Fluorescent, Electron Microscopic, and Immunoelectrophoretic Studies of Labeled Antibodies

Abstract. *Antibodies, produced in rabbits, to each of three bacterial species have been doubly labeled with fluorescein and ferritin. Irrespective of which label was conjugated to the antibody first, immunologic activity was maintained. Moreover, these preparations gave as high a degree of specificity in fluorescent and electron microscopic studies as did singly labeled antibodies. Immunoelectrophoretic analyses and other immunologic tests further confirmed that the antibodies were conjugated to both labels without loss of specific activity. The technique thus permits the relatively simple method of immunofluorescence to be used as an aid in selecting optimum ferritin antibody conjugates for localizing of antigen at the molecular level by means of electron microscopy.*

Singer's report (*1*) on the conjugation of the electron-dense label, ferritin, with antibody globulin stimulated workers in several fields to employ this technique in a variety of immunocytologic studies utilizing electron microscopy (*2*). Many factors, however, can affect the success of such experiments and negative findings may result from from an unsatisfactory labeling of globulin or from an antiserum of insufficient titer. Studies with ferritin-conjugated antibody would be facilitated if the same preparation of labeled antibody could be screened in a preliminary manner by the fluorescent antibody technique and subsequently employed in electron microscopy. Indeed, the possibility of a doubly labeled antibody was recently explored by Pepe, who reported the conjugation of fluorescein to a mercury-labeled antibody (*3*). The objectives of this report are to describe the technique for labeling antibodies with ferritin and fluorescein in a two-step reaction, and to present data to show that the double-labeling entails no loss of specificity.

Rabbits were immunized with heat-killed and pepsin-digested group A, type 4 streptococci (J17A4). The globulin fractions of the resulting antisera, containing antibody to the group-specific carbohydrate of the cell wall, were obtained by precipitation with 1.436*M* sodium sulfate (*4*). Globulin fractions of sera from rabbits immunized with pneumococcus types II and XVIII were kindly supplied by J. M. Ruegsegger, Lederle Laboratories. Horse spleen ferritin (Pentex Corp.) was purified by five to seven recrystallizations with cadmium sulfate, three precipitations with ammonium sulfate, and ultracentrifugation (*5*). In one procedure, purified ferritin was conjugated with rabbit immune globulins by a modification (*5*) of Singer's method employing xylylene metadiisocyanate (supplied by Carwin Co., West Haven, Conn.). The ferritin-conjugated antibodies were subsequently labeled with fluorescein according to the method of Riggs and Marshall (*6*). The doubly labeled conjugate was dialyzed against 0.05*M* phosphate buffer at *p*H 7.5 until the dialyzing fluid failed to fluoresce in ultraviolet light. The nondialyzable conjugate was ultracentrifuged at 100,000*g* for 4 hours and the pellet redissolved in one-third the volume of 0.05*M* phosphate buffer, *p*H 7.5. The reconstituted ferritin-fluorescein conjugated globulin was passed through a Millipore filter and stored at 4°C. In the second procedure, to reverse the order of labeling, fluorescein was first conjugated to the antibody globulin. Immunoelectrophoretic and electrophoretic analyses have indicated that virtually all of the globulin was labeled with fluorescein (*7*). The fluorescein-labeled globulin was passed through Sephadex columns to eliminate unbound fluorescein (*8*) and then conjugated to ferritin with xylylene metadiisocyanate. The doubly labeled fluorescein-ferritin conjugated globulin was ultracentrifuged twice, reconstituted, and sterilized by filtration, as already described. Since only ferritin-conjugated globulin and free ferritin are recovered after ultracentrifugation, it is apparent that in the final product virtually all globulin molecules are conjugated both with fluorescein and ferritin.

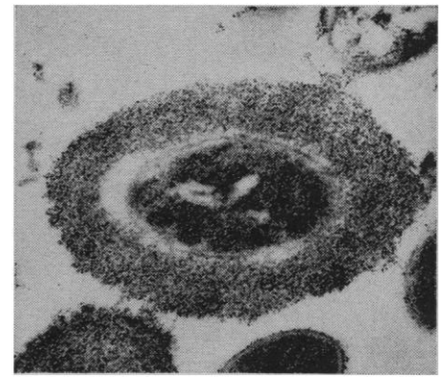


Fig. 1. Pneumococcus type II treated with fluorescein-ferritin doubly labeled specific antibody. Capsular material swollen as a result of the reaction with antibody. Ferritin-labeled antibody molecules are distributed throughout the capsule. (× 37,000)

