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Demonstration by immunoblotting of heterogeneity in the autoantibody response directed against fat cells in Graves' disease

A.P. Weetman, T.B. Nutman+, J.R. Baker, jr* and K.D. Burman*

Department of Medicine, Royal Postgraduate Medical School, Du Cane Road, London W12 OHS, England, +*Laboratory of Parasitic Diseases, National Institutes of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD 20892* and **Endocrine and Transplantation Services, Walter Reed Army Medical Center, Washington, DC 20307, USA*

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Guinea pig fat cell membranes (FCM) have been widely used in preference to thyroid membranes as a source of TSH receptors to investigate TSH receptor antibodies in Graves' disease, because FCM are ostensibly free of other thyroid antigens. However, by FCM immunoblotting we have found: (i) 8 of 10 normal sera bound to determinants at 38 and 190 kDa; (ii) 17 other determinants were recognised by 60% of Graves' or Hashimoto sera and by 20% of normal sera; (iii) three determinants at 65–90 kDa were recognised by 5 of 13 Graves' but by none of the normal or Hashimoto sera; and (iv) none of the determinants recognised appeared to be related to the TSH receptor.

Thyrotropin receptor antibody; Graves' disease; Autoantibody

1. INTRODUCTION

Graves' disease is almost certainly caused by antibodies directed against the thyrotropin (TSH) receptor [1]. The most frequently used assay for TSH receptor binding immunoglobulins (TBI), which may or may not have thyroid-stimulating properties, measures inhibition of TSH binding to thyroid membranes [2]. Because Graves' sera also contain antibodies which react with other thyroid components, such as thyroglobulin and microsomes, recently developed assays have used guinea pig fat cell membranes (FCM), which contain TSH receptors, but not Tg or M, to detect TBI [3–5]. FCM binding has also been used to investigate TBI interactions with the receptor and to purify these IgG [6–8]. However, the possibility that FCM contains other antigens recognised by Graves' sera (besides the TSH receptor), has not been investigated. We have therefore performed immunoblotting studies to address this point.

Correspondence address: A.P. Weetman, Dept of Medicine, Royal Postgraduate Medical School, Du Cane Rd, London W12 OHS, England

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2. MATERIALS AND METHODS

2.1. *Materials*

FCM were prepared exactly as described in [4] from guinea pig subcutaneous and epididymal fat. This preparation was shown to be active in an FCM ELISA prior to use [4]. Protein concentration was estimated by the Coomassie blue dye binding microassay [9]. Bovine TSH was from Armour (Kankakee, IL), human chorionic gonadotropin (HCG) from Ayerst (New York) and ^{125}I-TSH from Clinetics (Tustin, CA).

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2.2. *Methods*

SDS-polyacrylamide gel electrophoresis (5–15% gradient) was performed as in [10]. FCM were run at 800 μg/gel (gels 15 ml volume, 1.5 mm thickness) with 5% β-mercaptoethanol. Immunoblotting was performed according to [11]. Sera from controls, or patients with Graves' or Hashimoto's disease, were used at 1:50 dilution in 50 mM Tris, 150 mM NaCl buffer (pH 7.4) with 3% Tween and 0.5% Triton X-100. Bound IgG was detected with ^{125}I-protein A and autoradiography. Molecular masses were assigned using an NA2 sequence analyzer [12]. The FCM ELISA was as described [4], results being expressed as U/ml from a standard curve generated with a strongly positive serum.

3. RESULTS

Over 40 separate protein bands were identified on Coomassie blue staining of FCM after polyacrylamide gel electrophoresis (fig.1). Representative immunoblots are shown in fig.2 and the frequency distribution of the determinants recognised by these sera is shown in fig.3. Two bands of 190 and 38 kDa were recognised by 8 of 10 normal sera, and by all the Graves' and Hashimoto sera. One normal serum also reacted with an additional 9 bands in the range 27–115 kDa, and a second normal serum reacted with 4 bands between 30 and 40 kDa. Sera from the Graves' patients reacted with 17 discrete determinants between 15 and 115 kDa but any single determinant was never recognised by more than 8 of the 13 sera. Five Graves' sera showed no other reactivity than at 190 and 38 kDa. The Hashimoto sera gave a reactivity pattern between that of the Graves' and normals. However, none of the Hashimoto or normal sera reacted with three determinants identified up to five of the Graves' sera between 65 and 90 kDa ($p < 0.05$, Fisher's exact test).

