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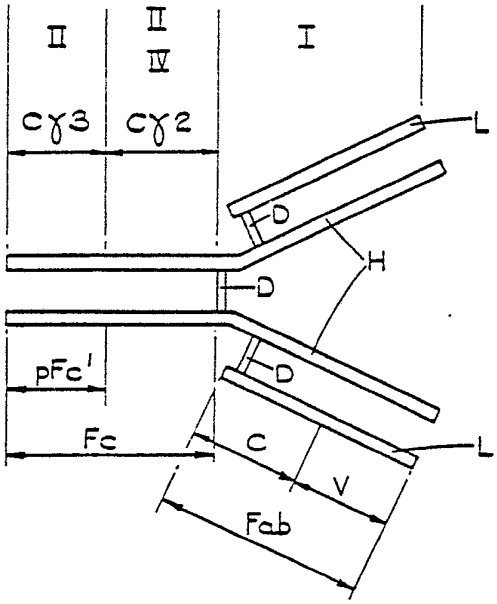
(54) Title: METHODS OF IMMUNO-ANALYSIS USING MONOCLONAL ANTIBODIES

(57) Abstract

An antigen/antibody precipitate is obtained, using monoclonal antibodies, the monoclonal antibodies (samples I or II or III or IV) being selected so as to be specific to two distinct antigenic binding sites (L or C 2 or C 3) on a protein (IgG) in a sample under test. The proportions of sub-populations of immunoglobulins (IgG kappa, IgG lambda) in a sample is determined by reacting the sample with a combination of antibodies (II and IV) both of which are specific to the heavy chains (H) of both sub-populations (IgG kappa, IgG lambda) and reacting the sample with an antibody combination (I and II) specific to said heavy chain (H) and to an antigenic determinant expressed by only one (IgG kappa) of the sub-populations.



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Methods Of Immuno-Analysis
Using Monoclonal Antibodies.

Technical Field

This invention relates to methods of immuno-analysis using monoclonal antibodies. Specific antisera are widely applied to the detection and quantitation of a target antigen when it is present in a complex mixture of molecules, and many quantitative methods depend upon precipitation resulting from the formation of insoluble antigen/antibody complexes. In the early stages of antigen/antibody interaction, the insoluble complexes are of relatively small size and are held in suspension. Several techniques of specific protein determination depend upon the light scattering or absorption properties of such complexes. One such, automated, method is based on difference turbidimetric measurements resulting from the increased (apparent) absorption properties of such complexes, held in suspension when a polyclonal antibody interacts with a multivalent antigen.

Background Art

In 1975 Kohler and Milstein (Nature, 256, pp 495-497) reported a method of producing monoclonal antibodies directed against a single antigenic determinant. Amongst the advantages of monoclonal antibodies are (1) their unique specificity and (2) their potential to allow the development of perpetually reproducible standard reagents. However, since monoclonal antibodies are specific to a single determinant, or interaction with antigen they can only form soluble linear complexes, rather than cross-linked complexes which might be insoluble (precipitating). Therefore, individual monoclonal antibodies have not hitherto appeared to be applicable to techniques that are dependent on the formation of insoluble immune complexes.

It is an object of the present invention to provide a method by means of which monoclonal antibodies may be reacted with proteins to produce insoluble antigen/antibody complexes that will allow the application of monoclonal

antibodies to techniques which require formation of the aforesaid insoluble complexes.

It is a particular object to provide a method by means of which monoclonal antibodies may be used to assay the total immunoglobulin population or selected immunoglobulin sub-populations present in complex mixtures viz. serum or other body fluids.

Disclosure of the Invention

According to the invention a method of determining the amount of a particular protein in a sample comprises reacting the sample with a combination of two monoclonal antibody preparations which are respectively specific to two distinct antigenic sites (determinants) on the macromolecule of the protein under investigation, and determining from the resulting antigen/antibody complexes formed a quantitative measure of the original protein concentration.

In a particular embodiment of the method the aforesaid two monoclonal antibodies are selected so as to be specifically directed against relatively widely spaced antigenic determinants present on a given macromolecule, whereby the binding of one monoclonal antibody to its specific antigenic determinant does not interfere, spatially, with the binding of the second monoclonal antibody to the antigenic determinant for which said second antibody has specificity.

In a further particular embodiment the macromolecule comprises an immunoglobulin and the monoclonal antibodies are selected so as to be specific to antigenic determinants expressed by the light and heavy chains respectively of the immunoglobulin.