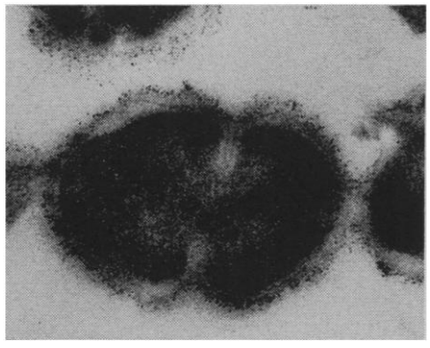
Five-milliliter samples of 18-hour broth cultures of pneumococcus, types II and XVIII, and of streptococcus J17A4 were centrifuged and the sediments resuspended in 4 to 5 ml of 0.5-percent formalin in saline buffered with phosphate at *p*H 7.2. Smears of the bacteria were made on slides, fixed in 95-percent ethanol for 30 seconds, and incubated at room temperature for 30 minutes with twofold serial dilutions of specific and nonspecific conjugates, doubly labeled in either sequence. The excess antibody was washed off with buffer and the slides were examined in ultraviolet light. For blocking experiments the bacterial smears were treated with the unconjugated specific antibody for 30 minutes before the application of the specific doubly labeled conjugate.

For electron microscopic study bacterial sediments from broth cultures of the microorganisms were washed in ice-chilled, 0.5-percent formalin in buffered saline and recentrifuged in the cold.

Table 1. Examination by ultraviolet light and electron microscopy of bacteria treated with antibodies double labeled with fluorescein and ferritin, in either sequence.

Bacteria	Nonconjugated antibody	Conjugated antibody	Results*	
			Fluorescence	Visible ferritin
PnXVIII		A-PnXVIII-FF	+	+
PnXVIII		A-PnII-FF	−	−
PnXVIII		A-J17A4-FF	−	−
PnII		A-PnII-FF	+	+
PnII		A-PnXVIII-FF	−	−
PnII		A-J17A4-FF	−	†
J17A4		A-J17A4-FF	+	+
J17A4		A-PnXVIII-FF	−	−
J17A4		A-PnII-FF	−	−
PnXVIII	A-PnXVIII	A-PnXVIII-FF	−	−
PnII	A-PnII	A-PnII-FF	−	−
J17A4	A-J17A4	A-J17A4-FF	−	−

* The results were the same, irrespective of the order of labeling. † Not tested.



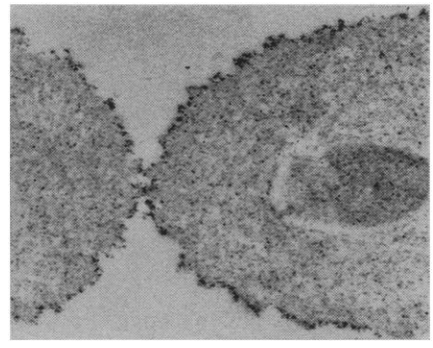


Fig. 2 (left). A cell of group A streptococcus (J17A4), in process of division, treated with fluorescein-ferritin doubly labeled antibody specific for the cell wall carbohydrate. The bacterial cell wall displays tagging with ferritin (× 54,000). Fig. 3 (right). Portions of two pneumococci, type XVIII, exposed to unconjugated antiserum prior to staining with ferritin-labeled specific globulin. The capsules are swollen and appear granular presumably due to the interaction with unconjugated antiserum. The extremely dense ferritin particles are observed only at the periphery of the capsules, which suggests some exchange of conjugated for unconjugated antiserum (× 30,000).

The sediments were resuspended in 0.2 ml of formalinized buffered saline and mixed with 0.2 ml of either ferritin-conjugated or doubly labeled specific antibodies. After 15 to 30 minutes of incubation at room temperature the mixtures were centrifuged and then washed with 0.01*M* phosphate-buffered saline at *p*H 7.2 and then centrifuged again. For homologous blocking experiments the bacterial suspensions were incubated with the unconjugated specific antibody for 15 minutes and washed with formalinized buffered saline before application of the specific conjugates for another 15 to 30 minutes. In other control experiments bacterial suspensions were incubated with heterologous conjugates. The pellets, after final centrifugation, were treated with phosphate-buffered osmium tetraoxide and embedded in methacrylate.

