

PURIFICATION OF *COWDRIA RUMINANTIIUM* BY IMMUNOADSORBENT AFFINITY CHROMATOGRAPHY

A. W. H. NEITZ and N. M. J. VERMEULEN, Department of Biochemistry, University of Pretoria, Pretoria 0002

ABSTRACT

NEITZ, A. W. H. & VERMEULEN, N. M. J., 1987. Purification of *Cowdria ruminantium* by immuno-adsorbent affinity chromatography. *Onderstepoort Journal of Veterinary Research*, 54, 247-253 (1987).

Immunoselective methods with special reference to immuno-adsorbent affinity chromatography as a means for the isolation of *Cowdria ruminantium* are reviewed. Attention is given to the source of the organism, immunization, purification of antibodies, coupling of antibodies to insoluble matrixes and desorption procedures.

INTRODUCTION

Immuno-adsorbent affinity chromatography is based upon biospecific interaction between immobilized antibodies or antigens and antigens and antibodies, respectively. The principles of affinity chromatography are reviewed by Vermeulen, Neitz & Viljoen (1987). In this review the application of immunoselective methods for the isolation of *Cowdria ruminantium* is discussed. The need to obtain pure *C. ruminantium* organisms has been enumerated (Viljoen, Vermeulen, Oberem, Prozesky, Verschoor, Bezuidenhout, Putterill, Visser & Neitz, 1985; Neitz, Vermeulen & Viljoen, 1987).

The selectivity of immuno-adsorbent affinity chromatography is as the result of antibody molecules that are directed against antigenic determinants (Campbell, Luescher & Lerman, 1951; Jagendorf, Patchornik & Sela, 1963).

Antibody reagents are used either to specifically select and purify a particular component from a heterogeneous mixture (positive selection) or to deplete a heterogeneous mixture of contaminating components (negative selection). The application of these approaches to the purification of *C. ruminantium* is depicted schematically in Fig. 1.

