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QUANTITATION AND PURIFICATION OF ANTIBODIES AND ANTIGENS BY IMMUNOADSORBENTS

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Abstract - For the last two decades immunoadsorption technique has been widely used for quantitation and preparative isolation of antibodies and antigens. The procedure usually involves immobilization of antibodies or antigens (ligands), adsorption of counter-ligands, washing from nonspecific proteins, and elution. For preparation of immunoadsorbents, the ligands are fixed on insoluble supports, cross-linked by bifunctional reagents or mechanically entrapped in inert polymers. Two types of carriers are often used: agarose in a beaded form (Sepharose) and small cellulose particles. The immunoadsorbents prepared on these matrices are stable, have high capacity and minimum nonspecific adsorption. The ligands fixed on the small cellulose particles are tightly packed and prove to be useful for the batch experiments. The beaded agarose possesses good flow characteristics and is very efficient for the isolation of one or another counterpart of the antigen-antibody reaction by column technique. The fixed antigens have been recently used for specific fractionation of lymphoid cells. In such experiments the antigens fixed on nylon fibers are especially effective.

INTRODUCTION

Nowadays the immunoadsorption method (or immunospecific affinity chromatography) has found a wide application in many fields of biological research. This is accounted for by a variety of reasons, and first of all by a wide use of immunological approaches and methods in solving different problems of molecular biology and bioorganic chemistry. By means of immunoadsorption it is possible to determine the quantity of antibodies or antigens and to isolate them in a pure form. Another important problem which can be achieved by the method is the elimination of a counter-ligand from a composite mixture. This methodological approach is very effective for removal of a particular type of antibodies from the polyvalent immune serum or of a given antigen from the tissue homogenate. Presumably, this method may also be useful for elimination of the toxic substances from blood of patients.

IMMUNOADSORBENTS

The main element of the immunoadsorption method is antigen or antibody directly polymerized by cross-linking agents or covalently coupled to the insoluble carrier (matrix). Good immunoadsorbents fulfil the following requirements: nonspecific adsorption should be negligible and specific capacity high, a fixed ligand should not leak into solution and the carrier should be chemically and mechanically stable. For successful use of column technique the immunoadsorbent must have good flow characteristics. If small volumes of solutions are investigated by batch technique the immunoadsorbent should pack well during centrifugation.

Beginning from the 1920-1930's many different materials have been used as insoluble supports for adsorption or chemical fixation of antigens as well as antibodies (for review of the early investigations see refs.1 and 2). In the last years the agarose derivative-Sepharose activated with cyanogen bromide (BrCN) has found a wide application in the immunochemical studies. Such adsorbents were first used in the Porath laboratory (3,4) and the method was modified afterwards (5). It has several advantages: the pre-

paration of immunoadsorbents with this matrix is rather easy, their capacity high and the nonspecific adsorption minimal. Finally, the high flow properties of the adsorbents are most suitable for column operations. The Swedish firm Pharmacia produces the BrCN-activated Sepharose as commercial product and this also contributes to the popularity of immunoadsorbents with Sepharose matrix. The immunoadsorbents with similar properties can be easily prepared from aminohexyl-Sepharose after its activation with glutaraldehyde (6).

Small cellulose particles prepared according to Gurvich (7) have been successively used as a matrix for the past fifteen years. The immunoadsorbents prepared with this carrier also have high capacity and low nonspecific adsorption, pack very well and are adequate for analytical as well as preparative batchwise experiments (8). For polymerization of macromolecular antigens bifunctional agents are employed, ethylchloroformate and glutaraldehyde frequently among them (9). The preparation of cross-linked antigens is simple and the products obtained have the highest capacity per unit volume. Crosslinking of protein antigen by glutaraldehyde can be performed in the presence of rigid particles of polyester (diameter less than 10 μm, specific surface 80-100 m^2/g). The resulting fine suspension of particles coated by the cross-linked protein is useful particularly for large scale operations employing batch technique (10). It must be taken into account that chemically aggregated immunoglobulin G has a significant capacity to bind immunoglobulins G nonspecifically (11).

Proteins possess many different functional groups (12) and their fixation on matrices could be achieved in several ways, for instance, on diazo-cellulose, on BrCN-activated Sepharose, on polyacrylamide beads using glutaraldehyde or water soluble carbodiimide. Before coupling polysaccharides have to be modified this way or another to obtain free amino groups, and thereafter they are coupled to the activated Sepharose. According to another method polysaccharides are first treated with periodate leading to appearance of the highly reactive aldehyde groups. These can react with the NH_2 groups of the carriers (13). For fixation of nucleic acids the newly developed aminooxyadsorbents (14) could be used even in the presence of proteins. Nucleic acids are coupled to matrices also in other ways (15). The method of immobilization of low-molecular ligands depends on their chemical structure. In some cases a spacer (bridge-compound) is introduced between the fixed ligand and the matrix. It contains one or more reactive groups and is used to increase the distance between the ligand and the matrix. This additional space can facilitate the interaction between the counter-parts of the immunological reaction.