In yet another embodiment the method comprises reacting a sample including first and second immunoglobulin populations with a combination of first and second monoclonal antibody preparations, both of which are specific to antigenic determinants expressed on the heavy chains of both immunoglobulins, determining from the resulting complex the total immunoglobulin level in the sampe reacting the sample with a combination of said first monoclonal antibody and a third monoclonal antibody which is specific for an antigenic determinant expressed by only one of said immunoglobulin population, and determining from the resulting complex the quantity of said one immunoglobulin population.

There will now be described by way of example only and with reference to the accompanying drawings, procedures incorporating the methods of the invention.

Brief Description of the Drawings

Figure 1 is a representation of an IgG molecule, indicating the regions against which test antibodies were directed;

Figure 2 is a graph of turbidity against time, for several antigen/antibody reactions; and

Figure 3 is a graph of change in turbidity obtained from antigen/antibody reactions with varying antibody concentrations.

Best mode of carrying out the invention

Figure 1 represents a macromolecule of immunoglobulin G (IgG) having two "heavy" polypeptide chains H and two "light" polypeptide chains L, the chains H and L being linked by disulphide bonds. The molecule is symmetrical and has two antigen binding fragments Fab, each of which includes a light chain L and a portion of the heavy chain H. A crystalisable fragment Fc of both heavy chains H

comprises $C_\gamma 2$ and $C_\gamma 3$ regions of equal lengths. The fragment Fc may be separated from the remainder of the molecule by enzyme action, and this fragment Fc may be subjected to further enzyme action to leave a residual fragment pFc' whose extent is effectively that of the $C_\gamma 3$ region. Each chain in the Fab fragment has a variable region V and a constant region C.

Normal human IgG includes sub-populations of IgG kappa and IgG lambda, the kappa and lambda characteristics being defined by the structures of the constant regions C of the molecules.

In the examples described antibodies were elicited using polyclonal human IgG as an immunogen, and the antibody products were selected on the basis of their ability to agglutinate sheep red blood cells sensitised with human IgG. The specificity of the cloned antibody products was further defined by their ability to agglutinate sheep red blood cells sensitised with kappa light chain, lambda light chain, Fc fragments, or pFc' fragments. By these methods monoclonal antibodies were selected having specificities as indicated below;

Sample	Specificity
I	Kappa light chain
II	
III	Fc
IV	

Sample II was also found to agglutinate cells sensitised with pFc' fragments, and this sample was thus directed against an antigenic determinant in the $C_\gamma 3$ region of the IgG molecule. In the absence of agglutination of pFc' sensitised cells by samples III and IV, these samples were presumed to be specific to antigenic determinants in the $C_\gamma 2$ region of the IgG molecule, or to be

conformational determinants dependent on the structural integrity of the Fc fragment of the molecule for expression.

The antigen with which the foregoing antibodies were reacted was an IgG kappa paraprotein.

Preparations of the antibody samples I to IV were reacted with the antigen, separately and in combinations of antibody. The reactions were carried out at 25°C in phosphate buffered solutions containing 4% polyethylene glycol (MW. 3000). The difference in turbidity of the fluid in which the reactions were performed was determined using light of wavelength 260 nm, (Jacobsen and Steensgaard, Immunology, 36 (1979) at 293-298).

As shown in Figure 1, monoclonal antibody samples I to IV were reacted separately against the antigen without a significant burbidity difference, D, when plotted againt a time T of up to 600 seconds. However, a combination of antibody samples II and IV produced a measurable turbidity change, whole reactions with a combination of samples I and II and a combinations of samples II and III which were equivalent to that normally obtainable in reactions using polyclonal antibody reagents.

It will be seen from Figure 1 that a reaction with a combination of samples I, II and III resulted in a turbidity difference less than that obtained with the combination of samples II and III. This result is probably due to antibody excess in the reaction.

Figure 2 shows the turbidity differences, D, obtained for a number of values of antigen concentration, C, expressed in mg/ml. The curve shown was obtained by titration of the IgG kappa paraprotein, at differenting concentrations, against a standard concentration of an antibody preparation. The antibodies used in this case were those from samples I

and II, that is those directed against the spatially distinct kappa light chain and C 3 region of the antigen.

As indicated above the IgG content of human serum is made up of sub-populations of IgG kappa and IgG lambda. In normal serum the ratio of IgG kappa to IgG lambda lies between 3:1 and 1:1, a typical ratio being approximately 1:8:1. A significant departure from this range of ratios may indicate an early stage of myeloma, which otherwise may develop for up to twenty years before showing symptoms. Since detection and treatment at an early stage may significantly prolong life expectancy, it is clearly advantageous that the quantities of the above sub-populations should be capable of being determined.

