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Novel separation method for serum immunoglobulins

Application to thyroid related antibodies

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A dye-based affinity chromatographic system using Remazol yellow GGL-Sepharose is described for the fractionation of serum immunoglobulins. Immunoglobulins are sequentially eluted from the gel columns using gradients of pH and salt with >88% recovery. Specific immunoglobulin activities were identified as discrete peaks and antibodies raised against the same antigen were separated. Biological properties of antibodies were retained following chromatography. The method is applicable to both human and animal immunoglobulins.

Thyroid autoantibody; Immunoglobulin; Affinity chromatography; Remazol yellow GGL

1. INTRODUCTION

Separation of a particular antibody activity from the multitude of immunoglobulins found in serum is a particularly difficult task. Classical chromatographic techniques have been used to extract the immunoglobulin fraction from serum but only limited subfractionation has been possible. Affinity chromatography techniques using immobilized antigen to extract desired antibodies from serum have been developed but are not applicable in situations where the antigen is not available or is unknown. Such methods also have disadvantages when polyclonal mixtures of antibodies with a range of affinities are studied; high-affinity antibodies may be difficult to recover intact from the adsorbent and low-affinity antibodies may not be adsorbed. Thus, antigen-based affinity chromatography is unsuitable in situations such as autoimmune diseases where the antigen may be unknown or is difficult to obtain, and particularly when all species of active immunoglobulin are of interest.

Dye-based affinity chromatography systems have been developed for the purification of a number of serum proteins [1,2]. Preliminary studies [3] demonstrated the potential of such conjugates as adsorbents to subfractionate serum immunoglobulin and this communication describes the development of a chromatographic system using Remazol yellow GGL-Sepharose.

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2. MATERIALS AND METHODS

The textile dye Remazol yellow GGL (Hoechst, Halifax, England) was conjugated to Sepharose-4B as described [3] to make the yellow gel. Ammonium sulphate precipitates from serum [4] were redissolved in water, dialysed exhaustively against 20 mM sodium phosphate buffer, pH 5, and applied to columns (1 × 20 cm) of the yellow gel, previously equilibrated in sodium phosphate buffer (20 mM, pH 5). Immunoglobulins were eluted by gradients of pH 5–7.5 in 20 mM sodium phosphate buffer (60 ml) followed by salt; 0–0.3 M NaCl in 20 mM sodium phosphate buf-

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fer, pH 7.5 (120 ml). Fractions (2 ml) were collected, the pH of each was recorded and the sodium concentration determined by flame photometry. Immunoglobulin concentrations in serum and column fractions were determined by nephelometry using anti-human IgG (Fc) serum (Dako-Immunoglobulins, Denmark). Protein was measured as described [5]. The presence of subclasses 1–4 of IgG and of $\varkappa$ and λ light chains was detected by Ouchterlony diffusion-type experiments. Appropriate antisera were obtained from The Netherlands Red Cross blood transfusion service, Amsterdam, The Netherlands (IgG1–4) and Dako-Immunoglobulins, Denmark ($\varkappa$ and λ).

Thyroglobulin antibodies which cross react with iodothyronines were detected by their ability to bind radiolabelled thyroid hormones and were quantified (in microlitre equivalents) by comparison with dilutions of the original serum [3]. Antibodies to chicken thyroglobulin were identified by an ELISA [6] using chicken thyroglobulin immobilized on microtitre plates and an alkaline phosphatase conjugated rabbit anti-chicken IgG antibody as the second reagent. Thyrotrophin (TSH)-receptor antibodies were detected by their ability to inhibit the binding of ^{125}I-TSH (RSR, Cardiff, Wales) to solubilized porcine thyroid TSH-receptors in a radioreceptor assay based on that described by Southgate et al. [7]. Bovine TSH (Thytropar, Armour Pharmaceuticals) was the standard. The biological activity of TSH receptor antibodies was determined by their ability to stimulate $^{125}I^-$ uptake in FRTL-5 cells, a continuously growing functional line of rat thyroid cells [8].

3. RESULTS AND DISCUSSION

Previous studies at pH 7.4 [3] showed that serum proteins may interfere with the adsorption of immunoglobulins onto dye-Sepharose gels. However, using immunoglobulins prepared from serum by ammonium sulphate (2.5 mol/l) precipitation and applying the sample in buffer at pH 5 to the yellow gel complete adsorption was achieved.

Immunoglobulins prepared from human serum were eluted as two broad bands of protein by the pH and salt gradients (fig.1). For all serum samples investigated, with the exception of those containing myeloma proteins, the elution profile of total IgG was similar. Segments of eluted bands ran in identical positions when re-chromatographed indicating that the interaction of particular immunoglobulins with the dye was specific and characteristic. The distribution of IgG1–4 and $\varkappa$ and λ was the same as that of total IgG, hence, separation of immunoglobulins does not depend

