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Contents of Volume 1

Contents of Volume 2

Contents

The Significance of the "Dying Back" Process in Experimental and Human Neurological Disease

J. B. CAVANAGH

Intravascular Clotting: Focal and Systemic

GEORGE D. PENICK AND HAROLD R. ROBERTS

Experimental Production of Malformations of the Limbs by Means of Chemical Substances

BERTHE SALZGEBER AND ETIENNE WOLFF

Teratogenic Effects of Ionizing Radiations on the Embryonic Development of the Higher Vertebrates

JEAN-MICHEL KIRRMANN AND ETIENNE WOLFF

The Use of Labeled Antibodies in Ultrastructural Studies[1]

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I. Introduction

Many controversies in the field of biology are the result of the limited resolution obtainable by light microscopy, the nonspecificity of histochemical reactions, and the lack of purity of isolates from homogenized cells. The problems of resolution were greatly reduced with the development of electron microscopy, but pictorial demonstrations of location and structure of organelles, of secretion granules, and of

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various inclusions have, in turn, given rise to new questions. It would be desirable not only to know the location and structure of cell organelles, but also to comprehend their functions and to locate their macromolecular components. The answers to many of these questions lie in the identification and localization of chemical species, whether protein, polysaccharide, nucleic acid, etc. This can be accomplished if the molecules of the chemical species under consideration are easily identifiable with the electron microscope by virtue of some characteristic pattern that is either naturally present or artificially induced. Epstein (1962) demonstrated the location of the nucleic acid in herpes virus by means of electron microscopy after enzymatic digestion with specific nucleases. Enzymes may be localized when the reaction products of histochemical procedures are sufficiently dense to electrons (Sheldon *et al.*, 1955; Novikoff, 1962; Holt and Hicks, 1962). Although the best electron microscopes are capable of resolving about 5 Å, the molecules in the tissues are usually indistinguishable from each other owing to insufficient contrast. Ferritin is perhaps the only molecule that can be identified with certainty in intact cells (Richter, 1963).

A variety of reagents have been developed to increase the ability of proteins to scatter electrons relative to the background matrix. Some of these are phosphotungstic acid, osmium tetroxide, uranyl acetate, salts of bismuth, and thallium (Gersh, 1959). In addition to being nonspecific, most of these agents also denature proteins.

In the parallel situation in light microscopy, histologists have employed a variety of stains and reagents in order to identify and localize various constituents of tissues and cells. Many of these procedures also lack specificity. The periodic-acid Schiff (PAS) reaction, for example, localizes free aldehyde groups irrespective of the origin or kind of macromolecules involved (McManus and Mowry, 1960). Unfortunately, much more than aldehyde localization has been read into the results of PAS staining reactions.

II. Antibodies as Cytological Tools

In cases in which the substance under investigation is a macromolecule against which specific antibodies may be obtained, antibodies, suitably labeled either with a fluorescent dye (Coons *et al.*, 1942) or with a radioactive isotope (Belanger and Leblond, 1946; Ficq, 1959), have been used as specific histochemical tools. Coons and his co-workers pioneered in immunohistochemical studies when they successfully conjugated antibody to fluorescein, and then used the complex as a stain for tracing antigens in tissues. Antibodies have proved to be valuable

tools for securing information concerning cell structure and function because of the sensitivity and specificity of antigen-antibody reactions. Nevertheless, the occurrence of antigen-antibody reactions that are either unrelated to the system being studied or are nonspecific could lead to erroneous conclusions. The establishment of the purity of antibodies and the removal of labeled components that can be bound nonspecifically to tissues are essential parts of the methodology (Coons, 1956; Mellors, 1959; Nairn, 1962).

The application of immunohistochemistry to electron microscopy is a natural extension of the fruitful developments that have resulted from the use of immunological techniques in light microscopy. Pepe *et al.* (1961) examined the feasibility of visualizing nonlabeled antibody-antigen precipitates directly in tissue structures. Striated muscle, reacted with anti-myosin and anti-actin, was washed to remove nonprecipitated antigen and antibody, and was examined under the electron microscope. The results were equivocal because of the insufficient electron-scattering power of the immune precipitates. Lack of recognition of individual protein molecules necessitated a search for suitable labels with high electron-scattering power, with which antibodies could be tagged.