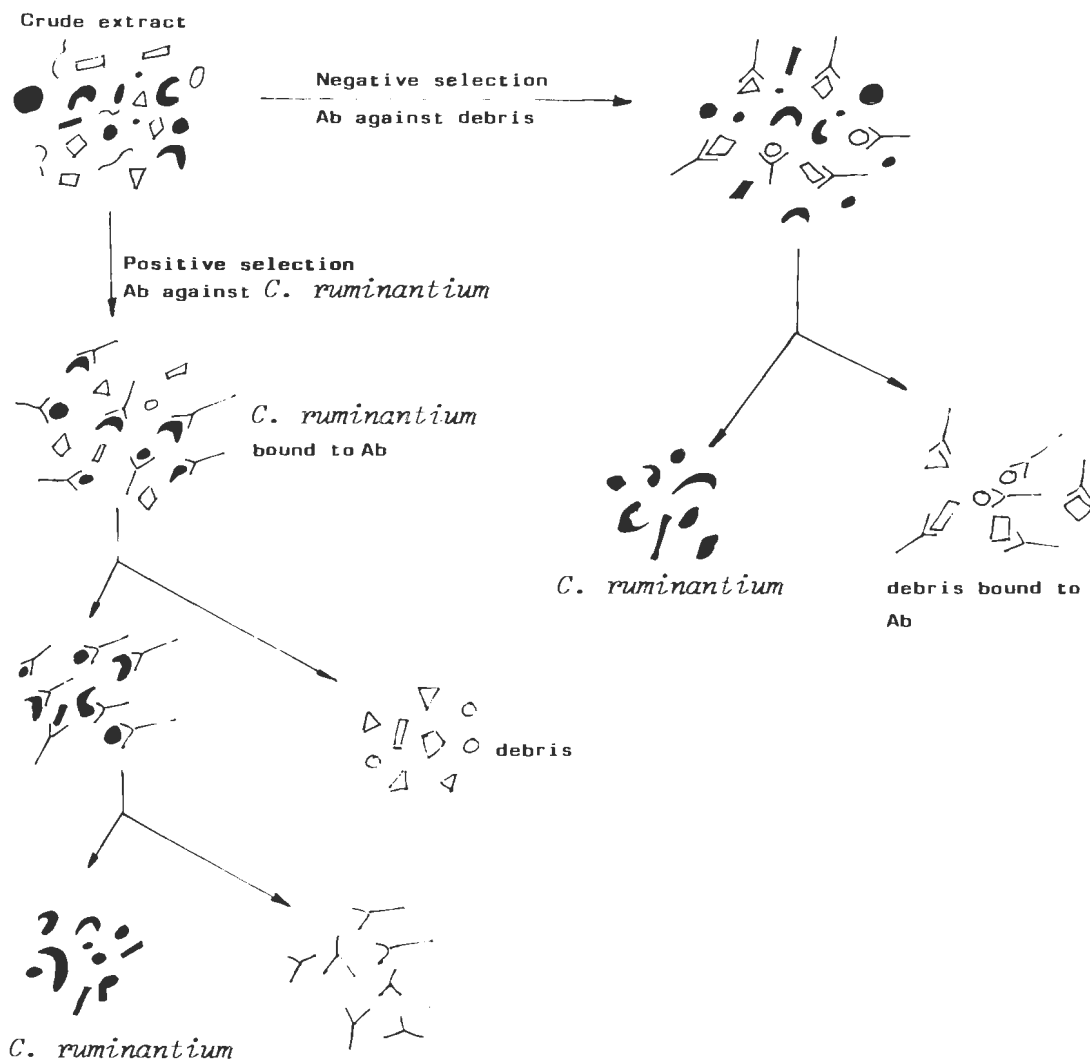


FIG. 1 Schematic depiction of positive and negative immunoselective methods for the purification of *Cowdria ruminantium*