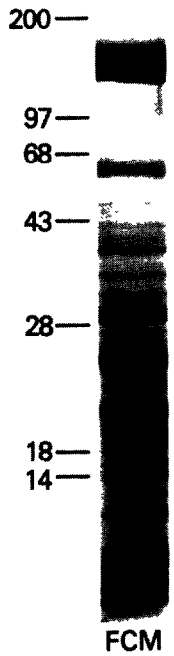

Fig.1. SDS-polyacrylamide gel electrophoresis of guinea pig FCM after Coomassie blue staining. Numbers on the left indicate molecular masses (in kDa).

The sera from the two Graves' patients which reacted with the greatest number of determinants (12 and 15) reacted most strongly in the FCM ELISA (980 and 738 U/ml). Moreover the two normal sera which reacted by immunoblotting were also reactive in the ELISA (206 and 190 U/ml; mean ± SD of other 8 normal sera 28 ± 23 U/ml). The Graves' sera which did not react with immunoblots had ELISA values from 67 to 224 U/ml.

To assess the effect of reducing conditions on antigen reactivity, the FCM was run on a single gel with or without β-mercaptoethanol; the binding of a normal and four Graves' sera was the same in each case. In another experiment TSH (10 or 100 mU/ml) and HCG (5, 50 or 500 USP U/ml) were shown to have no effect on the immunoblots of the two most reactive Graves' sera. With TSH (1 U/ml), there was a reduction in intensity of a single band (32 or 28 kDa) with each serum (shown for one serum in fig.4). TSH at any concentration had no effect on the intensity or number of bands produced by a pool of five other Graves' sera or on the two bands identified by a pool of five control sera. Furthermore, we found no binding of ^{125}I-TSH (added at up to 5×10^5 cpm) to blotted FCM on the nitrocellulose strips.

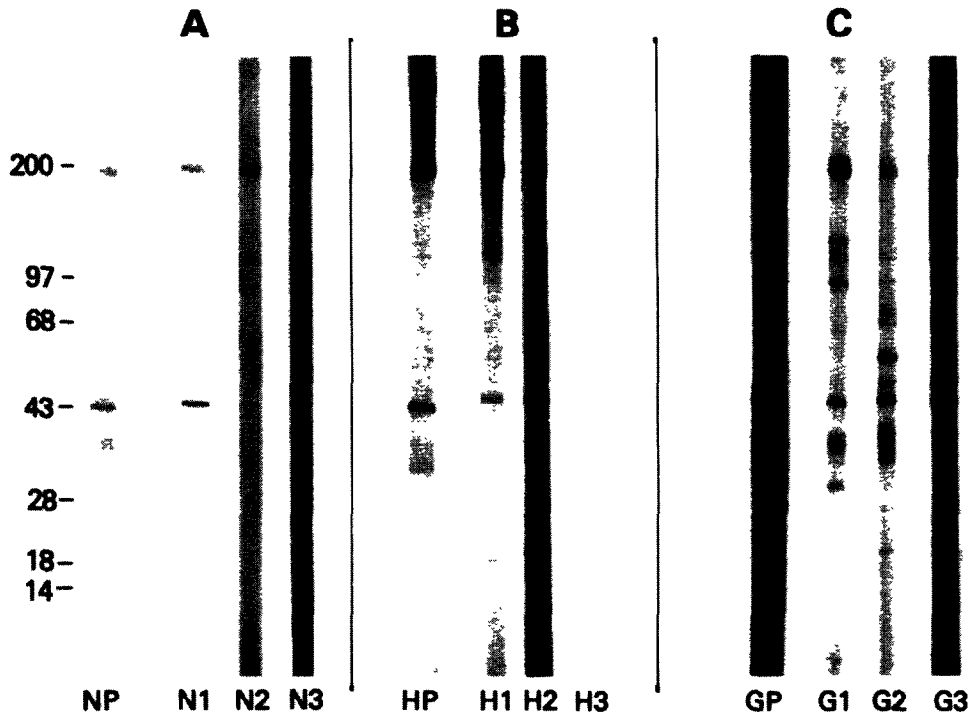

Fig.2. Immunoblots of normal and autoimmune sera against FCM. N, normal; H, Hashimoto's thyroiditis; G, Graves' disease; P, pool of sera; numbers refer to representative individuals (nos 1–3). Molecular masses (in kDa) are indicated on the left.

