0273-2289/86/1302-0133$02.60

REVIEW

Immunoaffinity Purification of Subcellular Particles and Organelles

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Received October 7, 1985; Accepted November 18, 1985

ABSTRACT

The application of immunoaffinity techniques to subcellular fractionation is reviewed and the basic principles underlying the various methods that have been successfully employed, identified. The requirement for organelle-specific antigens and high-avidity antibodies is discussed, as is the widespread use of indirect immunoadsorbents. Approaches for the optimization of immunoaffinity-based subcellular fractionation are suggested.

Index Entries: Immunoaffinity; immunoadsorption; immunoisolation; subcellular fractionation methods; subcellular particles, immunoaffinity purification of; organelles, immunoaffinity purification of; subcellular fractionation, application of immunoaffinity techniques to.

INTRODUCTION

Immunoaffinity techniques for subcellular fractionation are attractive, in principle, since they rely on biological differences (i.e., the expression of different antigens) between subcellular organelles. This is in contrast to classical centrifugation techniques (*1,2*) and, also, more recent methods, including electrophoretic separation (*3*) and polymer-phase partition systems, (*4*) all of which depend on differences in physical parameters (e.g., size, shape, density, charge, and hydrophobicity) between the organelles to be separated. In addition, immunoaffinity tech-

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niques offer the prospect of fast purification in high yields and may be particularly valuable in situations in which only a small amount of cell material is available (e.g., tissue culture cells). They also have the advantage of being applicable in physiological salt solutions and do not expose the organelles to unusual media nor to osmotic and electric shock. Although it is some time since an immunoaffinity approach to subcellular fractionation was first discussed (*5*) and successful methods for particular organelle isolation were reported (*6–8*), it is only recently that immunoaffinity techniques have been used widely and successfully in a variety of biological systems. Thus, it is now possible to review these techniques, discuss the differences in approach, and identify those parameters that have caused the most difficulty in establishing successful protocols. In this article we discuss the requirement for antibody and for the solid-phase matrix to which antibody is covalently coupled. In addition, we comment on methods of maximizing the yield and purity of the subcellular organelle to be isolated.

ANTIBODY REQUIREMENTS

Immunoaffinity techniques for subcellular fractionation can be divided into the use of either direct immunoadsorbents, in which anti-organelle antibody is itself covalently bound to the solid phase, or indirect immunoadsorbents, in which an antibody-binding reagent is covalently bound to the solid phase, providing an immunoadsorbent capable of isolating the antibody–organelle complex (Fig. 1). In practice, direct immunoadsorbents have rarely proven successful (but, *see* refs. *8,9*), and it has been argued by analogy with affinity chromatography that an indirect technique is required either to provide a more-flexible spacer arm or for steric reasons, because of difficulties inherent in the binding of two large particles. The antibody binding reagent coupled to the solid support may be either an anti-immunoglobulin second antibody (polyclonal or monoclonal) or protein A from *Staphylococcus aureus*, which binds to the F_c portion of many immunoglobulin molecules (*10*). Occasionally, fixed, heat-inactivated *S. aureus* bacteria are themselves used as the solid support (*11–13*). The use of the indirect technique makes it possible to standardize the solid phase for use in the preparation of many organelles with different antibodies.

The choice of first and second antibodies may be dictated by availability, although it is often possible to incorporate some element of design into the choice. In principle, the first antibody should be specific to the organelle or membrane fragment of interest, and be of high affinity. In practice, polyclonal antibodies prepared to isolated organelles are unlikely to be specific because of the impurity of the organelle preparations and because common antigens exist on different membranes. This may not prove an impossible obstacle, since, for example, in isolating plasma