The present review will be limited to a discussion of the relative merits and limitations of the various labels, the methods of conjugating antibodies with suitable tags, and the methods of utilizing the tagged antibodies. The use of ferritin as a label for antibodies will receive special attention because of our own experience with it. Although only a limited number of studies that utilized the ferritin-antibody technique have appeared, it is appropriate to review methods and to stress technical problems, limitations, and controls in the critical application of electron microscopy in immunopathology. Studies on ultrastructural changes associated with immune processes have been reviewed recently (Feldman, 1964).

III. Materials Possessing High Electron-Scattering Power as Labels for Antibodies

The ideal electron-scattering label for antibodies is yet to be discovered. This label would have to satisfy the following criteria (Singer, 1959; Pepe, 1961; Sternberger *et al.*, 1963): (*a*) It should be able to be attached to the antibody by stable covalent linkages in sufficient quantity, under conditions and by methods that would not cause significant denaturation of the protein, or alteration of biological activity or physicochemical properties (e.g., of solubility). The labeled product should not exhibit unspecific interactions with other proteins or chemicals (e.g.,

interactions due to charge). (*b*) The label, while possessing sufficient electron-scattering power, should not be too large, in order to avoid steric effects that might interfere with the mobility of the antibody in inter- and intracellular spaces or with its interaction with the antigen. (*c*) The label must remain stable during the rather drastic chemical procedures involved in fixation and processing of biological materials and must withstand the adverse physical conditions presented with electron microscopy, such as high vacuum and bombardment by the electron beam. The iron-rich protein, ferritin (Singer, 1959), and the heavy metals, mercury (Pepe, 1961) and uranium (Sternberger *et al.*, 1963), have been useful labels for antibodies, although each fails to satisfy one or more of the above criteria.

A. Ferritin

Singer (1959) first proposed the use of ferritin as a label for antibodies. Ferritin is a protein of molecular weight in excess of 650,000 that contains, on the average, about 23% iron in the form of ferric hydroxide micelles (Granick, 1946). It is identifiable, with certainty, by means of electron microscopy (Farrant, 1954; Richter, 1963) because of its unique properties. Farrant established that, in the ferritin molecule, the inorganic ferric hydroxide forms a core that is surrounded by a protein shell. The iron hydroxide micelles have a characteristic fine structure, and they occupy a space with a diameter of approximately 55 Å. The entire ferritin molecule, as seen in electron micrographs, has a diameter of about 105 Å, however, in the undehydrated condition, this diameter is about 120 Å. The fact that the dense inner core contains between 2000 and 3000 iron atoms in a spherical volume with a diameter of about 55 Å makes ferritin an excellent electron-scattering label that fulfills the necessary criteria except for the one imposed by the unique size and shape of the molecule. The rabbit globulin molecule, an ellipsoidal rod with major axes of approximately 40 and 250 Å (Hall *et al.*, 1959; Almeida and Cinader, 1963) and a molecular weight of about 160,000, diffuses readily through formalin-fixed tissues and cellular membranes. Ferritin-antibody-conjugates diffuse sluggishly. The chemical procedures described for the conjugation of ferritin to antibodies for ultrastructural studies are discussed in Section IV.

B. Mercury

Pepe suggested the use of mercury as a label for antibodies (Pepe, 1961; Pepe and Fink, 1961). They employed the organic mercurial, tetraacetoxymercuriarsanilic acid, which was diazotized and coupled to

the protein.[3] The coupling was variable, the maximum obtained was 32 atoms of mercury per molecule of antibody. Although this reaction involved little loss in antibody activity, other difficulties were encountered. The amount of mercury introduced was not sufficient to provide significant contrast to the antibody molecules. Furthermore, under the electron beam, mercury sublimated or formed dense globules which migrated in the section. This could be controlled only partially by sandwiching sections between layers of evaporated carbon. In addition, some nonspecific staining occurred because of the binding of mercury to sulfhydryl groups in the tissues, but this could have been prevented by pretreating the tissues with iodoacetic acid and formaldehyde; any nonspecific staining that still occurred was removed by brief washing with buffered thioglycollic acid. Pepe successfully employed anti-myosin antibodies labeled with mercury for localizing myosin in muscle. No other reports of investigations utilizing this technique have appeared.

