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GRAVES' IgG RECOGNIZES LINEAR EPITOPES IN THE HUMAN THYROTROPIN RECEPTOR*

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Twenty-nine peptides covering the full extracellular domain of the human thyrotropin receptor have been synthesized and tested for reactivity with Graves' patients' and normal sera in ELISA. Two peptides, amino acids 331-350 and the second extracellular loop of the transmembrane segment, bound IgG-s from 5 and 4 of 10 Graves' disease patients' sera, respectively. Neither of these two peptides showed enhanced binding to normal IgG. There were no apparent differences between the Graves' disease and normal group with respect to the other 27 peptides. We conclude that peptide 331-350 and the second extracellular loop carry important linear epitopes which may contribute to the disease process in selected Graves' patients.

Approximately 25 years ago, the human TSH receptor (hTSHR) was identified as the major autoantigen in Graves' disease (1). Since then, the spectrum of autoantibodies binding to the receptor in vivo has been intensively studied (2). However, the major epitopes which are critical in directing the immune response or which bind antibodies are not known. The hTSHR epitope(s) which interacts with the TSH receptor antibodies may either be conformational (non contiguous), as are the majority of protein antigens including thyroid antigens (3), or alternatively, polypeptide sequences on the surface of the molecule may form distinct linear (contiguous) epitopes. The coexistence of antibodies against contiguous and non-

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contiguous epitopes in the same individual is also a possibility. In the present study, we tested the sera of Graves' disease patients for IgG binding to a complete series of peptides systematically covering the entire extracellular portion of the hTSHR. We demonstrate that there are peptides which preferentially bind IgG-s from Graves' disease and not from control patients' sera.

Materials and methods

Sera were collected from 10 patients with Graves' disease and from 10 euthyroid control subjects. Each patient gave informed consent. The patients demonstrated typical clinical and laboratory signs of Graves' disease, including an elevated TSH binding inhibiting immunoglobulin value (TBII) at the time of blood sampling; TBII assays were performed by Nichols Institute of Endocrinology, San Juan Capistrano, CA.

Peptides were synthesized by solid phase methods on an automated 430A peptide synthesizer (Applied Biosystems, Inc., Foster City, CA) on p-methyl-benzhydralamine copolystyrene resin. Each peptide contained twenty amino acids of the hTSHR, covering the entire extracellular domain, with 5 amino acids overlapping between adjacent peptides on each end. Peptide 1 (P1) stands for amino acids 1-20, P16 for 16-35, P31 for 31-50 etc. with P376 (376-395) being the last peptide studied from the extracellular domain. EC-1, EC-2 and EC-3 represent the extracellular loops between the respective transmembrane domains. The numbering does not include the signal peptide. When results of different groups are compared care was taken to convert all numbers to the numbering system used in the present study. The size and amino acid composition of each synthetic peptide were analyzed to conform the nature of the product.

Triplicate wells in ELISA plates (Immulon 2, Dynatech) were coated with 10 ug/ml of the peptides in carbonate-bicarbonate buffer pH 9.6 at 4 °C overnight, blocked with 1% human albumin and exposed to 1:600 dilutions of sera for 2 hours, followed by exposure to AP conjugated goat anti-human IgG (gamma chain specific) raised in goat (KPL, Gaithersburg, MD), diluted 1:1000 for one hour. After the addition of paranitrophenyl-phosphate substrate, readings were made at 405 nm. Vigorous washing between steps was performed using PBS containing 0.05 % Tween 20. Negative controls included wells with no coat, no blocking agent, or no serum. As a positive control, immune serum from a rabbit having high IgG titer against a 16 amino acid hTSHR peptide (331-346) has been included, and anti-rabbit IgG (KPL, Gaithersburg, MD) second antibody has been applied.

The calculated ELISA indices are the multiples of baseline readings for each individual. An individual was considered to be reactive against a peptide if the ELISA index was above the mean + 2SD calculated for that peptide in the control group. Also, comparisons of the ELISA indices between the Graves' disease and the control group were made using the two sample unpaired t test.

Results

The findings regarding IgG binding to each peptide of the extracellular domain of the hTSHR are summarized in Table 1. There

Table 1. IgG binding of peptides representing the extracellular domain of the human thyrotropin receptor