Monoclonal antibody preparations have been used to determine the relative proportions of sub-populations of immunoglobulins present in such polyclonal IgG. For example, a test sample including IgG kappa and IgG lambda which is reacted with antibodies from sample II and sample III, both of which are directed against the Fc portion of the IgG molecule, allows quantitation of the total IgG content of the test sample. An identical IgG test sample reacted with a combination of antibodies from samples I and II will allow quantitation of the IgG kappa content. Similarly an identical IgG test sample reacted with, for example, an antibody preparation from sample II in combination with a further monoclonal antibody preparation which is specific to IgG lambda light chain will allow quantitation of the IgG lambda content. It has been found that the sum of quantitation of IgG kappa and IgG lambda carried out as above, have a high correspondence with the quantitation of total IgG content.

CLAIMS:

1. A method of determining the amount of a particular protein (IgG) in a sample, comprising reacting the sample with antibodies to said protein to obtain antigen/antibody precipitate, characterised in that said antibodies comprise two monoclonal antibody preparations (I or II or III or IV) which are respectively specific to two distinct antigenic sites (L or $C\gamma 2$ or $C\gamma 3$) on the macromolecule of the said protein (IgG).

2. A method as claimed in claim 1 in which said two antibody preparations (I or II or III or IV) are selected so as to be specific to relatively widely spaced ones of said antigenic sites (L or $C\gamma 2$ or $C\gamma 3$), whereby the binding of one monoclonal antibody to its specific determinant on the molecules of said protein IIgG) does not interfere spatially with the binding of the other monoclonal antibody to its specific determinant.

3. A method as claimed in claim 1 or claim 2 in which said protein comprises an immunoglobin (IgG) and said two monoclonal antibodies (I or II or III or IV) are selected so as to be specific to antigenic determinants expressed by the light (L) and heavy (H) chains respectively of the macromolecule of the immunoglobin (IgG).

4. A method as claimed in any preceding claim in which said sample includes populations of first and second immunoglobulins (IgG kappa and IgG lambda), and said monoclonal antibodies comprise first and second preparations (II and IV) which are both specific to antigenic determinants expressed on the heavy chains (H) of both said immunoglobulins (IgG kappa, IgG lambda), reacting a combination of said first and second preparations (II and IV) with said sample, determining fromthe resulting antigen/antibody complex the total level of the immunoglobulins (IgG kappa, IgG lambda) in the sample, reacting the sample with a combination of said first preparation (II) and a third monoclonal antibody preparation (I) which is specific to an antigen determinant expressed by only one (IgG kappa) of said immunoglobulins, and determining from the resulting complex the quantity of population of said one immunoglobulin (IgG kappa).

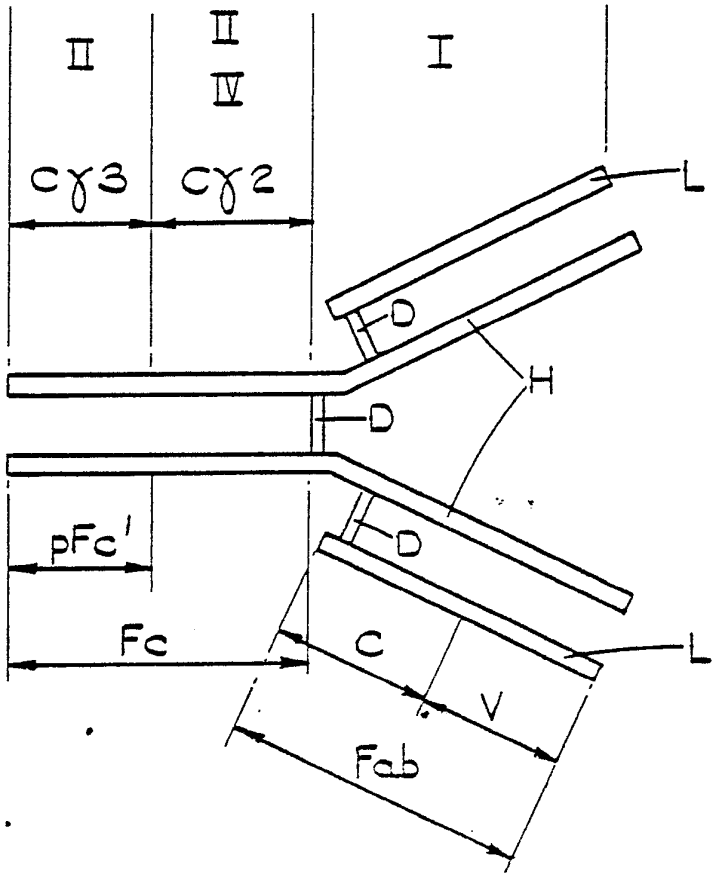

FIG.1.

