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Thyroid-Specific Autoantibodies Studied by Passive Cutaneous Anaphylaxis of Guinea Pig.* (24363)

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Thyroid-specific autoantibodies in animals have been demonstrated serologically by tanned cell hemagglutination, complement fixation, and precipitation technics(1,2,3.) Subsequently, circulating thyroid-specific autoantibodies in sera of patients suffering from chronic thyroiditis have been demonstrated by tanned cell hemagglutination(3,4) and precipitation(4,5,6) technics. Additional discussions have appeared(7,8,9,10). The present study reports detection and specificity of thyroid-specific autoantibodies by the method of passive cutaneous anaphylaxis of the guinea pig (PCA).

Materials and methods. Antisera. The following sera were studied by the PCA technic: rabbit anti-rabbit thyroid, guinea pig anti-guinea pig thyroid, and sera from patients with chronic thyroiditis. All these sera contained circulating antibody of high titer against the homologous antigen, as demonstrated by tanned cell hemagglutination. The procedure for animal immunization has been described(1). *Antigens.* Crude saline extracts were prepared by procedures previously described(11,12). The extracted organs (and extract concentrations in mg N/ml) included rabbit thyroid (2.5),§ guinea pig thyroid (0.92),§ human thyroid (7.8),§ beef thyroid (12.1), hog thyroid (10.4), human pancreas (3.6), human kidney (4.8), and beef muscle (1.7) Partially purified by human thyroid material (5.0 mg N/ml) was prepared by precipitation with sodium sulfate in a procedure similar to that of Heidelberger and Palmer(13), but omitting the preliminary acidification. This material was used only as

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§ Based on determination of protein by light absorption and an arbitrary conversion factor for nitrogen of 6.0.

challenging antigen. A more highly purified preparation of human thyroglobulin (300 μg N/ml) was made by a potassium phosphate procedure(14).‖ This purified thyroglobulin was used only for the absorption experiments. Other antigens and test materials included packed washed human erythrocytes (equal amounts from groups A, B, and O, all Rh positive, 3,5-diiodo-L-tyrosine (Eastman Kodak Co.), DL-triiodothyronine,¶ and DL-thyroxine.¶ *PCA technic.* Dilutions of serum (0.1 ml) were injected intradermally at various sites in shaved dorsal skin of albino guinea pigs, weighing 200-250 g. Several injections were made in the same animal. After a latent period of 3 to 5 hours, the animal was challenged intravenously with 0.5 ml of the antigen dilution mixed with 0.5 ml of a 1% Evans blue solution. Antigen excess does not inhibit response in the PCA technic, therefore, in all experiments antigen was used in excess (by 10-fold) of the minimum amount necessary to provoke a reaction. Reactions became visible as blue spots within a few minutes after challenge. Animals were sacrificed 30 minutes later and size of colored area on internal surface of guinea pig skin measured. Technical details of the PCA technic have been described(15). *Neutralization procedure.* Neutralization experiments were performed with human thyroiditis sera. The technics for these PCA experiments varied somewhat, depending on the antigen used. Samples (1 ml) of an appropriate dilution of thyroiditis serum were mixed with 0.1 ml of the following dilutions of saline extracts: human kidney (1:10), human pancreas (1:10), beef thyroid (1:10), hog thyroid (1:10), beef muscle (1:10), and human thyroid (1:100). The quantity of human thyroglobulin was 30 μg N. The powders of 3,5-diiodo-L-tyrosine, DL-triiodothyronine, and DL-thyroxine were added in 1 mg amounts to 1 ml of the dilution of thyroiditis serum. When human erythrocytes were used for absorption, 0.2 ml of packed washed cells was added to each 1 ml volume of the serum. The organ extracts were mixed with sera immediately before intradermal injection, whereas dry powders were mixed with serum dilutions one hour before intradermal injection, and the erythrocytes mixed with serum dilutions 30 minutes before injection and removed by centrifugation immediately before injection.

Results. Four groups of experiments were used to demonstrate intensity and specificity of the reactions of various thyroid autoantibodies with homologous and heterologous thyroid antigens by PCA.

In the first experiment, guinea pigs were injected intradermally with various dilutions of the following sera; rabbit anti-rabbit thyroid, guinea pig anti-guinea pig thyroid, and human thyroiditis, along with corresponding normal sera. The guinea pigs were challenged 4 hours later with 0.5 ml of a 1% Evans blue solution mixed with 0.5 ml of one of the following extracts: rabbit thyroid, guinea pig thyroid, and human thyroid. Two animals were tested for each antiserum dilution. The results are summarized in Table I. Strong positive results were obtained only with homologous antigens. No cross reactions were obtained. Normal serum controls were negative. The titers by tanned cell hemagglutination are provided for comparison.