C. Uranium

Sternberger *et al.* (1963) have reported a method for labeling antibodies with uranium. Since the direct treatment of antibodies with uranyl acetate resulted in the loss of antibody activity, the specific combining regions had to be protected by reacting the antibody with the antigen prior to the labeling procedure. The labeled antibody was later eluted from the antigen-antibody complexes by dissociation in alkaline media. In the studies of Sternberger *et al.*, rabbit antibodies were obtained against formalin-killed *Bordetella bronchiseptica*. The antibodies were removed from the crude antiserum by treatment (37°C for 4 hours and 1°C for 2 days) with alkali-treated, washed antigen suspension. The resulting antigen-antibody suspensions were washed and treated with uranyl acetate that had been dissolved in saline, for 18 hours at 1°C. The excess reagents were removed by centrifugation and washing. The precipitate was suspended in saline, the pH was brought to 12.6; it was centrifuged immediately, and the pH of the supernatant was brought back to 8.0 at once. The product was 80–100% pure, and contained 28–312 uranium atoms per antibody molecule under a variety of reaction conditions. The immunological specificity of the labeled antibody

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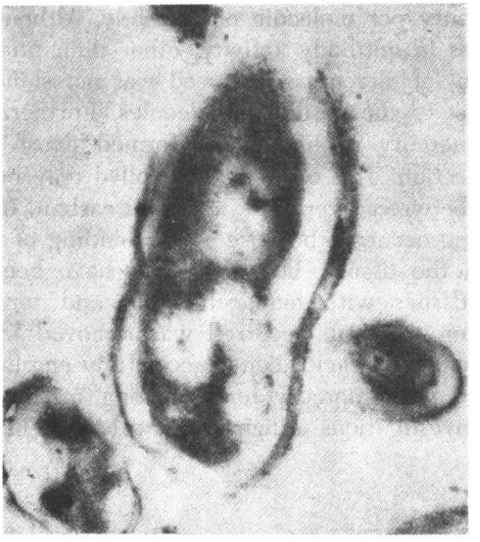
[3] The diazonium salt was obtained by adding to 15 mg of the organic mercurial, 3.7 ml of 20% acetic acid and a slight excess of $NaNO_2$, followed by stirring in the cold for 5 minutes. The resulting mixture was rapidly added to 4 ml of protein solution (0.016 *M* phosphate, 0.1 *M* KCl, pH 7.6) with stirring, and the pH was brought to 9–10 by the addition of alkali. After 1 hour, the soluble material was dialyzed against 100 volumes of 0.016 *M* phosphate buffer, 0.3 *M* KCl, pH 7.4.



(A)

(B)

FIG. 1A. *Bardetella bronchiseptica*, live broth culture, stained with uranium conjugated with antibodies to *B. bronchiseptica*, not fixed with formalin. Magnification: × 25,000. FIG. 1B. Same as Fig. 1A, but unstained and printed during twice the standard exposure time. Magnification: × 25,000. From Sternberger *et al.* (1963).

was confirmed by absorption and blocking experiments. Electron microscopic studies revealed that the labeled antibody specifically stained the cellular membranes of living or formalin killed *B. bronchiseptica* and the cytoplasm of the living bacterium (Figs. 1a, 1b).

Sternberger *et al.* (1963) suggested that uranium-labeled antibodies, containing up to 32% of weight as uranium, may possess a greater degree of electron opacity than ferritin-labeled antibodies. Furthermore, being smaller than ferritin-labeled antibodies, they may be less subject to steric problems. Thus, a polyvalent antigen may bind more antibody molecules labeled with uranium than with ferritin. The problems of cellular penetration are also likely to be less serious with uranium-labeled antibodies. On the other hand, the need to protect the antibody by the presence of the antigen, during the labeling procedure, followed by elution to secure the labeled antibody, is a serious limitation. In the system of Sternberger *et al.* (1963), in which an insoluble antigen was used, it was possible to separate the antibody from the antigen, after alkaline dissociation, by simple centrifugation. With soluble antigens, other methods of fractionation, such as electrophoresis or ion exchange, may have to be employed under conditions in which the recombination of antigen and labeled antibody will not take place. With each soluble antigen, then, it will be necessary to work out appropriate separation procedures to prepare uranium-labeled antibodies in a pure state.