The main steps of the immunoadsorption procedure are: 1) adsorption of antibody or antigen (counter-ligand) from solution, 2) washing of the complex of counter-ligand and matrix from nonspecific proteins, 3) elution of the bound counter-ligand, and 4) separation of the eluted counter-ligand. The whole procedure is performed very easily by column technique. If batch method is used the separation of immunoadsorbents from washings and eluate is achieved by short centrifugation. Separation of the immunoadsorbent is simple if it has magnetic properties. For example, magnetic polyacrylamide-agarose beads containing iron oxide could be easily isolated from the liquid phase with the aid of a magnet (16). The solution is then removed while the magnetic beads remain on the test tube walls, and the time-consuming centrifugation is not necessary at all.

For desorption of the counter-ligand bound to the adsorbent the specific or the nonspecific methods are applied. The specific method consists in displacement of the antihapten antibodies with the low-molecular hapten added at high concentration. In that case however a small portion of the isolated antibodies is obtained in a complex with the hapten which is extremely difficult to remove even by a prolonged dialysis or by gelfiltration.

In many investigations the antibodies (or antigens) are eluted by addition of the buffer solutions with low pH values (pH 2.5-2.7) followed by a rapid neutralization of the eluate by strong neutral buffers. In some cases the gradient of pH is more efficient than a one-step addition of the acid buffer (17). Strong solutions of urea (6-8 M) and of chaotropic ions are effective eluants even at neutral pH. They are especially useful if the acid solutions destroy antigens. Among chaotropic ions, thiocyanate and iodide are most frequently used. Generally speaking, the properties of a given antigen-antibody system determine the choice of the desorption method (18). The effectiveness of elution depends on several factors and particularly on the

affinity of the antibodies used. A specific complex of the low-affinity antibodies with the antigen is decomposed under milder conditions (19), and for that reason it is recommended to use early immune sera of high potency (20).

QUANTITATION OF ANTIBODIES AND ANTIGENS BY IMMUNOADSORBENTS

Immunoadsorbents are a very useful tool for determination of the quantity of different antibodies and antigens. The method possesses many advantages. It is strictly quantitative since the final result of determinations is expressed in the weight units or the units of radioactivity when radioactive counter-ligands are determined. Since the covalently coupled ligand itself is insoluble, formation of a soluble antigen-antibody complex in excess of the antigen is ruled out. The method also allows to quantitate nonprecipitable antibodies. The reproducibility of determinations is good. This is largely explained by the standardization of the immunoadsorbent preparations. The same batch of the adsorbent can be used in the prolonged experiments since its suspension is stable in the cold for a very long time, especially in the presence of antiseptics. The method is simple and convenient and does not require any special equipment other than that usually found in a biochemical laboratory. The determination is rapid, and many samples can be analyzed in 2-3 hrs.

The sensitivity is dependent primarily on the method used to determine the amount of antibody or antigen adsorbed. If the Lowry's method is used the lower limit is 1-10 μg protein. It is possible to determine very small quantities of counter-ligands if they carry a radioactive label.

One of the most thoroughly elaborated analytical methods is that using ligands coupled to small cellulose particles through diazolinkage (or amino-group) according to Gurvich (7). Immunoadsorbents of this type have been successfully used for determination of different antigens and antibodies in our laboratory for many years (8). The nonspecific adsorption of these immunoadsorbents is comparatively low and the adsorbing capacity very high. They are stable and packed well. The ratio of the immunoadsorbent pellet volume to the sample volume is usually small, and this allows to deal with small quantities of solutions doing all the procedure in the centrifuge tubes.

During analytical determinations it is necessary to take into account the following considerations.

1. The results can be accepted with confidence only if the quantity of adsorbent used in the test is sufficient to remove all antibodies (antigens) from the sample. As a rule, during the work with the solutions containing an unknown quantity of antibodies (antigens) the ratio of the adsorbent and the adsorbing counter-ligand should be determined in a preliminary test with different dilutions of the sample, or the sample is adsorbed by several fresh portions of the immunoadsorbent, and finally the results of all determinations are summarized.

2. The nonspecific adsorption on the cellulose immunoadsorbents is usually small. To reduce it the samples ought to be centrifuged at a high speed. It is also highly recommended to add a control immunoadsorbent to the sample several times before the addition of the experimental immunoadsorbent. In that case only the adsorption in the last control test is considered as a true value of the nonspecific adsorption. Since the volume of the cellulose adsorbent pellet is small the losses of the sample are usually negligible despite the repeated additions of several portions of adsorbent.

