FERRITIN-CONJUGATED PROTEIN A

A new immunocytochemical reagent for electron microscopy

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1. Introduction

Ferritin-conjugated antibodies have been used to locate specific antigenic sites on cell surfaces [1—3] and on the surfaces of subcellular organelles [4]. However there have been difficulties in the use of ferritin—Immunoglobulin G (IgG) conjugates prepared with bifunctional reagents such as glutaraldehyde or toluene 2,4-diisocyanate. Such conjugates generally exhibit reduced antibody activity relative to the original antiserum sample. Thus either high antibody titers or preliminary purification of the Ig may be necessary to ensure that the residual activity of the conjugate is sufficient. In some cases high titers are difficult to obtain and specific purification may provide only low yields and/or select for antibody of low affinity.

Culture filtrates of some strains of *Staphylococcus aureus* contain a protein A which specifically binds to the Fc portion of the IgG molecule from many mammalian species [6]. Because of this property, ferritin coupled covalently to protein A could be expected to react with IgG bound specifically to antigen sites at the Fab regions of the antibody molecule. This type of indirect label would:

1. Avoid reduction in the amount of activity of antibody by conjugation.

2. Depend upon the immunologically specific uptake by IgG by specific antigen.

3. Be useful for IgG molecules from several mammalian species.

2. Materials and methods

Protein A was purchased from Pharmacia Fine Chemicals, Uppsala. Ferritin (EM Grade) was purchased from Polyscience, Warrington, PA.

Ferritin, 10 mg, was mixed with 2.5 mg protein A in 0.25 ml 0.1 M sodium phosphate buffer (PB), pH 7.2. Glutaraldehyde 50 μl, 0.25% v/v was added with gentle stirring, and the mixture was incubated at 23°C for 60 min. The mixture was then dialyzed against 100 ml 0.1 M ammonium carbonate, pH 8.8, for 4 h at 4°C to block any unreacted free aldehyde groups in the mixture. The solution was then dialyzed against 4 liters PB for 12 h at 4°C.

To separate unreacted protein A, the mixture was made to 10 ml with 0.1 M PB and spun for 2 h in a Spinco L-2 ultracentrifuge at 78 000 × *g* on a cushion of 50% v/v glycerol. The supernatant fluid, containing

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protein A, was discarded, and the pellet and glycerol containing ferritin and ferritin conjugated protein A was dissolved in 1 ml 150 mM NaCl, 10 mM sodium phosphate pH 7.2 (PBS) and dialyzed for 12 h against 2 liters PBS.

In order to specifically purify the ferritin–protein A conjugate, 5 g CH–Sepharose 4 B was conjugated with 200 mg rabbit IgG using carbodiimide [6] and a 1.5 × 10 cm column prepared. The column was washed with 80 ml 0.1 M glycine–HCl buffer pH 2.3 (GB) until the absorbance at A_{280} was below 0.01. The column was then washed with 80 ml 0.5 M NaCl containing 0.05 M citric acid buffer, pH 5.6. The column was finally equilibrated with PB.

Of the ferritin plus ferritin–protein A conjugate mixture, 1 ml was added to the column and unbound material eluted with PB. The eluate was washed until A_{440} fell below 0.01. This eluate contained free ferritin and any conjugate unable to bind to IgG.

The column was then washed with GB which eluted a distinct yellow fraction from the Sepharose matrix. The eluate was immediately neutralized, to pH 7.2, with 0.5 M Na_2HPO_4 and dialyzed for 12 h at 4°C against 1 liter PBS. The solution was concentrated by centrifugation as described above and finally dissolved in 1 ml PBS.

Washed cells from nutrient agar slant cultures of an asporogenous strain of *Bacillus megaterium* strain KM were injected intravenously into rabbits to prepare antiserum. Preimmunization bleedings were used to provide normal serum (and normal IgG). IgG was recovered from serum by three ammonium sulfate precipitations at 40% saturation and finally suspended at 20 mg/ml in PBS.

Cells from a nutrient agar slant were scraped into PBS and sedimented for 1 min in an Eppendorf centrifuge. The cells were washed 5 times to remove residual medium, resuspended in 2 ml PBS and divided into 2 samples. To 1 sample, 5 ml immune serum or 10 mg IgG were added and to the control sample 5 ml non-immune serum or 10 mg normal IgG were added and allowed to react for 15 min at 23°C. The cells were sedimented in an Eppendorf centrifuge for 1 min and washed with PBS until supernatant fluid A_{280} was less than 0.01.

To each of the cell suspensions (in 1.0 ml PBS), 0.05 ml ferritin conjugate was added and allowed to react for 15 min at 23°C. The cells were sedimented in the Eppendorf centrifuge and washed until A_{440} (the maximum absorption of ferritin) was less than 0.01.

The cells were fixed by adding 1 ml 2% v/v glutaraldehyde in 0.1 M sodium phosphate buffer, pH 7.2, washed twice with buffer and stained for 60 min at 4°C with a solution of 2% v/v osmic acid in PB and block stained with 1% w/v uranyl acetate in 25% ethanol. Cells were dehydrated in graded ethanol (25%, 50%, 80%, 95% and 100%), washed with propylene oxide and embedded in Spurr resin [7] in Beem capsules.

Light gold to silver sections were cut on a Sorvall MT-2 ultramicrotome and mounted on bare 400 mesh copper grids. No post stain was used to enhance contrast. Specimens were examined in a Philips EM 300 operated at 40 kV.

