Origin and Structure of the Group-Specific, Complement-Fixing Antigen of *Rickettsia rickettsii*

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*Rickettsia rickettsii* was treated with ether and examined by negative-contrast electron microscopy. Group-specific complement-fixing antigen was seen to be originating from the cell wall. The antigen was composed predominately of round particles 10 to 60 nm in diameter. Intact *R. rickettsii* and antigen from ether-treated organisms were purified by density gradient centrifugation and analyzed by polyacrylamide gel electrophoresis. The whole rickettsial cell was composed of a minimum of 30 proteins which ranged in molecular weight from about 23,000 to 155,000. The “soluble” antigen contained nine proteins ranging in molecular weight from about 28,000 to 150,000.

In 1944 Topping and Shear (16) reported that a group-specific “soluble” complement-fixing (CF) antigen was released from rickettsiae after these organisms were treated with ether. Since their report, group-specific CF antigens prepared by ether treatment have been used in CF tests for the serological diagnosis of rickettsial disease. In 1946 Shepard and Wycoff (13) presented electron microscope evidence showing that the “soluble” antigen was composed of droplets of varying size which appeared to be derived from the rickettsial capsule. However, later workers reported that the “soluble” antigen contains sugars, nucleic acids, and protein (6, 11, 18). This chemical complexity suggests that the capsule may not be the singular origin of the antigen. In the present study, we have examined preparations of ether-treated *Rickettsia rickettsii* by modern electron microscope techniques to more accurately define the origin and morphology of the “soluble" antigen. In addition, we have purified these rickettsiae and the "soluble" antigen, and compared their protein composition by polyacrylamide gel electrophoresis (PAGE). The results of these studies are presented in this paper.

**MATERIALS AND METHODS**

**Propagation and purification of *R. rickettsii*.** *R. rickettsii* (strain Sheila Smith) was propagated in 4.5-day-old embryonated eggs from chickens reared on antibiotic-free feed. The inoculum was adjusted to a concentration that killed almost all of the chicken embryos by 104 h after inoculation. Inoculated eggs were incubated at 35 C until the embryos died. They were further incubated at 33 C for 48 h (15). Yolk sacs were harvested aseptically and each was stained by the method of Giménez (7) to demonstrate rickettsiae. Only those yolk sacs that had large numbers of organisms were processed for further use. They were purified by differential and density gradient centrifugation as described previously (J. F. Objeski, E. L. Palmer, and T. Tzianabos, Microbios, in press).

**Preparation of CF antigen.** Yolk sacs infected with *R. rickettsii* were blended into a 20% suspension by using 0.1% formalin in 0.01 M sodium phosphate buffered saline (PBS) at pH 7.0. The suspension was held at 4 C overnight for inactivation of infectivity. It was then mixed with an equal volume of 50% (wt/wt) sucrose in PBS and centrifuged in a no. 30 Spinco rotor at 15,000 rpm for 1 h (C. L. Wiseman, Jr., personal communication). The supernatant and lipid pellicle from this centrifugation were discarded and the interior of each tube was wiped clean. The pellets were resuspended using 1 ml of PBS for every gram of original yolk sac. Ether was then added in a ratio of three volumes to one volume of yolk sac suspension. This suspension was then shaken several times in a separatory funnel and allowed to remain at room temperature overnight. The resulting aqueous phase was collected and residual ether was removed by bubbling nitrogen through the suspension. The ether-free suspension was used as initial group-specific *R. rickettsii* CF antigen. “Soluble" antigen was separated from the above CF antigen by density gradient centrifugation. Antigen was placed onto 30 to 50% glycerol-potassium tartrate density gradients (9) and centrifuged for 16 h in an SW 41 Spinco rotor at 40,000 rpm.

Fractions of 0.8 ml were collected from one of the tubes for CF analysis. The visible bands were also collected, dialyzed against PBS, and rebanded in the same type gradient. Bands from the second gradient were collected and dialyzed against PBS. These were used as antigens in the CF tests and for electron microscopy. Microtiter CF tests were performed in block titration by the Laboratory Branch Complement Fixation (LBCF) method (5). The CF control
reagents were obtained from the Biological Products Division, Center for Disease Control, Atlanta, Ga. Guinea pig convalescent serum was used for CF control antiserum. It contained no antibody to chicken egg yolk material.