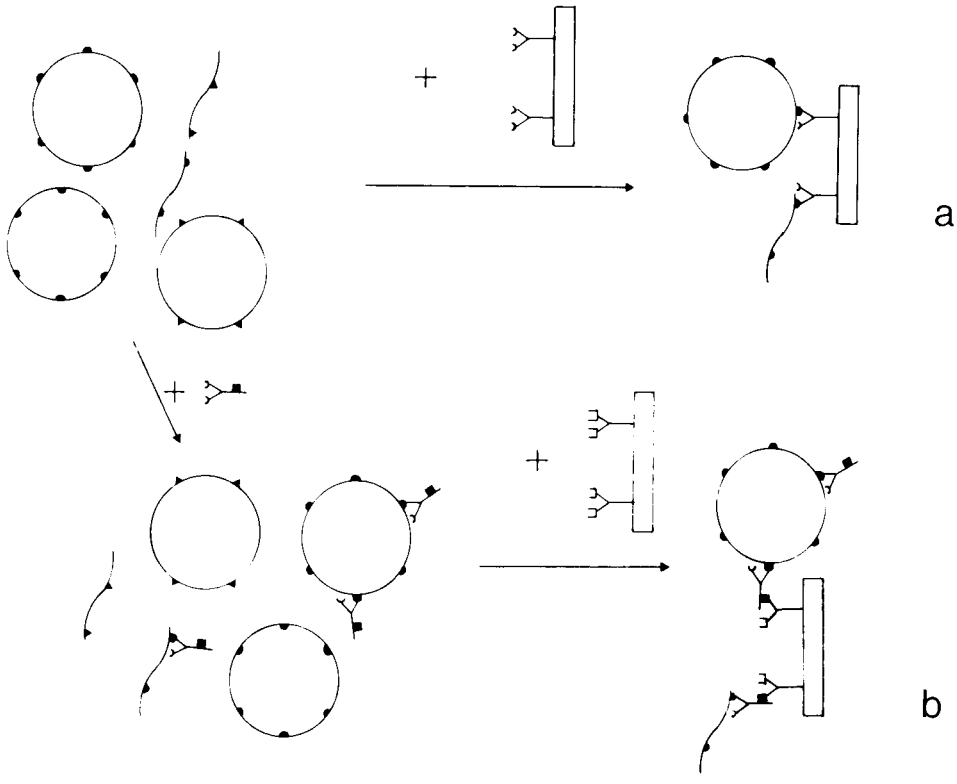

Fig. 1. Schematic representation of direct (a) and indirect (b) immunoaffinity techniques for the preparation of subcellular organelles and membranes. In the direct method (a), the homogenate or partially purified subcellular fraction is reacted with the anti-organelle antibody immobilized to give a solid-phase immunoadsorbent. In the indirect method (b), the free anti-organelle antibody is reacted with the homogenate (or fraction), the unbound antibody washed away, and the organelle then isolated on an immunoglobulin-binding immunoadsorbent. In the diagram (b), this immunoadsorbent is shown as an anti-immunoglobulin antibody coupled to a solid phase and capable of reacting with an antigenic site (■) on the F_c region of the anti-organelle antibody. The diagram shows that both direct and indirect immunoaffinity techniques will select only those membranes and organelles showing the correct antigenic topography, ignoring those with other antigens or those that are "inside out."

membrane, a polyclonal antiserum can be reacted with intact cells before homogenization so that only the plasma membrane is antibody labeled (*7*). Performing such an operation in the cold prevents endocytosis of antibody (*14*), and the kinetics of antibody binding with fast on rates and slow off rates militates against the subsequent redistribution of antibody.

Polyclonal anti-organelle antibodies may be prepared to specific antigens (protein, lipid, or carbohydrate) and/or be immunoaffinity purified on the antigen before use. Such purification considerably improves the specificity of the reagent and removes unwanted immunoglobulin that

will interfere with the use of the solid-phase antibody-binding immunoadsorbent. Removal of the nonbound immunoglobulin after the first antibody step normally necessitates washing, but the use of a suitable dilution of affinity-purified antibody may preclude the need for this. The use of affinity-purified anti-organelle antibodies also allows for the possibility of a single-purification step, since they may be prebound to the anti-immunoglobulin immunoadsorbent before incubation with the cell homogenate or partially purified subcellular fraction (*15*).

