fluorescent antibody staining reactions of rabbit spinal cord sections obtained with 4 sera from rabbits immunized with rabbit spinal cord suspensions are summarized in Table I together with complement fixation titers of these sera and observations on presence or absence of paralysis. Definite positive staining reactions were consistently obtained with one of the 4 antisera. The results with a second serum were not quite as intense or consistent and were therefore interpreted as questionable. Staining reactions with 2 other sera were interpreted as negative. Interestingly enough, there appears to be a correlation between staining reaction and complement fixation titer. However, there appeared to be no correlation between serologic tests and presence of paralysis. Strongly positive staining reactions and complement

TABLE II. Summary of *In Vitro* Reactions of Rabbit Thyroid Autoantibodies with Rabbit Thyroid Antigens and Observed Degree of Thyroiditis.

Sera	*In vitro* reactions: Fluorescent antibody reaction	*In vitro* reactions: Tanned cell hemagglutination titers	*In vitro* reactions: Complement fixation test	Degree of thyroiditis
515	Positive*	2560	Pos.	T.X.‡
558	" *	1280	"	Considerable
521	" †	320	Neg.	T.X.
555	Negative	81	"	Questionable
561	"	0	"	Neg.

* Positive staining reactions with serum dilutions up to 1:9.
† *Idem* 1:3.
‡ T.X. = Thyroidectomized. Rabbits' own thyroids were used as source of antigen for immunization.

fixation tests were obtained with 2 rabbit antisera to beef brain, using sections of rabbit brain or rabbit spinal cord as test material. Neither of the rabbits showed signs of paralysis.

Results of staining reactions with rabbit thyroid autoantisera are summarized in Table II. Of 5 rabbit sera described 3 with highest thyroid autoantibody titers regularly gave positive staining. Therefore, 2 systems of experimentally producible autoantibody tissue reactions are available, namely, thyroid autoantibody system and brain autoantibody system, which may serve as useful specificity controls for each other. Results of comparative experiments carried out to test specificity of systems are summarized in Table III. Specificity of autoantibody reactions for their homologous tissue is evident. Sera containing thyroid autoantibodies stain only the thyroid and not the spinal cord. In contrast, serum containing spinal cord autoantibodies stain only sections of spinal cord and not the thyroid.

Typical examples of staining reactions observed are shown in Fig. 1 to 4. Details of localization of staining reaction of rabbit spinal cord sections will have to be subjected to further scrutiny. However, as far as white matter is concerned, the axons appear as oval or round, dark, unstained areas surrounded or partially surrounded by brightly stained myelin sheaths. Such structures are seen in Fig. 1 in several places. No greenish staining was observed in spinal cord sections treated with rabbit thyroid autoantiserum or with normal rabbit sera.

Rabbit thyroid sections treated with selected thyroid autoantisera were intensely stained. Most of this staining is localized in the colloid. (Fig. 4). A thin rim of epithelium is also usually brightly stained. This is readily discernible in sections from which the colloid has been removed accidentally or intentionally by mechanical manipulation in the cryostat. Stroma of thyroid appears essentially unstained. Rabbit thyroid sections treated with antisera against rabbit spinal cord or with normal rabbit sera do not exhibit positive staining reactions.

The staining reactions referred to so far were all obtained by the 2-step method. Essentially, this method consists of adding rabbit sera containing autoantibody to sections of their homologous tissue as a first step, followed by addition of fluorescein-labeled goat antiserum containing antibody against rabbit immune globulin. Further experiments were carried out to obtain fluorescent staining by a one-step procedure. To this end a rabbit serum containing thyroid autoantibodies was fractionated and conjugated, as described pre-

TABLE III. Test of Specificity of Fluorescent Antibody Staining of Rabbit Thyroid and Rabbit Spinal Cord with Autoantibodies.