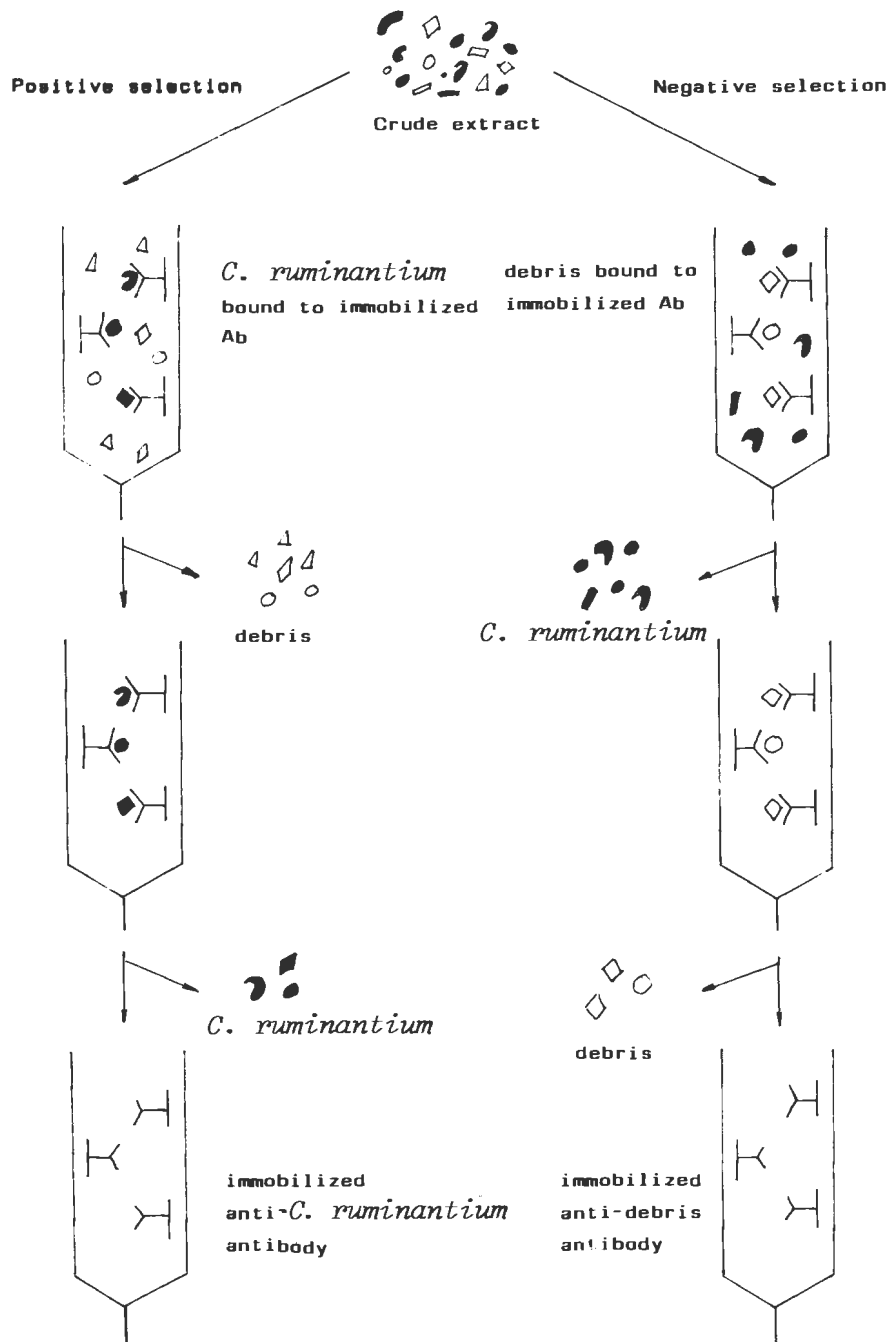


FIG. 2 Schematic representation of the use of immunoadsorption affinity chromatography for the purification of *Cowdria ruminantium*

With the positive selection method it may be difficult to recover bound antigen since desorption conditions may be detrimental to the antigen. This is especially true for labile bound cells. Methods which may overcome this difficulty are dealt with below. Whatever the method employed, the antibody-antigen complex must be separated from the sought after antigen or contaminating antigens (Fig. 1). This is greatly facilitated by insolubilizing the antibody. The antibody in its insoluble form is known as an immunoadsorbent (Jagendorf *et al.*, 1963) and may be packed into a column. Its use in antigen purification is known as immunoadsorbent affinity chromatography or immunoaffinity chromatography (Wilchek, 1984). Conversely, for the purification of antibodies, the antigen is insolubilized (Campbell *et al.*, 1951). Alternatively, antibody or antigen can be adsorbed or coupled onto fibres and meshes (Edelman & Rutishauser, 1974), polystyrene tissue culture dishes

(Mage, 1984), magnetic beads (Hubbard, Schluter & Marchalonis, 1984) and polyurethane foam (Evans, Mage & Peterson, 1969; Mage, Evans & Peterson, 1969). In addition, antisera can be induced to form gels by treatment with aqueous solutions of glutaraldehyde (Ladipo & De Zoeten, 1971).

APPLICATION OF THE METHOD

The use of immunoadsorbent affinity chromatography as a means for the isolation of *C. ruminantium* is shown schematically in Fig. 2.

It is evident that several steps are involved in immunoaffinity chromatography:

1. Finding a suitable source of the organism.
2. Immunization and purification of antibodies.
3. Coupling of antibody to the insoluble matrix.

4. Desorption of the organism (positive selection) or contaminating antigen (negative selection) to regenerate the column.

These steps are discussed below.

Preparation of a *C. ruminantium* source

Infected tick tissue (Bezuidenhout, 1981), brain (Viljoen *et al.*, 1985), blood fractions (Neitz, Viljoen, Bezuidenhout, Oberem, Visser & Vermeulen, 1986), mouse tissue (Du Plessis & Kümm, 1971; Du Plessis, 1982; Mackenzie & McHardy, 1984) or endothelial tissue cultures (Bezuidenhout, Paterson & Barnard, 1985) should be suitable sources of the organism for purification by means of immunosorbent affinity chromatography. It is imperative to test the sources for proteolytic activity as these may lead to digestion of the immobilized antibodies (Calton, 1984).

Preparation of antiserum and purification of antibodies

Immunization protocols to obtain suitable antiserum is well documented (Johnstone & Thorpe, 1982; Hudson & Hay, 1983). However, adjustments need to be made to meet the requirements of the method employed and the nature of the antigen to be purified. For negative selection methods, it is imperative to employ antibodies with high affinity and high avidity, whereas for positive selection methods this could render desorption of antigens difficult (Calton, 1984).

Progressive increase of antibody-antigen association constants have been shown to occur with time after immunization (Eisen & Siskind, 1964). This phenomenon has been termed the maturation of the immune response (Siskind & Benacerraf, 1969). Affinity maturation has been shown to be the result of changes in the cell population producing antibody rather than selective removal of high affinity antibody by the excess antigen present early in the response (Steiner & Eisen, 1967). The temporal changes in the affinity of antibody is affected substantially by the nature of the immunogenic stimulus. The antigen-depot effect produced by immunization with Freund's complete adjuvant results in a slow release of antigen over extended periods and in sustained maturation of affinity (Steward, 1977). Thus immunization in saline results in the relatively rapid elimination of the antigen and subsequent termination of stimulation by the antigen (Steward, 1977). The antigen dose is also known to influence the affinity of the antibody produced. Immunization with large doses of antigen in Freund's complete adjuvant results in a reduction in the rate of maturation of affinity compared to that when small doses are administered (Eisen & Siskind, 1964). This effect has been ascribed to the stimulation of also low affinity cells in the presence of excess antigen. Furthermore, in such situations it may be possible that the high affinity cells may be rendered tolerant (Steward, 1977).