IV. Preparation and Purification of Ferritin-Labeled Antibodies

Singer (1959) was the first to suggest the use of a bifunctional reagent for the conjugation of antibodies to ferritin. A variety of other reagents were soon tried and described (Borek, 1961; Borek and Silverstein, 1961; Ram *et al.*, 1963). Some of these procedures are compared in Table I. Of the various conjugating reagents, Singer's first one, *m*-xylylene diisocyanate, has been used most often, although Singer later recommended the use of 2,4-toluene diisocyanate (Singer and Schick, 1961). Smith and Metzger (1961a) described a method of increasing the amount of ferritin conjugated to the antibody with the former reagent. Our own choice has been *p,p'*-difluoro-*m,m'*-dinitrodiphenylsulfone (FNPS), which appears to offer a milder and simpler procedure of conjugation (Tawde and Ram, 1962; Tawde *et al.*, 1963; Ram *et al.*, 1963).

In the conjugation procedure with *m*-xylylene diisocyanate, Schick and Singer (1961) found significant noncovalent binding of ferritin to globulin, therefore they prefer the use of 2,4-toluene diisocyanate which binds the two proteins covalently. In studies with antibodies to bovine serum

TABLE I

METHODS FOR CONJUGATION OF FERRITIN (FE) TO RABBIT GLOBULINS (RGG)

Reagent	Conjugation procedure	Characterization of the conjugate	General remarks
m-Xylylene diisocyanate (XC) (Singer and Schick, 1961)	Stir a mixture of 5 ml of 1.5% FE in sodium borate buffer (pH 9.5, $\Gamma/2 = 0.1$) + 0.1 ml of XC for 45 minutes at 0°C, centrifuge; let the supernatant stand for 1 hour at 0°C, and then add 5 ml of 1.5% RGG in the same borate buffer; stir for 2 days at 6°C; dialyze against 0.1 *M* ammonium carbonate and then against phospate buffer. Discard the small ppt. About ⅓ of the proteins were conjugated	1:1 complex	(a) Considerable noncovalent association of the proteins reported to take place in addition to covalent coupling (b) Direct contact between specific antibody and XC or TC caused loss of antibody activity, necessitating the two-step reaction (c) Some polymerization of XC on the proteins appears to take place
Toluene-2,4-diisocyanate (TC) (Singer and Schick, 1961)	Stir a mixture of 5 ml of 1.5% FE in phosphate buffer (pH 7.5, $\Gamma/2 = 0.1$) + 0.1 ml of TC vigorously for 25 minutes at 0°C, centrifuge and let the supernatant stand for 1 hour at 0°C and add to equal volume of 1.5% RGG in borate buffer (pH 9.5, $\Gamma/2 = 0.1$) at 37°C. After 1 hour dialyze against 0.1 *M* $(NH_4)_2\ CO_3$. About ⅓ of the proteins were conjugated	Primarily 1:1 complex	Conjugation entirely covalent
Dianisidine (DA) (Borek, 1961)	Treat a mixture of 160 mg of FE + 80 mg of RGG in 7 ml of 0.1 *M* citrate buffer (pH 5.0) at 4°C, with 1 ml of a solution containing 6.7 mg of DA and 3.8 mg of $NaNO_3$ in 10 ml of 0.017 *N* HCl; stir for 2 hours and then dialyze overnight against 0.08 *M* borate, pH 9.4, and finally against neutral saline		A two-step reaction involving change of pH; one of the reactive groups couples at the acid pH, and the second one is activated at the alkaline pH

TABLE I (*Continued*)

Reagent	Conjugation procedure	Characterization of the conjugate	General remarks
p,p'-Difluoro-*m,m'*-dinitrodiphenylsulfone (FNPS) (Ram *et al.*, 1963)	Dissolve 460 mg of FE and 160 mg RGG in sufficient cold 2% Na_2CO_3 to make a 4% protein solution, add 1 ml of chilled acetone containing 5 mg of FNPS to this solution; stir mixture for 24 hours and dialyze against phosphate-buffered (pH 7.0) saline. Discard small ppt. formed. About ½ of the proteins were conjugated	Primarily 1:1 complex	(a) Conjugation entirely covalent (b) One-step reaction