The second experiment demonstrates the titer of human thyroiditis sera by endpoint dilution with the PCA technic. Guinea pigs were injected intradermally with serial dilutions of thyroiditis sera. The animals were challenged 3 hours later with 0.5 ml of 1% Evans blue solution mixed with 0.5 ml of either crude human thyroid saline extract or human thyroid extract partially purified by sodium sulfate fractionation. Four animals were tested for each antiserum dilution. The results are summarized in Table II. Both sera gave skin reactions in dilution of 250. The 2 antigens produced identical results. An additional 3 sera showed a variable range of titers.

In a third experiment, the specificity of the reaction of the 5 thyroiditis sera with various antigens was examined. Four groups of 4 guinea pigs each were injected with human sera mentioned in Table II, in dilution 10

‖ We are indebted to the House Staff, Pathology Dept., The Johns Hopkins Hospital, for human thyroid glands.

¶ Our thanks are due to Hoffmann-LaRoche, Inc. for this material.

TABLE I. PCA Reactions of Thyroid-Specific Autoantibodies.

Intradermal inj.	Challenging antigen: Rabbit thyroid extract	Guinea pig thyroid extract	Human thyroid extract	Saline	Hemagglutination titer
Rabbit anti-rabbit thyroid #690 (undiluted)	38	0	0	0	1:20,000
Rabbit anti-rabbit thyroid #690 (1:100)	20	0	0	0	1:20,000
Rabbit anti-rabbit thyroid #689 (1:100)	12	0	0	0	1: 2,000
Guinea pig anti-guinea pig thyroid #89	0	25	0	0	1: 625
Human thyroiditis serum (Rog.) (undiluted)	0	0	25	0	1: 3,000
Normal rabbit serum (undiluted)	0	0	0	0	
Normal guinea pig serum (undiluted)	0	0	0	0	
Normal human serum (undiluted)	0	0	0	0	

Numbers given are avg diameter for 2 animals, expressed in mm, for reaction area.

times more concentrated than the threshold dilution; *i.e.*, the greatest dilution which regularly gave a response. These dilutions were, respectively, 1:25, 1:25, 1:2.5, 1:10, and 1:100. After a latent period of 3 to 4 hours each group of guinea pigs was challenged with 0.5 ml of 1% Evans blue solution and 0.5 ml of one of the following saline extracts: human kidney, human pancreas, beef thyroid, or hog thyroid. No positive reactions were visible. To confirm the validity of this negative response, 2 of the 4 animals in each group were sacrificed 1 hour after the challenge for closer scrutiny of the skin. The conclusions were unaltered. The other members, however, were additionally challenged with 0.5 ml of 1% Evans blue solution mixed with 0.5 ml of human thyroid extract purified by sodium sulfate fractionation. Positive reactions with the 5 human sera were obtained in all animals of this latter group, thereby demonstrating that human thyroid autoantibodies were available for homologous reaction only.

TABLE II. PCA Titration of Human Thyroiditis Sera* by Endpoint Dilution.

Dilution of serum used for intradermal inj.	Rog.	Cas.
1: 10	21	19
1:100	13	19
1:250	9	12
1:500	0	0

Challenging antigen: Human thyroid extract. Numbers given are avg diameter for 4 animals, expressed in mm, for reaction area.

* An additional 3 human sera collected in Baltimore gave titers of 25, 100, and 1000, respectively. We are indebted to Dr. Samuel Asper of the Endocrine Clinic, Johns Hopkins Hospital, for these sera.

The next experiment once more shows specificity of human thyroiditis sera, utilizing PCA and neutralization technics. The 5 human sera were neutralized *in vitro* as described above, again using serum dilutions which were 10 times more concentrated than the threshold dilution. Each serum was injected intradermally into 4 guinea pigs. The antigens used for neutralization were saline extracts, as described above, of human kidney, human pancreas, beef thyroid, hog thyroid, beef muscle, human thyroid, as well as purified human thyroglobulin, and powders of 3,5-diiodo-L-tyrosine, DL-triiodothyronine, and DL-thyroxine. An absorption experiment was performed with human erythrocytes. Three or 4 hours following intradermal serum injection, the animals were challenged with 0.5 ml of 1% Evans blue solution mixed with 0.5 ml of human thyroid extract. The results are presented in Table III. Only homologous human thyroid extract and human thyroglobulin inhibited the reaction. No inhibition occurred with the other antigens. Some reactions were smaller but this is within the limit of normal variability of the reaction(16).

Discussion. It has been shown that thyroid autoantibodies, whether of human, rab-

TABLE III. PCA Reactions of Neutralized Human Thyroiditis Sera.