In addition to the time factor, dose rate and nature of the immunogenic stimulus, it is possible that other variables determine the affinity of antibody. They include genetic factors, reticulo endothelial system function, dietary factors, quantitative and qualitative aspects of lymphocyte function and the effects of free antibody, antigen or immune complexes (Steward, 1977).

From these observations, it is clear that the obtainment of antibodies with suitable affinity may require much effort. This is especially true for the negative selection method for which numerous antibodies with distinct characteristics are needed.

With monoclonal antibodies many of the problems can be overcome since it is possible to select the degree of affinity that is desired (Calton, 1984). Purification of immunoglobulins from antiserum for immunoabsorbent

affinity chromatography is desirable to ensure a high capacity immunoabsorbent with minimum non-specific adsorption. This is achieved by ammonium sulfate or sodium sulfate precipitation followed by dialysis and anion-exchange chromatography (Livingston, 1974; Johnstone & Thorpe, 1982). Alternatively, Protein A coupled to cross-linked agarose beads may be used. Protein A has the property of binding the Fc region of Ig, usually IgG from mammalian species, with high specificity (Kronvall & Williams, 1969; Lindmark, Thoren-Tollig & Sjoquist, 1983). Alternatively, bifunctional affinity gels (Lowe & Pearson, 1984) containing diethylaminoethyl or carboxymethyl exchange groups and Cibacron Blue F3GA (DEAE Affi-Gel Blue and CM-Affi-Gel Blue from Bio-Rad respectively) may be used. These gels effectively remove proteases in serum. A comparison of ion-exchange antibody purification methods are summarized in Table 1 (Bio-Rad Catalogue, L., 1986).

TABLE 1 Comparison of ion exchange purification methods for IgG. (Bio-Rad Catalogue, L., 1986)

Method	Yield of IgG (%)	Protease activity
DEAE cellulose	60	positive
Ammonium sulfate, DEAE cellulose	65	positive
DEAE Affi-gel Blue	65	negative
CM Affi-gel Blue, ammoniumsulfate	90	negative

Coupling of antibody to insoluble support

Ideally the support or matrix should meet the following requirements (Bio-Rad Technical Bulletin No. 1099, 1983):

1. Be porous enough to allow both the antigen and the antibody to penetrate the pores. For cell separation, it is imperative that the matrix should pack evenly, leaving sufficient space between beads of the matrix for cells to pass through freely without being physically trapped (Pharmacia Technical Bulletin, 1984);
2. be stable to all reagents used in the coupling and elution steps;
3. contain no chemically reactive groups after coupling and should show minimum non-specific adsorption;
4. for use in a chromatographic column, the matrix should have good flow properties which are determined by the size, shape and rigidity of the matrix particles. Good flow properties are important if labile components (for example, cells) are to be eluted in the shortest possible time.

Numerous supports for immunoabsorbent chromatography are available for attachment of antibodies and other proteins. These are generally in a bead form since they pack evenly and allow uniform flow. Plastic, (Hubbard *et al.*, 1984), glass (Abdou & Richter, 1969), polyacrylamide and agarose beads (Cuatrecasas, 1970) have been used. In addition, gelatin (Schlossman & Hudson, 1973), cellulose (Campbell *et al.*, 1951) starch and cross-linked polysaccharides (Axen, Porath, Ernback, 1967) have been employed.

The procedure for coupling the antibody to a particular matrix will depend on the chemical groups available on the support and the amino acid residues involved in the coupling. Examples are coupling to cellulose, starch and cross-linked dextran by means of cyanogen halides (Axen *et al.*, 1967), organic sulfonyl chlorides (Nilsson & Mosbach, 1984), disulfides (Stephan, Gallop & Smith, 1966; Sweet, Stephan & Smith, 1974), N-hydroxysuccinimide esters (Wilchek, Miron & Kohn, 1984) and s-triazinyl compounds (Kay & Lilly, 1970).