A modification of using a polyclonal antiserum directed at an endogenous component of an organelle is to alter the antigenic structure of the organelle. In the case of the plasma membrane, this has been achieved by haptenization of the cell surface (*16*) and then using antihapten antibodies to isolate membrane proteins (though not intact membrane vesicles) and by incorporating a viral-protein antigen (*13*). In the latter case, the viral surface glycoprotein-G of vesicular stomatis virus was incorporated into the red blood cell membrane simply by lowering extracellular pH and the membranes then isolated by reaction with anti-G protein antibodies and anti-IgG immunoadsorbent. Since the amount of viral protein incorporated into the membrane can be titrated, and it freely diffuses two dimensionally in the plane of the phospholipid bilayer, it was possible to show that indirect immunoaffinity isolation of the membrane was dependent on the antigen density, being >50 molecules/μm^2. This may be a general rule (*13,17*) and of importance in selecting an antigen for preparation of the anti-organelle antibody. It may also allow immunoaffinity isolation techniques to be used to prepare membranes and organelles that are particularly rich in an antigen that is present at lower concentration elsewhere.

Monoclonal antibodies directed to organelle antigens may be useful first antibodies for immunoaffinity isolation. Unlike polyclonal antisera, which are unlikely to contain >5% of total IgG directed against a specific antigen, a monoclonal antibody can be prepared as a pure chemical reagent. However, in any set of cell fusions for the preparation of monoclonal antibodies, it can be expected that the majority of such antibodies will be of low affinity ($Ka \leq 0.5/nM$), reflecting the fact that in the range of antibodies produced in a polyclonal antiserum, it is unusual to have more than 25% that are of high affinity ($Ka \geq 10/nM$) (*1*· Such high-affinity antibodies are likely to be of most use for subcel··lar fractionation. It is not known whether the fact that monoclonal antibodies each react with only one antigenic site affects their general use as first antibodies for immunoaffinity isolation, though it should be possible to overcome any difficulties by using mixtures of monoclonal antibodies. A more serious objection to their use is the problem of a suitable screening protocol with which to select antibody-secreting hybridomas. Proof that an antibody is directed to a specific organelle may require the use of immunofluorescence microscopy or even immunoelectron microscopy, which can be time consuming, but simpler protocols using binding as-

says to organelle and membrane fractions have been developed and may be satisfactory.

Further development is likely in generating good anti-organelle antibodies for subcellular fractionation. Since more antigens specific to particular organelles are described, they can be isolated and used to produce specific antibodies. Moreover, antibodies directed against specific polypeptide epitopes may be prepared, using synthetic peptides (*19,20*) or cDNA cloned in bacterial expression vectors (*21,22*).

The requirement for the solid-phase immunoadsorbent to have a high binding capacity is discussed below and to a great extent depends on the chemical nature of the solid phase. However, the anti-immunoglobulin second antibody used also determines the binding capacity. Ideally, this antibody should be of high affinity, react with many immunoglobulin subclasses, and be available in large amounts. Appropriately, selected monoclonal antibodies are particularly useful since essentially unlimited quantities of antibody can be produced from a single hybridoma cell-line. For some species of polyclonal first antibody (e.g., sheep and rabbit), suitable mouse monoclonal second antibodies are available, and, if mouse monoclonal first antibodies are used, rat anti-mouse IgG monoclonal antibodies may be coupled to the solid support. *Staphylococcus aureus* protein A, used on the solid support, binds to some species of immunoglobulin better than others (*10*) and is especially poor at binding sheep and mouse IgG, though binding of the latter can be improved by using slightly alkaline pH (*23*). Since protein A binds to the F_c portion of immunoglobulins, directional single-step immunoadsorbents may be prepared by reacting with affinity-purified polyclonal antibodies and may be stabilized by chemical cross-linking (*24*).

In any specific immunoaffinity protocol for isolating an organelle or membrane fragment, the time course and dose–response curve for binding of first antibody and immunoadsorbent must be experimentally determined to find the most appropriate conditions for specific isolation and high yield in the shortest possible time.

THE NATURE OF THE SOLID SUPPORT

The choice of solid support to be used in the affinity purification of subcellular particles is determined by a number of criteria; the most important of which are high binding capacity, nonporous nature, adequate spacer arms, low nonspecific binding, ease of separation from unbound particles, and availability.