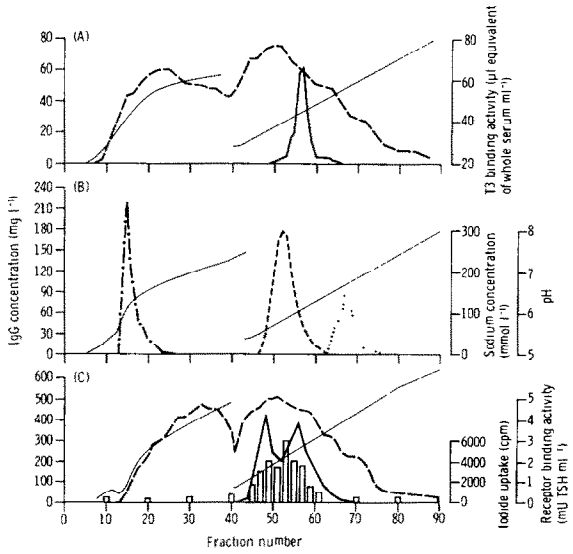

Fig.1. Elution of serum immunoglobulins from Remazol yellow GGL-Sepharose by gradients of pH and ionic strength. (A) Identification of a single peak of T3-binding thyroglobulin antibody at sodium concentration of 127 mmol·l^{-1} within the elution profile of total immunoglobulin. The ammonium sulphate precipitate from 750 μl serum from a patient with Hashimotos disease was chromatographed as described in section 2. (– – –) IgG, (——) T3-binding antibody. (B) Composite diagram showing the elution positions of myeloma proteins from three patients; using in each case the ammonium sulphate precipitate from 100 μl serum. Peaks of antibody eluted at pH 6.1, and at sodium concentrations of 84 mmol·l^{-1} and 169 mmol·l^{-1} on the salt gradient. Levels of other serum immunoglobulins were proportionally too low to be illustrated. (– – – –) K.W. IgG$\varkappa$, (– – –) H.B. IgGλ, (···) J.B. IgG$\varkappa$. (C) Separation of TSH-receptor binding antibodies in serum from a patient with Graves' disease (ammonium sulphate precipitate from 5 ml serum). Two peaks of receptor-binding activity were detected by the in vitro binding assay both of which were also found to stimulate iodide uptake in the in vitro bioassay. (——) Receptor-binding activity, (□) iodide uptake.

on structures which define these subclasses or light chain types. Using the mild conditions described, recovery of IgG was always greater than 88% and, as the following results demonstrate, biological and binding properties of antibodies were unaffected by chromatography.

The behaviour of individual components of total immunoglobulins was examined using samples prepared from human and animal serum containing identifiable activities. The elution of triiodothyronine (T3)-binding activity in the serum of a patient with Hashimoto's disease is shown in fig.1A. A single discrete peak was detected within the broad bands of total IgG in this and two other cases, each with approx. 10-fold increase in specific activity in terms of [^{125}I]T3 bound/mg IgG. Although having the same hapten-binding property a different active immunoglobulin was present in each patient as shown by the different elution positions (sodium concentrations 67, 73, 125 mmol/l). Monoclonal antibodies present in high concentrations in the serum of patients with myelomas were also eluted as discrete peaks confirming that the broad bands from whole serum represent the sequential elution of a multitude of individual immunoglobulins (fig.1B).

The retention of biological activities, other than hapten binding, during the chromatographic procedure was examined using a sample from a patient with Graves' disease. In Graves' disease a population of antibodies against the thyroid cell TSH-receptor is found which interact with the TSH-binding site and stimulate thyroid cell function. An immunoglobulin preparation from the serum of one such patient produced a normal profile for total immunoglobulins on elution from a yellow gel column (fig.1C). Analysis of the fractions with the radioreceptor binding assay revealed two peaks of antibody which interacted with the TSH-binding site, each capable of stimulating the incorporation of $^{125}I^-$ into cultured rat thyroid cells. Thus two antibodies with similar biological properties raised against a common antigen, the TSH receptor, were identified and separated.

The potential of the technique for the subfractionation of polyclonal antisera was demonstrated using immunoglobulins prepared from the serum of a chicken immunised with chicken thyroglobulin. A broad band of anti-thyroglobulin activity was detected throughout the chromatogram after

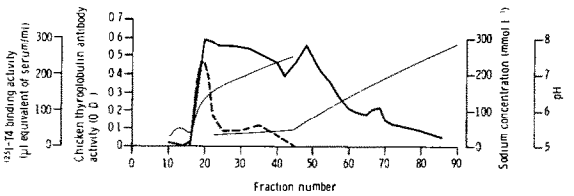

Fig.2. Elution of chicken anti-chicken thyroglobulin antibodies from a Remazol yellow GGL-Sepharose column (1 × 20 cm). Identification of a single major peak of [^{125}I]T4-binding activity at pH 6.0. The ammonium sulphate precipitate from 5 ml chicken serum was chromatographed as described in section 2. (——) Thyroglobulin antibodies, (– – –) T4-binding antibodies.

fractionation on the yellow gel (fig.2). However a subpopulation of thyroglobulin antibodies which bound thyroxine was detected as a single discrete peak. Similar results were obtained with a polyclonal rabbit antiserum to human thyroglobulin.

Several examples showed single peaks of activity within broad bands of immunoglobulins. In some instances other information indicated that they represented monoclonal products. However this cannot be said a priori for an individual peak whether found alone or as one of a mixture. Somatic mutation of a clone generates an oligoclonal mixture with respect to antigen binding but each member retains the same constant regions (dye-binding areas) on which the separation depends (Wells, C., personal communication) and such a mixture would co-elute.

As the method of separation is not dependent on the antigen-binding structure of molecules, closely related immunoglobulins with similar biological properties but different constant regions may be separated. The constant regions of the immunoglobulins are determined by the genotype of the secreting clone, thus identification of more than one peak may be evidence for a polyclonal immune response.

4. CONCLUSIONS

The development of an affinity chromatography system, for the subfractionation of immunoglobulins, which uses a synthetic dye as the ligand has considerable advantages over antigen-affinity chromatography. The adsorbents are cheap and easy to prepare, have high capacities [3],

are not easily degraded and may be re-used. Using the textile dye Remazol yellow GGL as the ligand we have successfully chromatographed immunoglobulins from human and animal sera achieving good recovery of biological and binding properties. A particular advantage will be in the study of antibodies against unknown or unavailable antigens. For example the investigation of autoantibodies against human TSH receptors which has not previously been possible due to the difficulty of purifying receptors and their scarcity.

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