Electron Microscopy of the Combination of Antibodies with Flagellar Antigen and with a Pyocene

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Micrographs are presented of antibodies in combination with flagella of Salmonella typhi and with a phage-bound pyocene Rnc, which is supposed to be the tail of a defective bacteriophage from Pseudomonas aeruginosa. The pyocene preparation seems to offer advantages for the study of antibody-antigen complexes. Under the conditions of our experiments, the surfaces of the antigenic structures are saturated with antibody layers approximately 95 A in thickness, i.e., slightly less than half the accepted lengths of 75 antibody molecules. Our interpretation is that the antibody is attached by combining sites at the ends of the molecules to form loops along the surface of the antigenic structures.

The first electron micrographs of antibody molecules in combination with their specific antigens were published in 1941 by Anderson and Stanley (3), Ardenne et al. (4), and Mudd and Anderson (17). By modern technical standards, these studies were primitive. Both electron microscopy and the chemistry of the immunoglobulins have advanced greatly in sophistication and in refinement of techniques. In addition, more recent electron-microscopic studies of antigen-antibody complexes have been published (1, 2, 7–9, 14, 15).

The objectives of the original work were to visualize antibody molecules in combination with antigen, and hence to discern shapes and orientation, to measure dimensions, and to infer the sites of antibody and antigen combination. These objectives seem as worth achieving as 26 years ago, and seem considerably closer to realization. It may be clarifying to state some desirable conditions. (i) Antigen-containing structures need to be selected in which individual antigen molecules can be resolved. The size and configuration of such antigenic structures need to be such that antibody molecules in combination can also be visualized. The latter requirement at once rules out such relatively gross structures as bacterial cells, whose electron opacity obscures the antibodies combined with them.

(ii) Resolution and contrast in electron microscopy need to be of such high order as to visualize individual protein molecules without undue distortion from drying or other conditions.

(iii) It is desirable that the antibody preparation used be well characterized and free from such sources of confusion as components of complement, conglutinin, or other antibodies of similar specificity but different molecular size, e.g., 7S, 12S, or 19S antibodies.

This brief article is offered as evidence that these conditions are by no means impossible of realization.

MATERIALS AND METHODS

Strains. Salmonella typhi, H901 and P32, were used.

The organism was cultivated at 37 C overnight in a synthetic medium consisting of 1% casein hydrolysate (Difco), 0.02% tryptophan, 0.5% glucose, 0.7% Na2HPO4, 0.3% KH2PO4, and 0.01% MgSO4 (pH being adjusted to 7).

P32 antiserum. Rabbits were immunized intravenously with a suspension of Formalin-killed P32 strain for 4 weeks. The antiserum had a titer of 1:1,280 in the agglutination test; no antiseptic was added to this serum.

Electron microscopy preparations. The preparations were made on copper grids covered with a thin film of colloidion, which was supported and strengthened with micro grids made of cellulose acetobutyrate (Triafol). This film was coated with a thin layer of carbon. The negatively and positively stained preparations were made as follows.

Carbon-coated grids were inverted in a drop of S. typhi culture with or without addition of Formalin at a concentration of 0.5%. After an interval of several minutes, the grids were touched with filter paper and left to dry. Immediately after drying, they were inverted in a drop of diluted serum for several minutes.
at room temperature. Then they were rinsed three or four times in distilled water and inverted in a drop of staining solution. After another minute, the grids were touched gently with filter paper to remove surplus fluid, were left to dry, and were examined in a Japan Electron Optics Lab. type M-7 electron microscope. The instrument was operated at an accelerating voltage of 80 kv. Specimens were examined at a magnification of 50,000.

As a staining solution, 1.5% phosphotungstic acid adjusted to pH 6 with 1 N KOH was used for the negatively stained preparations, and 1.5% uranyl acetate (pH 6), for the positively stained preparations. In the case of the control preparations, only the antibody exposure was omitted.

Preparation of phage-bound pyocine Rmc and antisem. The culture medium consisted of Na₂HPO₄, 10.5 g; KH₂PO₄, 4.5 g; NH₄Cl, 1.0 g; MgSO₄, 0.3 g; 1 M CaCl₂, 0.3 ml; Casamino Acids, 15 g; and glycerol, 30 g; water was added to 1,000 ml and the pH was adjusted to 7.2 (J. Y. Homma et al., in preparation).