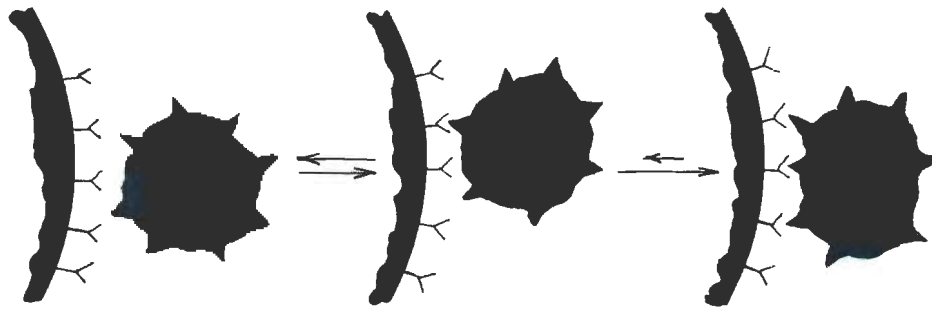


FIG. 3 Formation of multiple interaction between cell and antibody leading to very tight binding

Whatever the method employed, the degree of antibody substitution on the matrix will largely determine the success of antigen purification. For negative selection the level of antibodies must be high so to allow adequate adsorption of contaminating antigens. For positive selection the level must be low enough so that desorption of cells is not too difficult. The total antibody valency can be varied by changing the antibody concentration in the coupling solution and/or by mixing the antibody and irrelevant proteins in different proportions (Hubbard *et al.*, 1984).

Desorption of bound antigens

Various methods have been used to desorb bound components. Methods which do not involve dissociation of antibody-antigen complexes include digestion of the matrix, for example, dextran matrixes with dextrase (Schlossman & Hudson, 1973) and gelatin with collagenase (Webb, Teitelbaum, Rauch, Maoz, Arnon & Fuchs, 1975). If reducible disulfide bonds have been

used for the coupling of antibody to the matrix, these can be cleaved by mercaptoethanol (Kiefer, 1975) or alkaline buffers (Stephan *et al.*, 1966). All of these approaches result in the liberation of immuno-complexes which need to be dissociated to obtain free antigen. Furthermore, they do not permit the regeneration of the immuno-adsorbent.

Direct dissociation of the antigen-immunoadsorbent complex thus has advantages. For positive selection methods, the conditions used for dissociation should obviously be as mild as possible. This can pose many problems because of the generally strong antigen-antibody association with association constants higher than $10^{10} M^{-1}$ in some cases (Steward, 1977). The binding of antibody to antigen may become tighter with time (Campbell *et al.*, 1951). This can be a substantial problem in the case of cells through multi-point attachments to the adsorbent (Pharmacia Fine Chemicals Bulletin, 1984) as pictured in Fig. 3.