It is recommended, for a number of reasons, that immunoadsorbents of high binding capacity be used to isolate subcellular particles. For example, if the particle to be purified has been sensitized with a primary antibody (as is usual), there will be some free, unbound antibody present, despite extensive washing. Such free antibody will

then compete successfully with particle-bound antibody for binding sites on the immunoadsorbent. In addition, the binding of the immunoadsorbent and the subcellular particle is facilitated by the presence of potential binding sites over the entire surfaces of both. In contrast, one potential advantage of low-capacity immunoadsorbents is that elution of bound, subcellular particles may be easier, since extreme conditions (e.g., high or low pH, chaotropic ions) may not be required (*25*). However, elution is usually not necessary, since most immunoaffinity-purified particles [e.g., membranes and synaptosomes (*7,26*)] retain their normal biochemical functions while attached to the solid support.

The binding capacity of any immunoadsorbent is governed largely by the number of attachment sites for the IgG-binding molecule (hereafter referred to as the second antibody) and the degree of inactivation suffered during covalent bonding to the solid support. A problem encountered using one of the most popular methods, CNBr-activated supports, has been the multisite attachment of antibody molecules, resulting in their inactivation. Schneider et al. (*24*) surmounted this problem by prior attachment of protein A to the support (Sepharose) and subsequent binding of second antibody to the protein A, followed by covalent linking of the two, thus producing a high-capacity immunoadsorbent in which most of the second antibody molecules were oriented in the optimal direction for affinity binding. The capacity of this immunoadsorbent was consequently limited solely by the number of reactive groups present on the original support. This number can differ widely with different supports, e.g., Affi-Gel 10 (BioRad) and CNBr-activated Sepharose (Pharmacia) have approximately 15 mμ equivalents of active group/g, whereas cellulose, diazotized by the method of Gurvich et al. (*27,28*), has approximately 150 mμ equivalents/g.

The most favored supports for affinity purification of subcellular particles have been nonporous (e.g., Sepharose 6MB, polyacrylamide, cellulose) since these give the greatest surface-binding capacities. Although porous supports, e.g., Sepharose 4B, give high total-binding capacities, most of the binding sites are within the sepharose beads and so unavailable for the binding of relatively large subcellular organelles.

Table 1 illustrates the binding capacities of a number of immunoadsorbents used for subcellular fractionation. All except that used by de Kretser et al. (*29*) are nonporous, the highest capacity (as determined by IgG binding) being obtained with a monoclonal second antibody coupled to cellulose, modified by the method of Gurvich et al. (*27,28*), as described by Hales and Woodhead (*30*).

One of the greatest limitations of classical subcellular fractionation has been the heterogeneous (i.e., contaminated) nature of the individual fractions. Immunoaffinity techniques provide an approach that allows far greater degrees of purification. The amount of contamination must be assessed, both as the nonspecific binding of organelles to the immunoadsorbent in the absence of the primary antibody and also from the amount

TABLE 1
Immunoadsorbent Binding Capacities[a]

Matrix	Immunoglobulin binding reagent	Amount of immunoglobulin binding reagent coupled to solid support, μg/mg	Immunoglobulin binding capacity of immunoadsorbent, μg/mg	Purified particle	Reference
Sepharose 6MB	Protein A	3.4	≤14	Lymphocytes	25
Sepharose 4B	MAb	3.3	≤6.6	Lymphocytes	29
Polyacrylamide	Polyclonal (AP)	2	≤4	Synaptic membrane[b]	32
Polyacrylamide	Polyclonal (AP)	3	≤6	Golgi membranes	15
Polyacrylamide	Polyclonal (AP)	2	≤4	Erythrocyte membranes	13
Polyacrylamide	Protein A	15	≤75	Synaptic vesicles	40
Polyacrolein microspheres (magnetic)	Polyclonal (AP)	40	≤80	Cultured cell membranes	31
Cellulose	Polyclonal	300	≤1	Fat cell plasma membranes	7
Cellulose	MAb	287	250	Synaptosomes	39