Peptide	ELISA Indices, Mean ± SD		P	Number of positive cases			
	Graves' disease (n=10)	Controls (n=10)		Graves' disease (n=10)		Controls (n=10)	
1-20	1.25 ± 0.14	1.25 ± 0.10	0.899	0		0	
16-35	1.11 ± 0.08	1.26 ± 0.20	0.038	0		1	
31-50	1.15 ± 0.14	1.13 ± 0.09	0.683	1		0	
46-65	1.76 ± 0.57	1.54 ± 0.26	0.289	1		0	
61-80	1.32 ± 0.15	1.26 ± 0.13	0.343	1		0	
76-95	4.03 ± 2.02	3.32 ± 1.35	0.367	2		1	
91-110	1.12 ± 0.08	1.08 ± 0.04	0.205	2		0	
106-125	1.04 ± 0.06	1.11 ± 0.18	0.206	0		1	
121-140	2.80 ± 1.11	2.17 ± 0.92	0.189	2		1	
136-155	1.19 ± 0.17	1.15 ± 0.15	0.640	1		0	
151-170	1.27 ± 0.19	1.32 ± 0.14	0.486	1	16[1]	1	9[1]
166-185	1.33 ± 0.13	1.28 ± 0.19	0.535	0		1	
181-200	1.08 ± 0.07	1.08 ± 0.11	0.981	0		0	
196-215	1.12 ± 0.05	1.11 ± 0.08	0.938	0		0	
211-230	1.04 ± 0.08	1.03 ± 0.08	0.755	0		1	
226-245	1.12 ± 0.13	1.21 ± 0.17	0.199	0		1	
241-260	1.87 ± 0.60	1.73 ± 0.47	0.579	2		0	
256-275	2.38 ± 0.92	2.27 ± 0.68	0.768	2		0	
271-290	1.07 ± 0.09	1.08 ± 0.69	0.787	1		0	
286-305	1.14 ± 0.15	1.28 ± 0.25	0.144	0		1	
301-320	1.31 ± 0.13	1.29 ± 0.11	0.657	1		0	
316-335	2.60 ± 0.68	2.48 ± 0.50	0.642	1		0	
331-350	1.77 ± 0.30	1.38 ± 0.16	0.002*	5		0	
346-365	1.25 ± 0.14	1.20 ± 0.12	0.397	1		0	
361-380	1.32 ± 0.20	1.21 ± 0.15	0.195	1	15[2]	0	1[2]
376-394	1.36 ± 0.13	1.25 ± 0.12	0.078	1		0	
EC-1	1.00 ± 0.48	1.72 ± 0.42	0.194	1		1	
EC-2	1.24 ± 0.12	1.16 ± 0.05	0.065	4		0	
EC-3	1.24 ± 0.12	1.26 ± 0.11	0.681	0		0	

[1]The number of positive samples for peptides upstream from residue 300.

[2]The number of positive samples for peptides downstream from residue 300.

were many similarities in the binding pattern of Graves' disease patients and normal controls. Four peptides showed strong IgG binding in both groups (P76, P121, P256, P316), while other peptides had virtually no binding (P31, P91, P106, P136, P181,

P196, P211, P226, P271, P286). When the means of ELISA indices of Graves' disease patients were compared, the difference reached statistical significance only in the case of peptide P331. We believe that the peptides in this study actually bound to the wells of the ELISA plates as intended, and the readings are not a reflection of different degrees of coating. Our supporting evidence is as follows: (A) sequential coating with a low and a high IgG binding peptide always resulted in a reading close to the initial coating peptide; (B) co-coating with two peptides always resulted in the mean of the separate readings; (C) IgG binding did not show correlation with hydrophilicity (data not shown); and (D) positive and negative controls gave the expected results.

Two peptides, P331 and EC-2, showed high reactivity with 5 and 4 Graves' patients' sera, respectively, while they did not give significant binding with any of the control sera (Table 1). Moreover, the four individual Graves' disease patients with EC-2 binding also gave demonstrable binding to P331. Except for one case, each of the peptides which demonstrated binding in the control group were upstream from amino acid 300, whereas 48 % of the antibodies in the Graves' disease group were directed against sequences downstream from residue 300.

Discussion

Due to its recent cloning and sequencing, there have been further developments in our understanding of the structure and physiology of the hTSHR. Nevertheless, the precise mechanism by which the immune system interacts with the hTSHR in either patients with Graves' disease or in normals remains unclear. Both contiguous and non-contiguous epitopes may be important in antigenic recognition of the hTSHR. Though the former is the less probable (4), identifying a short linear epitope capable of blocking the binding of stimulatory IgG-s would be of therapeutic significance. In addition, T cells are known to recognize linear, rather than conformational epitopes. Distinct polypeptides of the primary structure may form epitopes in the TSH receptor. Successful immunization using short peptides of the hTSHR generated antibodies which were stimulatory in cAMP bioassays (5-9). No systematic screening of IgG interaction with peptides representing the entire extracellular domain has been previously reported.

Of the present series of 29 peptides, two exhibited markedly higher IgG binding in the Graves' disease group (P331 and EC-2 in

Table 1). P331 includes the 16 amino acid region which has sequence homology with a retroviral protein (10) and which was found to be highly immunogenic in rabbits, even without being coupled to a carrier (5). Other reports mention higher IgG binding to this region when Graves' disease patients and normals are compared (11). Also, antibodies raised in rabbits against this region carry thyroid stimulatory activity *in vitro* (5, 12). EC-2, representing the second extracellular loop, has also been reported to evoke rabbit antibodies which were stimulatory *in vitro* in the cAMP assay (9).

Recently, an explanation for the seemingly contradictory findings regarding the subunit structure of the hTSHR has been offered (13); these explanations take advantage of the predicted two proteolytic cleavage sites between amino acids 267-293. This location is close to residue 300, which we found to divide the extracellular domain of the hTSHR into two sets of linear epitopes; against the upstream set, both normals and Graves' patients had IgG-s, while against the downstream set, the controls did not (with one exception). This finding raises the possibility that, at least for linear epitope binding antibodies, the target is in the region of the hTSHR polypeptide chain closest to the cell membrane.

Even though particular individual peptides did not exhibit IgG binding, they may still participate in the formation of larger, composite, non-contiguous epitopes. The peptides which bound IgG to a lesser degree were often arranged as clusters in the extracellular domain (amino acids 1-50, 181-245). The finding that P331 and EC-2 bound IgG-s from 5 and 4 patients, respectively, but none of the controls' sera, points out that there are linear epitopes of the hTSHR involved in antibody binding.

In summary, we have analyzed Graves' IgG binding to peptides representing the entire extracellular domain of the hTSHR. We have identified two areas (amino acids 331-350 and the second extracellular loop of the transmembrane region) which bound Graves' IgG, suggesting that these linear epitopes may be important in generating or propagating the immune response in this disease. Further studies investigating the precise nature of this interaction, as well as the role of these peptides in T cell stimulation, are warranted.

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