Antigen used for neutralization	Serum neutralized				
	Rog. 1:25	Cas. 1:25	A-1 1:2.5	A-27 1:10	A-60 1:100
Untreated	20	19	19	24	27
Human kidney extract (1:10)	18	20	20	21	22
" pancreas " (1:10)	18	16	16	20	19
Beef thyroid extract (1:10)	18	20	20	22	25
Hog thyroid extract (1:10)	19	19	19	20	21
Beef muscle extract (1:10)	17	18	18	18	22
Human erythrocytes (AB)	18	18	18	21	24
3,5-Diiodo-L-tyrosine (1 mg)	19	20	16	18	23
DL-triiodothyronine (1 mg)	19	20	16	18	22
DL-thyroxine (1 mg)	20	19	18	18	24
Human thyroid extract (1:100)	0	0	0	0	0
" thyroglobulin (30 μg N)	0	0	0	0	0

Numbers given represent avg diameter for 4 animals, expressed in mm, for reaction area.

bit, or guinea pig origin, will give a positive PCA reaction when tested with corresponding homologous antigens. The highest dilution among the 5 human sera, which still gave a positive response was 1/1000. Specificity of the autoantibodies was examined in several ways. Only homologous thyroid preparation gave a positive reaction; there was no detectable cross reaction with thyroids of other species. The lack of such cross reaction may be due in part to lower sensitivity of this method, under these experimental conditions, compared with tanned cell hemagglutination, which does, in fact, reveal existence of autoantibody cross reaction among thyroids of several species, as does also the method of complement fixation(3,17). A higher antigen level might well have been succesful in revealing cross reactions by PCA, but the experiments were not designed to pursue this point intensively. On the other hand, the observed lack of cross reaction with extracts of other organs within the same species is fully substantiated by hemagglutination and complement fixation studies(4).

It should also be kept in mind that other factors may be involved in the observations on thyroid cross reactions. The precipitin studies of Stokinger and Heidelberger(18) showed cross reactions of 20% or more between thyroglobulins of various species and their antibodies. These antibodies, however, were heteroantibodies—an important point of difference from studies reported here. Furthermore, the precipitin reactions were done at low temperatures for long periods of time. It is well known that dissociation of cross reacting antibodies is much greater at higher temperatures, and one would not expect the same amount of cross reaction *in vivo* at 37°C as *in vitro* at 0°C. In fact, it has been observed in the PCA reaction of the rat(19) that cross reacting antibodies are considerably less efficient, on a weight basis, than homologous antibody in production of PCA reactions.

Special precautions must be followed in neutralization procedures. It was observed in preliminary experiments that if organ extracts were mixed with rabbit antibody to egg albumin and several hours permitted to pass before intradermal injection of this mixture, that PCA response to subsequent intravenous egg albumin was inhibited. On the other hand, if organ extract-antibody mixture was made a few minutes before intradermal injection, this non-specific and unexpected inhibition did not occur. For this reason organ extracts were always mixed with sera immediately before intradermal inoculation to avoid false negative responses, which would seem to indicate neutralization of the autoantibody. In spite of this short time interval, thyroid preparations were successful in eliminating subsequent responses. These observations serve to emphasize the almost instantaneous union between antibody and antigen, in contrast to non-specific interactions(20,21).

For a more precise quantitative study, not only will more animals be needed, but injection sites must be randomly distributed, and above all, latent periods must be identical. The purpose of the present report was, how-

ever, to establish the feasibility of the PCA test in study of autoantibodies.

It was seen by Snapper and Grunbaum that diiodotyrosine and thyroxine produced no inhibition in rabbit sera containing thyroid heteroantibodies(22). The fact that diiodotyrosine, triiodothyronine, and thyroxine do not neutralize the human thyroid autoantibody suggests that antibody is not simply directed against antigenic determinants with configurations similar to these substances. The further fact that human thyroglobulin completely neutralizes the antibody suggests that the antibody detected by these PCA experiments is directed against some other portion of the globulin structure.

Summary. 1. Passive cutaneous anaphylaxis of the guinea pig has been successfully used to study thyroid autoantibodies of rabbit, guinea pig, and man. 2. The thyroid autoantibody proved to be specific for the homologous antigen (thyroid extract and purified derivative) and did not give cross reactions with thyroid extracts of other species. 3. Five human sera with high titer of thyroid autoantibody were studied. The antibody was completely neutralized by human thyroid extract or human thyroglobulin, but was not significantly affected by any of a variety of other organ extracts or of iodinated amino acids, again indicating the specificity of this type of antibody.

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Agent in Ak Leukemic Tissues, Not Sedimented at 105,000 g, Causing Neoplastic and Non-neoplastic Lesions.*† (24364)

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Following earlier work suggesting that the leukemia agent of Gross(1) may be analogous to the transforming principle of microorganisms(2), an experiment was set up to identify

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