[a]AP, affinity purified; MAb; monoclonal antibody. Where the IgG-binding capacity was not quoted, affinity-purified polyclonal and monoclonal antibodies were assumed to bind 2 μg IgG/μg anti-IgG. Polyclonal antibodies were assumed to bind 0.02 μg IgG/μg anti-IgG, and protein A 5 μg IgG/μg protein A.
[b]Binding capacity taken from ref. (*13*).

of a given contaminant that copurifies with the particle. Nonspecific binding to supports is determined both by the quantity of the support used and the amount of protein already coupled. Although there are a number of ways of calculating the efficiency of any given adsorption process (*31*), the most informative is the ratio of specific:nonspecific binding, which illustrates the degree of purification achieved (Table 2). This ratio falls with increases in the ratio of support to particle used in the adsorption process (*32*). Consequently, high-capacity immunoadsorbents tend to increase the efficiency of the process by reducing the amount of solid support required.

Immunoadsosrbents are designed to facilitate the separation of bound and free particles. There are three commonly used methods of separation; column, centrifugation, and magnetism. Each separation method has its own advantages and disadvantages.

Column-based methods are often used to separate bound and free cells or organelles, using supports with good flow properties, e.g., Sepharose. The greatest restriction on the use of column methods is the constant danger of organelles becoming trapped in the immobile support (*33*). One way around this has been the binding of organelles to the solid suppport in the column, washing, followed by removal from the column support, and rewashing in a batch method (*34*).

Centrifugation methods are popular because most of the commonly used supports (Sepharose, cellulose, and polyacrylamide) are pelleted at low centrifugation speeds. Rapid separation and washing protocols have therefore been devised for the preparation of many subcellular organelles (*see* Table 3). Centrifugation protocols do, however, suffer from a number of disadvantages. For example, when optimizing any given affinity-purification procedure, it is tempting to aim for very-high-capacity immunoadsorbents and low solid support:organelle ratios. This can lead to very high binding or organelle to matrix, thus, considerably modifying the particle density and destroying the separation protocol (Richardson and Phillips, unpublished data). Such problems can be avoided by either increasing the solid support:organelle ratio or by using an alternative separation procedure, e.g., magnetism.

Magnetic-immunoaffinity purification of subcellular particles is achieved by using either solid supports containing magnetic iron or by ferritin labeling of the particles, i.e., ferritin covalently linked to antibodies or lectins could be used for the isolation of cells or organelles (*35*). One of the greatest advantages of magnetic-based separation is the ability to maintain the magnetic particles in suspension within a magnetic field (*31*). The resultant free-flow system should then give a significant reduction in the nonspecific binding. A variety of magnetic solid phases has been developed, including polyacrolein (*31,36*), dextran (*37*), polyacrylamide–agarose (*34*), and cellulose (*38*). However, magnetic separation in free flow may require strong magnetic fields [1 T (*37*)] or specifically designed magnetic "bottles" (*31*). Even the use of weak mag-

TABLE 2
Immunoadsorbent Purification Efficiencies

Matrix	% Binding[a]		Ratio	Purified particle	Reference
	Specific	Nonspecific			
Polyacrylamide	NR[c]	NR	7.2	Synaptic plasma membrane	32[b]
Cellulose	35	4	8.8	Fat-cell plasma membrane	7
Polyacrylamide	64	7	9.1	Golgi membranes	15
Cellulose	41	4	10.2	Liver-cell plasma membrane	41
Cellulose	48	4	12.0	Islet plasma membranes	42
S. aureus cells	73	5	14.6	Bile canalicular membrane	43
Sepharose	21	0.7	30.0	Lysosomes	9
S. aureus cells	68	2.2	31.0	Erythrocyte membrane	13
Polyacrylamide	76	2.0	38.0	Prelysosomes	44
Polyacrylamide	7	0.1	70.0	Erythrocyte membranes	13
Cellulose	17	0.2	85.0	Cholinergic synaptosomes	39
Cellulose	70	0.6	115.8	Fao-cell plasma membrane	17

[a]In cases in which more than one marker was assessed, the major marker of interest was used.
[b]Figure taken for a ratio of 7.5:1 for support:synaptosomes (Fig. 3 of this ref.) The ratio quoted is the binding ratio, i.e., specific:nonspecific. It should be noted that different authors incubated different input fractions with immunoadsorbent.
[c]NR Not reported.