**Electron microscopy.** Specimens were prepared for electron microscopy by the pseudoreplica technique (14). They were stained with 0.5% uranyl acetate (UA) at pH 4.5 and examined with a Philips 201 electron microscope.

**PAGE.** PAGE was performed in 8% polyacrylamide gels containing 0.1% sodium dodecyl sulphate (SDS) by the method of Maizel (8). Samples for electrophoresis were dialyzed against TE buffer (0.002 M tris(hydroxymethyl)aminomethane-hydrochloride, pH 7.4, and 0.002 M ethylenediaminetetraacetic acid) and precipitated in 10% (wt/vol) trichloroacetic acid. The precipitate was collected by centrifugation at 10,000 × g for 30 min at 4 C and washed once with absolute alcohol; this process was then repeated. The pellet was dissolved in 0.6 ml of 0.01 M sodium phosphate buffer at pH 7.0, which contained 3.0% SDS, 5.0% 2-mercaptoethanol, and 10% (vol/vol) glycerol, by heating in a boiling water bath for 3 min. The solution was mixed with bromophenol blue tracking dye (0.0001%) and samples containing 100 to 200 µg of protein were loaded onto cylindrical gels approximately 12 cm in length. Molecular weights were calculated as reported by Weber and Osborn (17) by using proteins of defined molecular weights (cyto-

**Fig. 1.** Purified preparation of *R. rickettsii* negatively stained with UA. The cytoplasmic membrane has pulled away from the cell wall and condensed into an electron-dense mass. The arrows point to the electron-lucent cell wall. Magnification ×32,076.
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chrome c, myoglobin, chymotrypsin, ovalbumin, bovine albumin, and human immunoglobulin G) as markers.

RESULTS

Figure 1 shows an electron micrograph of *R. rickettsii* as seen when stained with UA. This stain causes the organism to undergo morphological changes resembling plasmolysis. They appear with electron-dense centers surrounded by an electron-lucent cell wall (arrow). In comparison, Fig. 2 shows an electron micrograph of ether-treated *R. rickettsii*. It clearly depicts the action of ether on the cell and the formation of "soluble" antigen from the cell wall. The cell envelope is shown to be unraveling and fragmenting into round particles of varying sizes (arrow), whereas the center of the cell remains electron dense.

Figure 3 shows a photograph of a potassium tartrate-glycerol density gradient after centrifugation of an ether extract of *R. rickettsii* to equilibrium. Three bands are evident and each contained morphologically different forms. When examined by electron microscopy immediately after centrifugation, band 1 was found to contain a homogenous group of round particles ranging in size from about 10 to 60 nm in diameter (Fig. 4). Band 2 consisted of larger particles that appear to be more pleomorphic and membranous in nature than those in band 1. Many of them exhibited regularly occurring surface projections (arrow) (Fig. 5). However, after standing at 4°C for several hours or after dialysis against PBS, these pleomorphic particles became rounded and appeared similar in morphology to the spherical particles shown in

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**Fig. 2.** *R. rickettsii* treated with ether. Arrow points to cell wall material which is unraveling to form varying sizes of particle types. UA stained. Magnification x50,150.
serum. CF tests on the fractions collected from the gradient were reactive only in the regions where the visible bands occurred. The protein component of purified antigen fractions was analyzed by PAGE in 8% SDS gels. Figure 6 shows a photograph of comparative electropherograms of bands 1, 2, 3, and of purified whole R. rickettsii. The whole cell is composed of at least 30 resolvable proteins which range in molecular weight from approximately 23,000 to 155,000. Bands 1 and 2 of soluble antigen have identical electropherograms. They each consist of nine proteins corresponding to proteins 2, 3, 5, 6, 12, 19, 26, 28, and 29 of the whole cell. Band 3, which consists of partially disintegrated cells, has all the proteins present in whole cells. However, it can be seen that the concentration of proteins 2, 5, 26, and 28, which are major proteins of the soluble antigen, are markedly decreased. The molecular weights of these proteins are 150,000, 130,000, 39,000 and 35,000, respectively.