Table 1 summarizes the results obtained from studies with both ultraviolet light and electron microscopy. Column 4 shows that smears of the pneumococci and the streptococcus, when treated with specific, doubly conjugated antisera, exhibited specific fluorescence of the organisms in ultraviolet light. The antisera were tested in serial dilutions ranging from 1 : 10 to 1 : 160, and specific staining occurred at dilutions up to 1 : 80 or 1 : 160. Antisera labeled in either sequence were equally effective. No fluorescence was found in any of the smears treated with nonspecific antisera. It is shown in the last three lines of Table 1, column 4, that previous treatment of the smears with unlabeled specific antisera blocked "staining" with the fluorescein-labeled antibody. Column 5 of Table 1 summarizes the findings obtained in electron microscopic studies. In those experiments the conjugated sera were used in only one dilution, as previously indicated. The results paralleled those obtained by ultraviolet light microscopy. Specific antibodies, tagged with both labels in the sequence of ferritin-fluorescein or fluorescein-ferritin were as effective as those conjugated with ferritin alone (*9*). Figure 1 is an electron micrograph of pneumococcus type II, treated with fluorescein-ferritin doubly labeled specific antibody. Capsular swelling can be seen, and the presence of ferritin granules throughout the capsular material up to the cell wall indicates penetration of the antibody. Figure 2 shows a similar specific binding of a doubly labeled antistreptococcal globulin in the cell wall of streptococcus J17A4.

The specificities of the reactions seen in electron microscopy were demonstrated both by the inability of nonspecific antisera to "stain" the organisms (Table 1, column 5) and by the ability of unlabeled specific antisera to block the "staining." Figure 3 illustrates blocking of the "staining" of pneumococcus type XVIII with ferritin-conjugated specific antiserum by previous treatment with the unconjugated antibody. The prior reaction of unconjugated antibody with bacterial antigen is indicated by capsular swelling, but very few ferritin granules are present and these occur only at the surface, presumably due to exchange of unlabeled for labeled antibody.

Standard test tube agglutination tests and hanging drop capsular swelling tests were performed. The titer of specific agglutination for doubly labeled antibody to type XVIII pneumococcus was 1 : 80, and for type II, 1 : 20. The capsular swelling reaction was not titrated. A loopful of undiluted, doubly labeled antiserum in a drop of culture produced excellent, specific capsular swelling. No cross reactions with heterologous antibodies were observed either in the agglutination tests or capsular swelling reaction.

Two types of gel diffusion tests—Ouchterlony agar diffusion (*10*) and immunoelectrophoresis (*11*)—were employed for further characterization of the doubly labeled antibodies. In the Ouchterlony plates it was found that doubly labeled antibodies, singly labeled antibodies, and unconjugated antibodies to pneumococcus types II and XVIII, all produced lines of identity when the sera reacted with specific carbohydrate antigen. The antisera did not react with heterologous antigens.

The results of immunoelectrophoretic analysis of doubly labeled immune globulins were in general agreement with the observations of Borek and Silverstein (*12*), but the doubly labeled conjugates migrated faster than the singly labeled compounds. Figure 4 is a diagrammatic representation of precipitin lines developed when ferritin-fluorescein doubly labeled rabbit antibody to pneumococcus type II (RAPn2-Fer-Fl), in the two center wells, was subjected to electrophoresis and allowed to diffuse against duck antibody to rabbit globulin (DAR) in the upper trough, pneumococcus type II soluble polysaccharide (Pn2SS) in the middle trough, and rabbit antibody to ferritin (RAF) in the lower trough. Precipitin arc 1 produced against DAR is in the same relative position as its

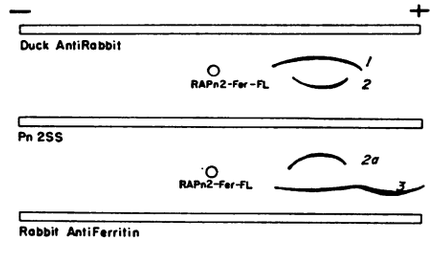


Fig. 4. Fluorescein-ferritin doubly labeled rabbit antibody to pneumococcus type II in center wells (RAPn2-Fer-Fl) was subjected to electrophoresis for 2½ hours at 10 ma. The pattern developed against antibody to rabbit globulin (in upper trough), pneumococcus type II soluble polysaccharide (Pn2SS, in middle trough), and antibody to ferritin (in lower trough) in 4 days.

counterpart, arc 2, precipitated by Pn2SS, which demonstrates that this doubly labeled rabbit antibody to pneumococcus retained its immunologic activity with the specific polysaccharide. Arc 2a is a mirror image of arc 2 formed by the same soluble substance in the middle trough. The long line of precipitation, arc 3, developed against RAF, is biphasic: the more rapid component has no counterpart in arc 1 or arc 2 and represents unconjugated ferritin, and the slower component exhibits the same mobility as arcs 1 and 2, and therefore contains ferritin in combination with immune rabbit globulin. The two or three sequential ultracentrifugations employed during the preparation of the double conjugates has removed any unconjugated rabbit globulin. The unconjugated ferritin can also be eliminated, if necessary, by starch-block or continuous-flow electrophoresis.