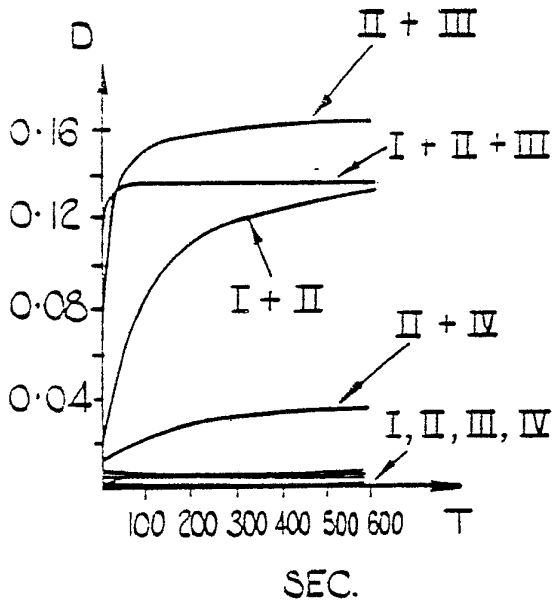

FIG.2.

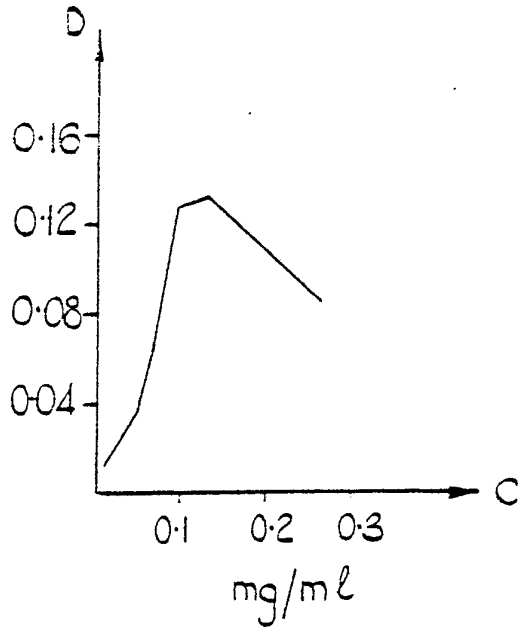

FIG.3.

INTERNATIONAL SEARCH REPORT

International Application No PCT/GB 81/00136

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [3]

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.Cl.[3]: G 01 N 33/54

II. FIELDS SEARCHED

Minimum Documentation Searched [4]

Classification System	Classification Symbols
Int.Cl.[3]:	G 01 N 33/54; G 01 N 33/56; A 61 K 39/395

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [5]

III. DOCUMENTS CONSIDERED TO SE RELEVANT [14]

Category *	Citation of Document, [16] with indication, where appropriate, of the relevant passages [17]	Relevant to Claim No. [13]
X	Chemical Abstracts, vol. 94, no. 11, published March 16, 1981 (Columbus, Ohio, US), R. Jefferis et al.: "Quantitation of human total IgG, kappa IgG and lambda IgG in serum using monoclonal antibodies" see page 537, column 2, abstract no. 81882e, J. Immunol. Methods, 1980, 39(4) 355-362	1,2
P	The Journal of Immunology, vol. 126, no. 2, published February 2, 1981 (Baltimore, US), V. Quaranta et al.: "Serological and immunochemical characterization of the specificity of four monoclonal antibodies to distinct antigenic determinants expressed on subpopulations of human Ia-like antigens", pages 548-552, see the complete article	1,2
P	Gastroenterology, vol. 79, no. 5, part 2, published in November 1980; J.R. Wands et al., "Identification of epitopes on HB_5Ag polypeptides by analysis with monoclonal anti-HB_5 antibodies", page	2 ./.

* Special categories of cited documents: [15]

"A" document defining the general state of the art

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IV. CERTIFICATION

Date of the Actual Completion of the International Search [3]	Date of Mailing of this International Search Report [3]
6th October 1981	22nd October 1981

International Searching Authority [1]	Signature of Authorized Officer [20]
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III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, [16] with indication, where appropriate, of the relevant passages [17]	Relevant to Claim No [18]
	1063, see the complete article	
A	Gastroenterology, vol. 77, no. 5, published in 1979, J.R. Wands et al.: "Production and characterisation of monoclonal antibodies to hepatitis B surface antigen (HB_sAg) by cellular hybridisation", page A46	
A	Nature, vol. 256, published August 7, 1975, G. Köhler et al.: "Continuous cultures of fused cells secreting antibody of predefined specificity", pages 495-497	