DISCUSSION

Early electron micrographs of metal shadow-cast preparations of ether-treated rickettsiae show that the “soluble” antigen is released from what appeared to be a capsule (13). Thin-section electron microscopy has shown evidence of a rickettsial capsule (1); however, a well-defined capsule has not been demonstrated by electron microscope examination of rickettsiae in suspension. We have examined scores of such preparations and none contain rickettsiae with discernible capsules. Others have suggested that the capsule is loosely bound and is rapidly lost when these organisms are subjected to laboratory manipulation (2). In addition, previous investigators have suggested that the major site of rickettsial antigenic activity is the cell wall (3, 4, 12, 18). This was confirmed by electron microscopic examination of ether-treated R. rickettsii and by CF. The data presented clearly show that the “soluble” antigen originates from the cell wall through the action of ether. It has previously been shown that when rickettsiae are stained with UA, they undergo morphological changes resembling plasmolysis. The cytoplasmic membrane pulls away from the cell wall and condenses into a darkly staining electron-dense mass, whereas the cell wall remains electron-lucent. In cells stained with UA, as shown in Fig. 1, the electron-lucent cell wall could easily be interpreted as a capsule. However, comparison of rickettsiae stained with UA and phosphotungstate has shown that it is the cell wall (10).
Fig. 4. Electron micrograph of band 1 after density gradient centrifugation of R. rickettsii CF antigen. UA stained. Magnification ×22,470.
FIG. 5. Electron micrograph of band 2 after density gradient centrifugation of R. rickettsii CF antigen. Arrows point to parts of cell wall with external membrane appearing as regularly spaced projections. UA stained. Magnification ×95,000.

TABLE 1. Complement-fixation titers of R. rickettsii antigen and component fractions

| Antigen                  | Guinea pig immune sera | Normal egg yolk sac |
|--------------------------|-------------------------|---------------------|
|                          | R. rickettsii           | R. monseri          |                     |
| Starting crude antigen   | 32          | <8          | 32          |
| Band 1                   | 16          | <8          | <8          |
| Band 2                   | 16          | <8          | <8          |
| Band 3                   | 8           | <8          | <8          |
| Recombined antigen       | 16          | ND         | ND          |

* LBCF optimal antigen titer.
* Not done.

When R. rickettsii was treated with ether, such preparations contained three morphologic forms which were separable by density gradient centrifugation. The predominant form was a round particle 10 to 60 nm in diameter. Larger and more pleomorphic forms were also seen, but when left standing in buffer, they became rounded and morphologically identical to the 10- to 60-nm particles. The particles derived from the cell wall are no doubt analogous to the droplets of "soluble" antigen described by Shepard and Wycoff (13) in 1946. Rickettsiae in various stages of disintegration were also separated from "soluble" antigen particles. However, most of these cells still had unreleased cell wall fragments even after extensive purification.

The "soluble" CF antigen particles were found to react with homologous antisera but not with antiserum to normal egg yolk. Disintegrated forms of R. rickettsii were found to be less reactive in CF tests and were frequently anticomplementary. In addition, CF antigen retained its optimal titer when these forms were removed by centrifugation.

Purified preparations of R. rickettsii and
Fig. 6. Photograph of comparative electropherograms of R. rickettsii and related antigens obtained by ether treatment and density gradient centrifugation. Eight percent polyacrylamide SDS gels stained with Coomassie blue. Gels were electrophoresed for 16 h at 3 to 5 mA per gel.
antigens obtained from ether extracts of these organisms were analyzed by PAGE and their resulting electropherograms were compared. Not surprisingly, *R. rickettsii* was found to be highly complex. It was separated into a minimum of 30 proteins. The two forms of “soluble” antigen had identical electropherograms, each composed of a minimum of nine proteins. This finding, along with morphological studies, indicates that the two forms represent the same cell component. Five of the “soluble” antigen proteins were present in relatively high concentrations as compared to the whole cell. Traces of proteins 15 and 30 were occasionally seen in some “soluble” antigen preparations. The integrated form of the rickettsial cell contained all of the proteins present in the whole cell, but those corresponding to proteins of the “soluble” antigen were markedly reduced. This was not surprising since we consistently observed that these forms retained “soluble” antigen on their surface after ether treatment and purification. When rickettsiae are subjected to PAGE, the resulting proteins fail to stain for lipid and carbohydrate by conventional staining techniques (J. F. Obijeski, E. L. Palmer, and T. Tzianabos, Microbios, in press). More sensitive techniques such as radiolabeling of rickettsiae in cell culture may subsequently reveal these organisms to contain glyco- or lipoproteins, or both.

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