TABLE 3
Organelles and Particles Successfully Isolated Using Immunoaffinity Techniques

Organelle	Cell type	Reference
Plasma-membrane		
From isolated cells	Rat adipocytes	*6,7,45*
	Rat hepatocytes	*41*
	Rat islets of Langerhans	*42*
Membrane domains	Rat liver	*43*
After insertion of viral protein		
Right-side-out and inside-out vesicles	Human erythrocytes	*13*
Apical membrane domain	Cultured canine kidney cells (MDCK)	*13*
From cultured cells	Cultured rat hepatocytes (Fao)	*17*
Neuronal components		
Presynaptic plasma membrane	Marine ray electric organ	*32*
Cholinergic synaptosomes	Rat brain	*39*
Synaptic vesicles	Rat brain	*40*
Adrenergic vesicles	Cultured pheochromocytoma cells	*40*
Endocytic pathway components		
Clathrin-coated vesicles	Porcine brain	*11*
	Bovine brain	*12*
Endosome-derived vesicles	Rat hepatocytes	*14*
Prelysosomes	Rat liver	*44*
Lysosomes	Rabbit liver	*9*
Endoplasmic reticulum microsomes	Rat liver	*8*
Golgi	Rat liver	*15*

nets in a batch process can still be advantageous, since density differences between particle and support are unnecessary.

CONCLUSIONS

A wide variety of subcellular organelles and membrane fractions have been isolated, using immunoaffinity techniques (Table 3). Their specificity, speed (fractions usually prepared within 2 h of adding antibody), and versatility enable immunoaffinity techniques to be a useful addition to the available battery of subcellular fractionation methods. Increasingly, the methods are used to prepared fractions that cannot be separated with conventional approaches. Thus, for example, cholinergic nerve terminals (synaptosomes) can be separated from all other species

present in the complex homogenates of mammalian brain (*39*). Another striking example is the separation of clathrin-coated vesicles carrying different transmembrane proteins, using an indirect immunoaffinity technique, with specific monoclonal antibody gaining access to the cytoplasmic tail of the target protein through the clathrin coat (*12*). A further advantage of the techniques is the ability to select right-side-out membrane vesicles (Fig. 1), which may be of importance in subsequent functional studies. Inside-out vesicles may also be selected, using a suitable antibody, and, in one study, both right-side-out and inside-out plasma-membrane vesicles were selected from erythrocytes after incorporating a viral glycoprotein into the membrane and separately using antibodies directed at its extracellular and cytoplasmic domains (*13*).

The versatility of immunoadsorbent techniques can be increased by their use in conjunction with more conventional methods of subcellular fractionation. Thus, antibodies can be added to fractions after their preliminary isolation by centrifugation methods, and, on some occasions, contaminating organelles can be removed with immunoadsorbent (*14*).

The present status of immunoaffinity approaches to subcellular fractionation allows some general recommendations to be made; first, that indirect immunoaffinity techniques are most likely to be successful. It is important to use a specific and high-avidity anti-organelle antibody (either monoclonal or affinity-purified polyclonal) directed to an antigen present at high density. The immunoadsorbent should be of high capacity, with a solid support capable of covalently binding large quantities of protein, and if a second antibody is used, this should be of high avidity and available in large amounts. Clearly, the solid support should demonstrate low levels of nonspecific binding. Incubation and washing conditions are also of importance and should be gentle to prevent disruption of organelles and vesiculation of membranes (*31*). The availability of more antibodies specific to particular organelles and better immunoadsorbents will lead, no doubt, to an even more widespread use of immunoaffinity techniques.

ACKNOWLEDGMENTS

We thank Professor C. N. Hales and Drs. K. Siddle and K. Howell for many useful and stimulating discussions. Experimental work by the authors reported in this article was supported by grants from the Medical Research Council, the British Diabetic Association, and Beit Foundation, St. John's College Cambridge, and Muscular Dystrophy Group of Great Britain and Northern Ireland.

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