To a growing culture of *Pseudomonas aeruginosa* strain P 15-X11, mitomycin C was added to a final concentration of 1 μg/ml; this culture was incubated for another 5 hr at 37 °C. After removing the bacterial debris by centrifugation, the lysate was made up to 50% saturation by adding ammonium sulfate. The resulting precipitate was collected and suspended in a proper volume of 0.005 M tris(hydroxymethyl)aminomethane buffer containing 0.1 M NaCl (pH 7.2). This was then centrifuged at 70,000 × g for 1 hr. Almost all the pyocine activity was found in the sediment. The extraction procedures of ammonium sulfate precipitation and ultracentrifugal centrifugation were repeated three times. The final sediment was suspended in buffered saline and was chromatographed on a diethylaminoethyl (DEAE) cellulose column (15 by 250 mm). The phage-bound pyocine was eluted with gradient concentrations of NaCl, was concentrated by ultracentrifugation, and was designated as Rmc.

To rabbits weighing 2.5 kg each were administered intravenous injections of phage-bound pyocine Rmc for a total volume containing 1 to 3 mg. No adjuvant was used. A 1-ml amount of the antiserum usually could neutralize 250,000 to 1,000,000 minimal doses of phage-bound pyocine Rmc detectable by the pyocine test.

**Results**

Flagella of *S. typhi*. Even in the early work of Mudd and Anderson (17), bacterial flagella were shown to be promising material for study of the antibody-antigen complex. Flagella have recently been subjected to analysis by refined instrumentation and preparative techniques (13, 16). In the latter studies, globular molecules of flagellin are resolved individually, and these are arranged in either of two recognizable patterns designated by Lowy and Hanson as A and B.

Figure 1 shows flagella of *S. typhi* prepared by the positive staining technique. In Fig. 1A, the flagellin globules are in pattern A of Lowy and Hanson. In the lower part of Fig. 1B, the flagellin globules are in pattern B; in the upper part of Fig. 1B, the pattern is less well defined. In Fig. 1A, several cross sections of flagella are shown, each having a hole with a diameter of about 90 Å. The widths of the flagellin molecules have been estimated at 45 Å (13, 16). The widths of our well-stained flagella were about 200 Å. No differences were detected in flagella of *S. typhi* strains H901 and P32.

Effects of normal rabbit serum, complement, and thimerosal on the flagellum of *S. typhi*, with or without Formalin treatment. There was no difference in shape of the flagellum, whether it was suspended in a solution containing Formalin at a concentration of 0.5% or not. No change was observed in the flagellum on a grid when it was exposed to normal rabbit serum or to the same serum containing thimerosal in a concentration of 0.01% and washed, before being subjected to electron microscopy examination. This was again the case when the flagellum was exposed to a mixture of normal rabbit serum and guinea pig serum as complement.

Effects of immune rabbit serum against the flagellum of *S. typhi*. When the flagella of H901 and P32 strains were exposed to specific antibodies, remarkable changes were observed in both cases in the state of the flagellum (Fig. 2A and B). After exposure to P32 antiserum, the apparent width was observed to be 390 Å (Fig. 2A and B and Table 1).

Pyocine particles. Homma and Suzuki (10, 11) have obtained from cells of *Pseudomonas aeruginosa* a phage-bound pyocine Rmc which resembles the tails of a bacteriophage. Electron micrographs of the bacteriophage tail-like particles of pyocine Rmc have been published by Ishii, Nishi, and Egami (12). These pyocine particles turn out to be even more favorable than flagella as antigenic structures suitable for visualizing antibodies in combination with antigen.

The photographs presented in Fig. 3A and 4C show contracted forms of the sheath and the core which were components of mitomycin C-induced pyocine Rmc. Specific antibodies against the sheath and the core were both clearly demonstrated in Fig. 3B, C, D, and 4A and B. The width of the sheath was estimated to be 180 Å and was not changed even when it was combined with specific antibodies. The subunit particles were definitely stained by the negative staining method. Moreover, the antibodies of pyocine itself were more distinctly demonstrated than those of the flagella. The width of the sheath was estimated as 180 Å, and the width of the sheath combined with its specific antibody was estimated.
FIG. 1. Flagella of H901 strain, prepared by positive staining method. (A) and (B). Neither serum nor complement was added. Pictures were taken at $\times 50,000$ and enlarged to $\times 280,000$. 

753
Fig. 2. Flagella of P32 strain, prepared by negative staining method. P32 antiserum was diluted to 0.1 of original. (A) Salmonella typhi culture medium with Formalin added at a concentration of 0.5%. (B) S. typhi culture medium was not exposed to any kind of antiseptic. Pictures were taken at $\times 50,000$ and enlarged to $\times 280,000$. 