Auto-antiserum		Tissue sections: Rabbit thyroid	Tissue sections: Rabbit spinal cord
Thyroid	515	Positive	Negative*
	521	"	"
	558	"	"
Spinal cord	59	Negative	Positive

* Negative reactions are defined as those which are indistinguishable from the ones obtained with normal rabbit serum.

viously, and added to sections of normal rabbit thyroid glands. (This conjugate was adsorbed with ion exchange resin only to remove unconjugated fluorescein derivatives.) Definite characteristic staining was observed, comparable to that observed using the 2-step reaction.

Staining reactions similar to those in experimental rabbit thyroiditis have been obtained with sections of normal human thyroids using sera from patients with chronic thyroiditis and a conjugated rabbit antiserum to human gamma globulin for second step of the reaction.

Discussion. The organ-specific nature of brain antigen was first observed by Brandt, Guth and Müller(13) using alcoholic brain extracts as antigens. Witebsky and Steinfeld (14,15) studied appearance of brain-specific antibodies in rabbits following injection of aqueous brain suspensions of foreign species. The autoantibody nature of these brain hetero-antibodies was demonstrated by the reaction of rabbit antisera with rabbit brain suspensions and extracts. Rivers and Schwentker(7) demonstrated appearance of paralysis in monkeys following prolonged immunization with aqueous emulsions and alcohol ether extracts of rabbit brain. Appearance of paralysis following immunization with brain extracts in Freund's adjuvant was demonstrated by Kabat and co-workers(8) and Morgan(9).

The relation of circulating antibodies to appearance of characteristic lesions in the central nervous system is still an unanswered question. The problem at present is whether circulating antibodies combine with brain matter *in vivo* or are just incidental findings. A similar problem presents itself in the case of chronic thyroiditis both experimentally produced in animals(1-5) as well as in patients suffering from this disease(3-6).

The Coons technic(10) allowing recognition of antibodies fixed to tissue cells seems to offer interesting possibilities for the study of localization and fixation of antibodies in certain tissues and even within certain parts of specific cells involved. White(16,17) reported staining reactions obtained with human thyroid tissue and serum of patients with thyroid antibodies. At the same time one of us (EW)(16) reported staining reactions obtained with rabbit thyroid sections and sera of rabbits immunized with rabbit thyroid suspensions. Hiramoto, Engle and Pressman (18) report staining reactions obtained with human thyroid tissue and serum of a patient with thyroid antibodies, using rhodamine conjugation method developed by them.

To determine whether positive results obtained by our fluorescent antibody technic, are really specific in nature and not due to non-specific adsorption of antibody globulins by themselves, comparison of the 2 autoantibody systems with completely different specificities seemed to constitute excellent mutual controls. The results here reported demonstrate the feasibility of application of Coons technic to specific combination of autoantibodies and their corresponding tissue constituents.

Summary. (1) Sections of normal spinal cords of rabbits were treated with rabbit antisera produced by immunization of rabbits with rabbit spinal cord suspensions; the treated sections were stained with fluorescein conjugated goat antiserum to rabbit immune globulin. Selected areas stained suggest the myelin sheath as at least one of the antibody combining sites, though further histological studies are needed. No staining was obtained with normal sera or with rabbit sera containing thyroid autoantibodies. (2) In the same manner sections of thyroid glands of normal rabbits were exposed to the action of rabbit sera containing rabbit thyroid autoantibodies and stained with fluorescein conjugated goat antiserum to rabbit immune globulin. Positive staining reactions were obtained in the colloid extending into adjoining epithelial layer. Interstitial tissue remained basically unstained. No positive staining reactions were obtained using normal rabbit serum or rabbit serum containing brain autoantibodies. (3) With thyroid autoantibodies, the positive staining reactions and antibody titers seemed to run roughly parallel. However, rabbit autoantisera failed to give staining reactions when diluted above ninefold. On the basis of limited experience with spinal cord autoantisera, it is not possible to make a state-

ment regarding appearance of positive staining reactions and presence of demonstrable circulating antibodies, though the data obtained are suggestive of a correlation.

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