3. Another methodological problem is the solubilization (leakage) of a small part of the fixed ligand during a long-term storage. For that reason the immunoadsorbent suspension has to be washed several times by saline immediately before the experiment to remove the minute quantity of the leaked antigen (antibody).

By immunoadsorption method it is possible to solve some other problems. For example, using specific immunoadsorbents one can estimate relative immunogenicity of one or another part of the antigen molecule. In our previous experiments (21) we determined relative immunogenicity of the peptide chains and the proteolytic fragments of immunoglobulin G. To this end the specific capacity to adsorb antibodies by the cellulose fixed peptide chains and the

fixed fragments from the anti-immunoglobulin G serum was measured quantitatively. The results obtained proved the fact that the antigenic determinants are distributed relatively uniformely among the subunits of the immunoglobulin G molecules under study.

As is mentioned above the elution of the antihapten antibodies is achieved by solutions of the corresponding hapten. By different concentrations of hapten and using successive elution it is possible to obtain fractions with different affinity properties. If the antibodies are radioiodinated, all the procedure can be performed analytically. The fractions obtained could be used for further investigations, for example, for identification of their allotypes (22).

For the quantitation of the antigens by immunoadsorbents several different approaches are used. The immobilization of antibodies is achieved by their cross-linking or by fixation on the insoluble supports either directly (23) or through antibodies bound to a fixed antigen (sandwich-adsorbent) (24). The sensitivity of test will be, of course, higher if the antigen carries a radioactive label. In this case a portion of immunoadsorbent with the bound antigen is put directly into a counting vial after washing and subsequent drying. The bound nonradioactive antigen can be treated by antiserum containing corresponding antibodies labeled with radioactive iodine. The amount of the radioactivity bound will indicate the quantity of the adsorbed antigen (25).

The quantitation of antigens can be performed indirectly measuring the inhibition of binding of the radioactive antigen to the matrix-bound antibodies (26-28). The dependence of inhibition on the quantity of the added unlabeled antigen must be found in the preliminary test. The inhibition of radioimmunoadsorption can reveal very small antigenic differences between proteins. If the unlabeled protein to be studied slightly differs in its antigenic properties from the radioactive test protein, subsequent binding of the radioactive antigen to the homologous immobilized antibodies cannot be completely prevented with the unlabeled protein, even if it presents in a large excess. This method is particularly suitable for comparing the antigenic properties of a large number of proteins, e.g. for detecting the allotypic antigenic differences (22).

The antigen under study presents either in a free form or in a complex with other antigen(s). By corresponding antibody immunoadsorbents it is possible to find what portion of antigen is free and what is bound to other antigen(s). In that way we have found how many light and heavy immunoglobulin peptide chains synthesized in a cell free system are in a free and in a complexed form (23). To this end, the supernatant of the incubation mixture was divided into two equal parts, the first part being initially treated with the anti-light chain adsorbent and then with the anti-heavy chain adsorbent, and the second part undergoing a reverse order of treatment. Evidently, in both probes the first adsorption indicated to the existence of the chain complexes and the second adsorption pointed to the amount of the free chains.

Several modifications of immunoadsorption technique have been developed for the measurements of the reaginic antibodies belonging to immunoglobulins E. To this end the anti-immunoglobulin E antibodies are fixed on agarose or bromacetyl cellulose and the quantity of immunoglobulin E in sera of the allergic patients is determined usually by indirect inhibition assay (3, 29).

To perform simultaneously a large number of determinations all the procedures can be automatized. For example, such technique has been developed for the solid-phase double-antibody radioimmunoassay for the β_2-microglobulin and allowed processing of a more than thousand samples daily. The method can also be applied for other antigens (30).

PREPARATIVE ISOLATIONS OF ANTIBODIES AND ANTIGENS

The ligands fixed on small cellulose particles are also successively used for the preparative isolations of antibodies or antigens by batch technique. Naturally, it is very convenient to perform not only analytical determinations but also large scale isolations with the same set of immunoadsorbents.

To apply column technique it is convinient to use the ligands coupled with agarose beads. In these experiments the Sepharose activated with BrCN is used more frequently. Clear advantages of the above-mentioned method have won wide popularity for the Sepharose immunoadsorbents nowadays (3,4,31). For the last years the antibodies against many proteins, polysaccharide and synthetic antigens are purified by Sepharose immunoadsorbents (for review see ref. 10). This method is also effectively applied for isolation of the antibodies to single antigenic determinants of the protein molecules as,for example, to the "loop" peptide of lysozyme (32).