754
molecules are evidence of the present nation seems depressions antibodies flagellar from this agglutinated flagella study the antibody and offer material visualization strain R. We and their sheaths material the molecules al. (17) Anderson electron microscopic study as in the out antigenic to 65 A and that of the width of antigen-antibody complex 7S minus antigen)/2 = antibody layer.

to be 370 A. Similarly, the width of the core was 65 A and that of the core combined with its specific antibody, 255 A (Table 1).

**DISCUSSION**

The flagella of *Salmonella* species have been chosen as antigenic structures favorable for the electron microscopic study of antibody-antigen complexes in the early work of Mudd and Anderson (17) and in the recent work of Elek et al. (8). The latter investigators particularly point out advantages of minute cylinders of antigenic molecules for such studies.

Even more minute cylinders of antigenic material are herewith presented for the first time for the study of antibody-antigen complexes. These are phage-bound pyocine particles, the sheaths and cores of pyocine Rmc from *P. aeruginosa* strain R. We suggest that bacteriophages and their component structures (5) in general offer material favorable for electron-microscopy visualization of antibody-antigen complexes.

Evidence was presented in the early studies of Anderson and Stanley (3) and Mudd and Anderson (17) to suggest that the orientation of antibody in combination with antigen might change with varying ratios of antibody to antigen. This suggestion has been confirmed in the later study of Lafferty and Oertelis (15).

Elek et al. (8) find that the distance between agglutinated flagella of *Salmonella* species is less than the length of a 7S antibody molecule as deduced from physical-chemical measurements (6). The explanation they offer is that the antigenic antibodies are imbedded in "pits" or depressions in the flagellar surface. This explanation seems very improbable to us.

At the ratios of antibody to antigen used in the present experiments, we estimate that the thickness of the layer of combined antibody is slightly less than one-half the accepted length of a 7S antibody. We believe it is more compatible with the electron pictures in Fig. 2 and 4 and with other evidence to suppose that the antibody molecules are combined with flagellin at their tips and are bent into loops. Such loops are shown in Fig. 4B.

Almeida et al. (1, Fig. 10) have shown a loop of antibody attached with both combining sites to the same particle of wart virus. Lafferty and Oertelis (15, Fig. 2) have shown the two-site attachment of antibody to influenza virus observed when the virus reacts with low concentrations of antibody, and also (15, Fig. 3) the saturation of the surface of influenza virus with antibody attached through both active sites of the molecule. The same authors (15, Fig. 6) show that the particles may be held together by divalent antibody molecules attached to the subunits on adjacent virus particles, and that some of the antibody molecules visible in the same micrograph appear to be attached through both active sites to the same virus particle.

The third desideratum mentioned in the Introduction, namely, the use of purified antibodies, has rarely been met in the study of antibody-antigen complexes by electron microscopy. An exception is the study of Elek et al. (8), who used 7S γ-globulins prepared by use of a DEAE column. Constant amounts of the flagellar antigen were treated with varying dilutions of this agglutinin.

Concerning the ultrastructure of bacterial flagella, Lowy and Hanson (16) have shown that this may occur in two patterns, A and B. They suggest the possibility that these patterns might be characteristic of certain bacterial species, or, alternatively, that the two patterns may represent different states of contraction of the flagella. The flagella of *S. typhi* in our study occur in both A and B patterns.

In studying the beautiful micrographs of phage-bound pyocine published by Ishii, Nishi, and Egami (12), we note that extended sheaths of pyocine closely resemble the A pattern of Lowy and Hanson, and contracted sheaths, the B patttern. The Japanese authors, however, make no mention of the similarities we have noted. These observations seem to us to strengthen the suggestion that these patterns of ultrastructure may represent different contraction states.
FIG. 3. Pyocine Rmc components, prepared by negative staining method. Sera were diluted to 0.1 of original. (A) Control without being exposed to antiserum. (B, C, D) Pyocine components, exposed to rabbit antiserum. Pictures were taken at $\times 50,000$ and enlarged to $\times 280,000$. 
**Fig. 4.** Pyocine Rmc components, prepared by negative staining method. Sera were diluted to 0.1 of original. (A and B) Pyocine components, exposed to rabbit antiserum. The arrow indicates antibody "loops." (C) Control without being exposed to antiserum. Pictures were taken at $\times\ 50,000$ and enlarged to $\times 280,000$. 

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{Image: Diagram of Pyocine Rmc components with arrow indicating antibody "loops" and pictures enlarged to $\times 280,000$.}
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