Experiments with fluorescein-ferritin–conjugated antisera described in this report indicate that by the tests employed there is no significant alteration in the specific activity of such doubly labeled antibody. It is anticipated that this technique will facilitate the task of the electron microscopist in the fine-structural study of antigen-antibody localization (*13*).

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Inhibition of Growth of Chick Embryo by Inhibition of Deoxycytidylate Deaminase

Abstract. *Deoxyguanylate when added to chick embryos grown in explant inhibited growth and development. Deoxycytidylate deaminase activity was inhibited both in the explants and in vitro; since the effect was quite specific, it is suggested that this may represent another control mechanism for deoxynucleotide synthesis.*

There appears to be a general correlation between the activity of dCMP (*1*) deaminase and the rate of cell proliferation or growth. Thus, high activities have been reported in embryonic tissues (*2*), in certain tumors (*3*, *4*), and in regenerating liver (*5*). This correlation is not surprising, since it has been shown that the product of dCMP deaminase action, deoxyuridylate, is utilized in the synthesis of thymidylate (dTMP), a DNA precursor.

Considering these observations, it is possible that the specific inhibition of dCMP deaminase in a rapidly growing system might inhibit growth.

In this report, some effects of deoxynucleotides on growth and dCMP deaminase activity of chick embryos grown in vitro and in the egg are presented.

The technique of explanting chick embryos of 11 to 13 somites with small extra-embryonic membranes has been described (*6*). Embryos were cultured on a whole-egg homogenate medium with a gas mixture consisting of 25 percent O_2 plus 75 percent air for 0 to 24 hours and 95 percent O_2 plus 5 percent CO_2 for 24 to 48 hours (*7*). After 48 hours of cultivation in vitro, between 9 and 15 embryos (or single embryos) from each group were homogenized in cold 0.25*M* sucrose solution in a 1-ml Ten Broeck homogenizer, and assays for total protein and dCMP deaminase (*4*, *8*) were carried out immediately.

Initial experiments with 2 μmole of dGMP (*9*) per milliliter of medium resulted in death of the embryos. The concentration was therefore reduced to a maximum of 0.1 μmole per milliliter (Table 1).

Embryos explanted in the presence of 0.05 μmole of dGMP per milliliter of medium were strikingly inhibited in growth and development, and the protein content of individual embryos was only one-half that of the controls (*10*). The specific activity of dCMP deaminase was only slightly reduced, however. Treatment with 0.10 μmole of dGMP led to a further reduction in protein content, and a marked reduction in dCMP deaminase specific activity. These effects were completely reversed by addition of dCMP to the medium. At a concentration of 0.10 μmole per milliliter, dAMP had no significant effect on the embryos, and caused no reversal of the inhibitory effects of dGMP when added to the reaction mixture with this latter nucleotide.

At a concentration of 0.10 μmole per milliliter, dG was also inhibitory to the growth of the embryos, giving about the same results as 0.05 μmole of dGMP.

These observations are in agreement with those of Karnovsky and Lacon (*11*) who reported a severe toxic effect when dG was injected into eggs. They also reported that this effect could be reversed by injection of dC.

Table 1. The effect of deoxynucleotides on the growth and enzyme activity of explanted chick embryos.

Total protein per embryo (mg)		dCMP deaminase specific activity (μmole/g prot/hr)	
Expt. 1	Expt. 2†	Expt. 1	Expt. 2†
	*None (control)**		
0.344	0.321	622	635
	dGMP (0.05 μmole)		
.142	.182	344	486
	dGMP (0.10 μmole)		
.104	.107	0	136
dGMP (0.05 μmole) + *dCMP (0.05 μmole)*			
.372		550	
dGMP (0.05 μmole) + *dCMP (0.10 μmole)*			
	.297		639
	dTTP (0.10 μmole)		
.280		1100	
	dG (0.10 μmole)		
	.184		261

*Total nucleotide in 1 ml of culture medium. Whole homogenate was used for assay of dCMP deaminase activity. †Average of two separate determinations in duplicate on single embryos performed by a micro modification of the Conway procedure.