The column technique is especially useful if fractionation of the isolated antibodies is to be performed simultaneously. To this end the pH gradient (33) or different salt concentrations (34) can be applied. If an antigen builds up from the repeating subuntis (polyaminoacids or polysaccharides) the fractionation is carried out by successive additions of oligomers of the increasing length (35). The antibody molecules of the separated fractions differ from each other by their combining sites.

The antibody preparations isolated by immunoadsorption technique are not identical to the whole spectrum of the antibody molecules present in the original immune serum. Denaturation of a small part of the isolated antibodies is not the only reason for that. The measurements of affinity of the purified antihapten antibodies and the serum supernatants indicate that in the isolated preparations the high affinity molecules are absent (36). These molecules bind to the immunoadsorbent so strongly that only concentrated denaturating agents - such as guanidinium chloride - are effective for elution. For that reason the final results of the analytical measurements must be evaluated according to the total amount of the protein adsorbed, but not according to the amount of the eluted protein.

The quantity of isolated antibodies is not always proportional to the amount of the coupled antigen in a volume unit of immunoadsorbents. It is more likely that the antibody molecule will react with the both combining sites if the density of the antigen molecules on the immunoadsorbent surface is very high. The bigamic antibody interaction with the multivalent antigen is much stronger than the interaction with the monovalent antigen of the same specificity (37), and the elution of the antibodies may appear even impossible due to the cooperative effect of the multivalent attachment of the antibodies to the adsorbent. Hence the increase of the amount of the fixed antigen is not always effective.

The isolation of antigens by the immunoadsorbents is far more complicated partly due to the lower capacity of the fixed antibodies as compared to that of the fixed antigens. Nevertheless, during the last years the method has been successively applied for purification of the antigens of different nature including virus antigens (3,10). In some cases immunoadsorption technique is especially effective. For example , with the matrix- bound antibodies the isolation of a peptide can be performed from a tryptic hydrolysate of the selectively modified or the affinity-labeled proteins. Givol et al. (38) have isolated DNP-peptide from the combining site of the anti-DNP antibodies on the anti-DNP agarose after affinity labeling. The problem of isolation of the specific and the modified peptides has been recently reviewed by Wilchek (39). The best eluant in these experiments was 1M NH_4OH since it does not change very much the capacity of the antibody immunoadsorbent. For a better yield it is recommended to saturate the immunoadsorbent to its maximal binding capacity (20).

ISOLATION OF LYMPHOCYTES AND THEIR ANTIGEN RECEPTORS

Lymphoid cells have antigen specific receptors on their surface, and during the last years several immunological laboratories have brought the immunoadsorbents into use for separation of a particular type of lymphocytes from other cells. This approach has been described by Wigzell and Andersson (40) and later elaborated by Wofsy and coworkers (41) who made use of polyacrylamide beads as a carrier. Cells of immunized animals as well as normal lymphocytes are selectively bound on the fixed antigens placed in the columns and after washing eluted by passing the solution of specific low-molecular antigen (hapten). If there is a free antigen in the column it competes with the fixed antigen, and specific retention of the cells does not take place.

Edelman and Rutishauser (42) have developed a very effective procedure

according to which the cells bind to nylon fibers derivatized with the antigen molecules. This method has several advantages (43). The bound cells can be visualized, quantitated and manipulated individually and released mechanically into solution by plucking the fibers with a needle. For adsorbtion of the cells the antigen-coated nylon fibers are put directly into the cell suspensions. Successive additions of fibers with different antigens allow effective fractionation of the lymphoid cells according their specificity. Competition between the free antigen and the fiber-fixed antigen can be used for selective adsorption of the cells according to their affinity. This method is less harmful to the cells than the passing through columns. The fibers may be modified in different ways to introduce a variety of molecules between the fixed antigen and the matrix which allows the removal of the bound cells by chemical or enzymatic cleavage (42).

One of the most intriguing problems of the present-day cellular immunology is the nature of the antigen recognition system(s) of T-lymphocyte cells. The search for the T cell antigen receptor(s) is being intensively performed in several laboratories. Isolation of the receptors should be the first step of such studies, and to this end specific immunoadsorbents are used (44,45). The results obtained are very encouraging. As is shown the variable region of the heavy chain is an integral part of the T cell receptors. Neither the constant regions of the heavy or the light chains nor the variable region of the light chains have been found in the isolated material.

CONCLUSION

Our brief discussion of the immunoadsorption technique shows that it has become a very important and powerful tool for the immunological researches. Beyond question the method will contribute a lot to different areas of molecular and cellular biology as well as bioorganic chemistry since quantitation and purification of any immunogenic chemical compound and cells with specific receptors can be easily achieved in that way. All the procedure is characterized by simplicity, effectiveness, specificity, and in some cases can be automatized.

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