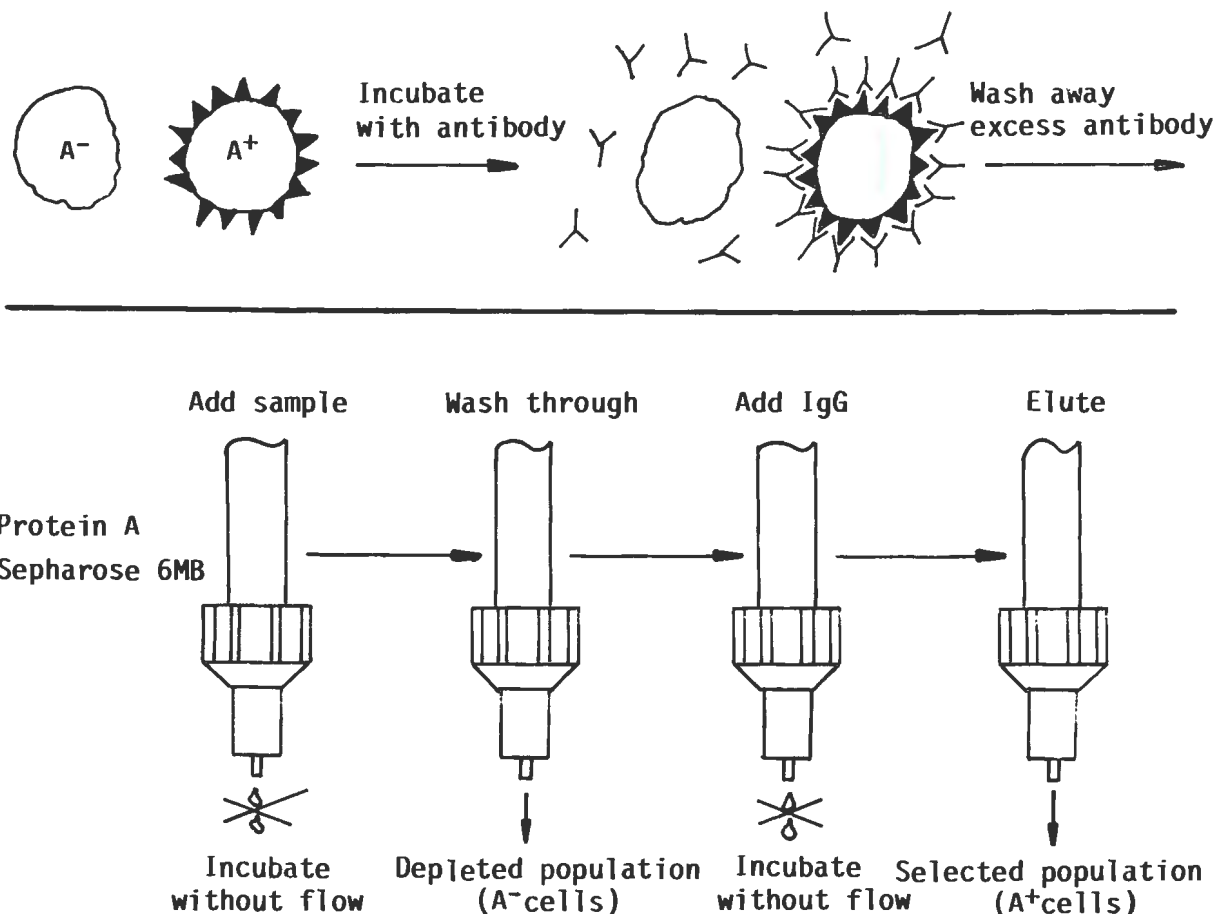


FIG. 4 Separation of cells bearing a specific surface antigen (A^+ cells) from cells lacking the antigen using Protein A-Sepharose 6MB. (Pharmacia Fine Chemicals, 1984)

Although it has been observed that the association constant between an antibody and antigen is decreased when the antigen (or antibody) is insolubilized (Ternynck & Avrameas, 1971), desorption of cells from immunoadsorbents can be problematic. The antibody-antigen interaction probably involves hydrophobic, ionic and hydrogen bonds (Dandliker, Alonso, De Saussure, Kierzenbaum, Levison & Schapiro, 1967).

Prediction of conditions suitable for dissociation is difficult since the degree of association is dependant upon the number of the bonds and proportion of the hydrophilic and hydrophobic groups involved (Cohn & Ferry, 1943). Furthermore, the stability of these bonds are affected in opposite ways by ionic strength, type of ions and temperature (Hofstee, 1976). Thus, electrostatic interactions are decreased by increasing ionic strength, whereas hydrophobic bonding is increased. The latter bonding is reversed by polarity-reducing or chaotropic agents whereas electrostatic interaction is not. In addition, hydrophobic bonding is diminished with a decrease in temperature. The opposite is true for electrostatic interaction (Hjerten, Rosengren & Pahlman, 1974).

Methods for the desorption of antigens, antibodies and cells from immunoadsorbents have been the objective of several studies (Dandliker *et al.*, 1967; Boegman & Crumpton, 1970; Ternynck & Avrameas, 1971; Crom-

well, 1973; Ghettie, Mota & Sjoquist, 1978; Basch, Ber- man & Lakow, 1983). Chaotropic ions, acid and high ionic strength were investigated. In addition, mechanical treatment involving stirring (Manderino, Gooch & Stavitsky, 1978) or vortexing (Marshak-Rothstein, Fink, Gridley, Raullet, Bevan & Gefler, 1979) have been used. It has also been reported that elution may be easier at 37 °C than at lower temperature (Nicola, Burgess & Metcalf, 1978). Competitive elution is a gentle specific method for the elution although the released antigen is liberated as the antigen-antibody complex (Wilchek, 1984).

Alternative immunoadsorbent methods for the purification of cells

(a) Chromatography with insolubilized Protein A

When covalently coupled to a suitable support, protein A can be employed to adsorb cells that have been coated with specific antibody of the IgG type (Ghettie *et al.*, 1978). Desorption of bound cells may be achieved by competitive elution using excess soluble IgG (Fig. 4).