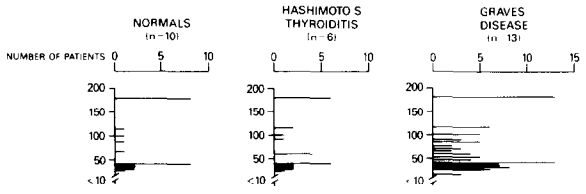

Fig.3. The frequency distribution of various FCM determinants recognised by the three groups of sera. Each line shows the number of sera which reacted with a given determinant identified by its molecular mass (in kDa, indicated to the left).

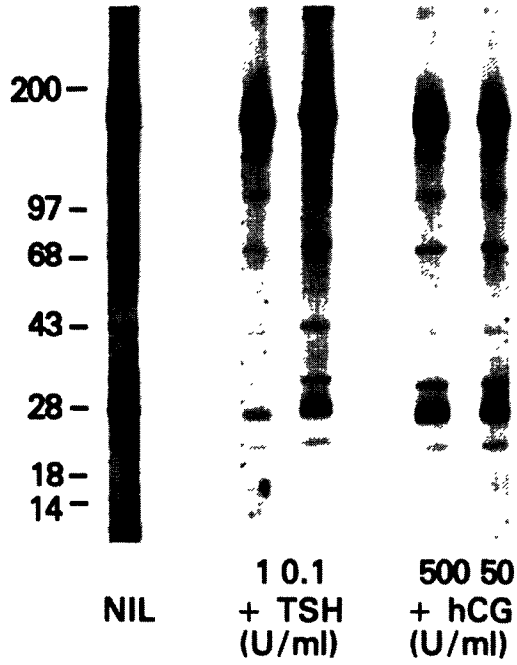

Fig.4. Effect of TSH and HCG on the binding of a Graves' serum to FCM by immunoblotting. Molecular masses (in kDa) indicated on the left.

4. DISCUSSION

These results show the presence of previously unsuspected antibody heterogeneity in the reaction of Graves' and Hashimoto sera with guinea pig FCM. It is unlikely that the determinants identified by immunoblotting were related to the TSH receptor. The molecular masses of the bands which discriminated Graves' from Hashimoto sera (65–90 kDa) do not correspond with the known size of the guinea pig FCM TSH receptor subunits [13], and antibody binding to the immunoblots could not be inhibited by TSH at up to 100 mU/ml. It is difficult to know whether the reduction in binding to bands at 28–32 kDa with two sera at 1 U TSH/ml is truly specific inhibition [14]. Even if this were to represent a TBI binding to the 30 kDa TSH receptor B subunit [13], it is clear that the other determinants found by immunoblotting cannot be related to this.

We also found reactivity of some normal and Hashimoto sera with FCM antigens by immunoblotting. These data are consistent with other reports showing normal and Hashimoto sera binding to FCM in solid-phase immunoassays [15,16]. Normal sera have been shown to immunoprecipitate non-specifically FCM proteins [14]. It is possible that some of the reactivity found with normal sera is associated with heterophile antibody activity against guinea pig species-related antigens [16].

In conclusion it is apparent that under these immunoblotting conditions TBI cannot be demonstrated with FCM, yet sera from Graves', Hashimoto, and to a lesser extent normal subjects contain antibodies directed against several other determinants in FCM. This reactivity could explain the lack of correlation with TBI in some FCM-based antibody assays [15,16] and questions the use of such material as a source of TSH receptors implicitly free from other contaminating antigens.

ACKNOWLEDGEMENT

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