3. Results and discussion

Figure 1A shows a *B. megaterium* cell reacted with non-immune serum and then with the ferritin–protein A conjugate. Only several electron dense ferritin particles were seen on the cell envelopes. Cells in fig.1B were reacted with immune serum and subsequently with the conjugate. Numerous electron dense particles of ferritin can be seen surrounding the envelope of the bacterium. The ferritin molecules of the conjugate are considered to have reacted with the Fc portion of IgG molecules combined specifically with the cell envelope. Since only several particles were observed on cells reacted with non-immune serum, it was concluded that:

1. Very little non-specific uptake of conjugate to bacterial cells did occur. The binding of conjugate required the presence of immune IgG.

2. No appreciable non-specific binding of normal IgG to the bacterial cells occurred (or if such did occur the Fc portions of such molecules were not available for reaction with the conjugate).

Non-specific binding has been a continuing problem with the immunoferritin technique, and it has been necessary to use highly purified ferritin. For this procedure we have found Polyscience EM grade ferritin satisfactory without further purification.

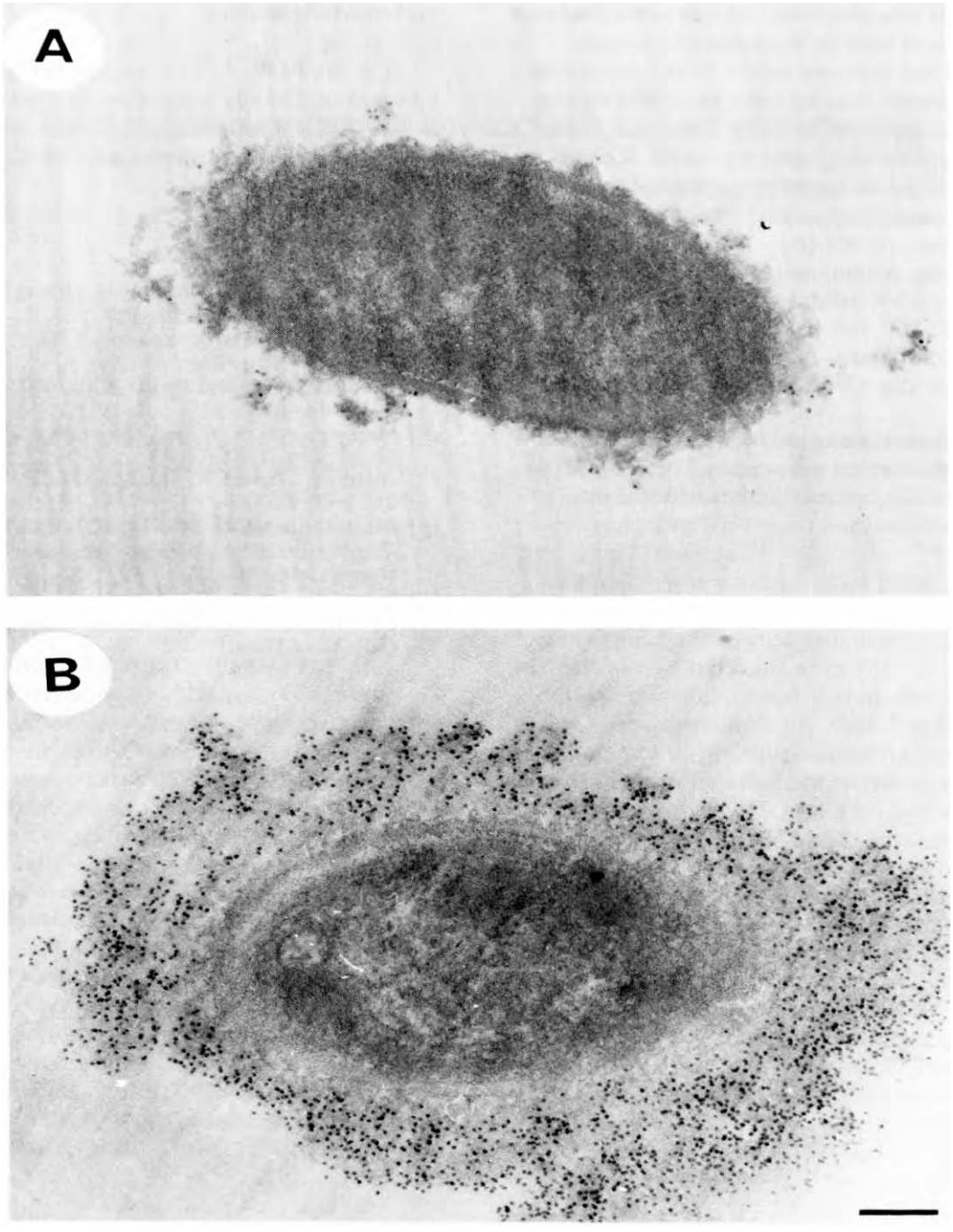

Fig.1A. *Bacillus megatarium* strain KM cell treated with nonimmune serum then with ferritin conjugated protein A. Very few electron dense ferritin particles can be seen on the cell envelope. Fig.1B. *B. megatarium* strain KM cell treated with immune serum then with ferritin conjugated protein A. A wide zone of ferritin particles can be seen surrounding the cell envelope. Total magnification 100 000 ×. Bar is 100 nm.

The use of this two-step method has several advantages over the direct ferritin conjugated antibody technique. Since the immune serum is not subjected to chemical treatment, one can use even sera of low titer, and the separation of Ig from the serum is not necessary. The ferritin-conjugated protein A can be prepared and purified in quantity, and used for antibodies of various specificities and, indeed, from various mammalian species [6].

Ferritin–protein A conjugates can be stored in the presence of 0.2% (w/v) sodium azide or filter sterilized and stored at 4°C. It is felt that this procedure can be widely applied particularly in view of the ease with which controls for the specificity of the reaction are carried out.

A similar conjugate using peroxidase conjugated to protein A might also be prepared to amplify antibody reactions using standard diaminobenzidine reactions [8].

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