(b) Chromatography with immobilized anti-lectin antibodies

In this method antibodies directed against a suitable lectin are coupled to a matrix to provide a lectin specific immunoadsorbent.

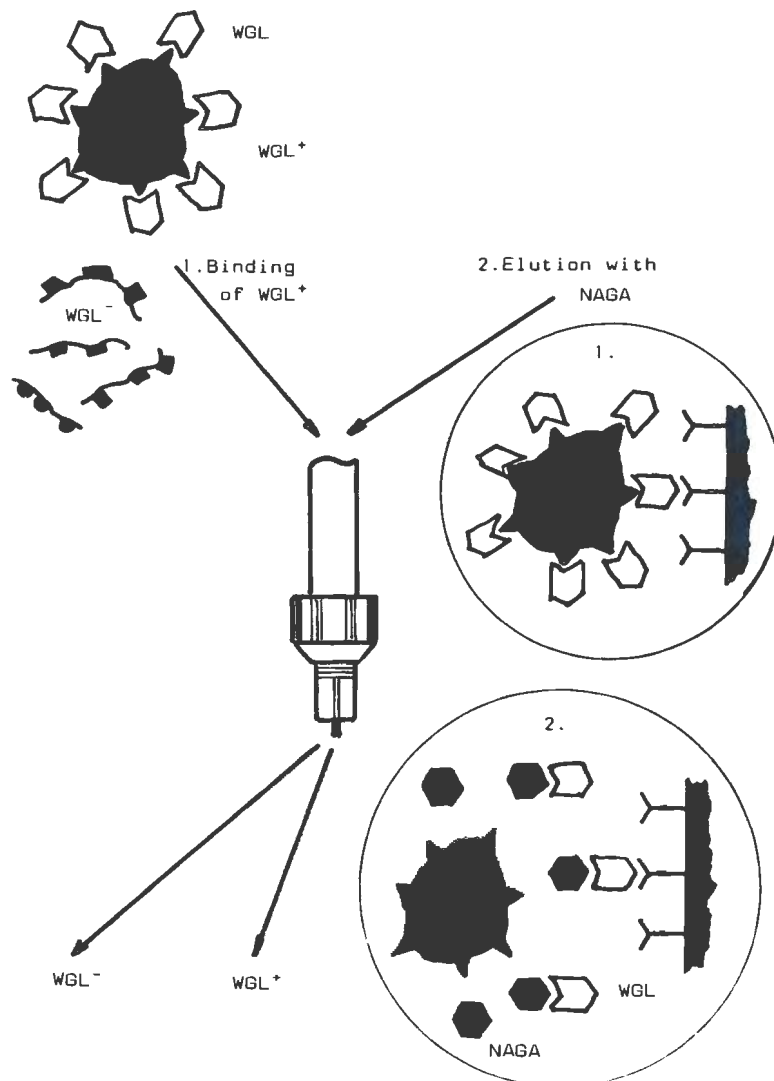


FIG. 5 Purification of wheat-germ lectin binding cells (WGL⁺) from non binding components (WGL⁻) by immobilized antibodies directed against wheat-germ lectin (WGL). NAGA: N-acetyl-D-glucosamine (Pharmacia Fine Chemicals, 1984)

Lectin binding cells are covered by treating them with free lectin and applying these cells to the column (Irle, Piguët & Vassali, 1978). Desorption is achieved by means of excess carbohydrate for which the lectin is specific. The potential application of this method for the isolation of *C. ruminantium* is depicted in Fig. 5.

(c) Chromatography with immobilized avidin

Biotinylated antibodies were used to label cells which are then removed by passing them over immobilized avidin (Jasiewicz, Schoenberg & Mueller, 1976). The binding has however been shown to be essentially irreversible. Basch *et al.* (1983) have prepared antibodies which were covalently modified with biotin analogues which have substantially lower binding constants with respect to avidin. Desorption is achieved with authentic biotin.

FUTURE PROSPECTS

Although the isolation of *Cowdria ruminantium* by immunoadsorbent affinity chromatography has to date not been successful, the method offers distinct advantages. These include, selectivity, mildness and short time required for the actual isolation. With the optimization of immunization protocols and ideally through use of monoclonal antibodies with carefully selected affinity, pure populations of the organism from various sources should be feasible.

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