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Antigen Specific for Bacteria Resistant to Tetracycline

IDA BOLDUR AND DAVID SOMPOLINSKY

The Laboratory of Basic Gerontology, Asaf Harofe Government Hospital, Zerifin, and Rappaport Laboratories of Microbiology, Bar Ilan University, Ramat Gan, Israel

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Antisera produced against envelope fractions of tetracycline-resistant *Escherichia coli* and supernatant fluid of disrupted cells of tetracycline-resistant *Salmonella typhi* were adsorbed with the corresponding fractions of susceptible cultures. With these sera, cross-reacting antigens were demonstrated in the following fractions obtained from tetracycline-resistant strains: envelopes of *E. coli*, supernatant fluid of *S. typhi*, and envelopes and supernatant fluid of *Pseudomonas aeruginosa*. These antigens were not demonstrable in the corresponding fractions from tetracycline-susceptible strains.

The mechanism of plasmid-mediated bacterial resistance to tetracycline (Tc) remains uncertain. All attempts to demonstrate inactivation of the drug by resistant microorganisms have been unsuccessful (1, 3, 4, 14, 15). Though it has been amply demonstrated that resistant strains of *Staphylococcus aureus* (1, 12), *Escherichia coli* (4, 5), *Proteus mirabilis* (5), and *Pseudomonas aeruginosa* (15) accumulate Tc to a lesser extent than their susceptible counterparts, the quantitative relationship between resistance level and uptake of Tc is complex (12). Nevertheless, the decreased accumulation of the drug is the probable basis from which a biochemical unraveling of the resistance mechanism is likely to emerge.

We have previously reported that resistant strains of *S. aureus* contain a particular antigen probably related to resistance (1). We now present evidence for a cross-reacting Tc-resistance antigen in *Salmonella typhi*, *E. coli*, and *P. aeruginosa*, with all carrying R factors. Recently, the presence of a particular protein in membranes of Tc-resistant, gram-negative organisms has been demonstrated (8).

MATERIALS AND METHODS

Abbreviations. Tc^R and Tc^S indicate resistance and susceptibility to tetracycline, respectively.

Bacterial strains, plasmids, and growth. The strains of *S. typhi* and *E. coli* K-12, W1485, *met*$^-$, *Flac*$^+$, as well as the R factor carrying Tc^R from *S. typhi* have been described previously (11). The *P. aeruginosa* strain used in this study was isolated from clinical material. Growth of this strain was inhibited on MacConkey agar with 50 μg of Tc per ml. To this strain we transferred, by conjugation with *E. coli* as an intermediate, the R factor K7475 from *P. aeruginosa* Ellsworth obtained from E. J. L. Lowbury, Birmingham, England. This R factor carries genes for resistance to Tc, kanamycin, carbenicillin, ampicillin, and cephaloridine (10). The bacteria were grown in brain heart infusion broth (Difco Laboratories, Detroit, Mich.). For the resistant strains, this medium was supplemented with 1 μg of Tc per ml. The bacteria were harvested in the exponential growth phase (0.2 optical density units at 660 nm) and washed three times with tris(hydroxymethyl)aminomethane (Tris)buffer (KCl, 0.06 M; ethylenediaminetetraacetate, 10^{-4} M; β-mercaptoethanol, 0.06 M; and Tris, 0.02 M, pH 7.5).

Fractionation of bacteria. The bacteria were resuspended in Tris buffer, about 1 g (wet weight) per 10 ml, and disintegrated by three passages through the French press (20,000 lb/in^2). Pancreatic deoxyribonuclease, 50 μg/ml (Worthington Biochemicals Corp., Freehold, N. J.), and magnesium acetate, 0.045 M, were added, and after 5 min at 4 C the extract was separated into whole cells (sedimented by centrifugation at 4,080 × *g* for 5 min), an envelope fraction which sedimented at 40,000 × *g* for 20 min, and the supernatant fluid. The envelope fraction and the supernatant fluid were subjected to dialysis against Tris buffer, three changes a day during a 3-day period. The protein content was determined as indicated by Lowry et al. (9). The purity of the envelope fraction was checked by plating for viable bacteria (a decrease to less than 0.01%), light microscopy (less than one bacterial cell per field with the oil-immersion objective), and electron microscopy after negative staining with 1% uranyl acetate (only vesicles were observed on the micrographs). On disc electrophoresis in 7.5% polyacrylamide, only two distinct bands were obtained from the envelopes, a fact indicating that it was not contaminated with cytoplasmatic proteins to a considerable degree (7). The envelope fraction was suspended in a solution of 1.6% sodium deoxycholate. In some cases, this suspension was used for immunodiffusion; in other cases, the suspension was left overnight at 4 C and sedimented at 22,000 × *g*, and the sediment was extracted twice in the same way

with deoxycholate solution. These deoxycholate extractions (extracts no. 1 through 3) were used separately in immunodiffusion.

Ouchterlony double immunodiffusion (2). This was performed on glass plates covered with 1% purified agar (Oxoid Ltd., London). In some of the experiments, 0.8% deoxycholate was added to the agar to prevent turbidity due to the precipitation of lipid from the envelope fractions or extracts of envelopes. After 48 h of diffusion, the plates were washed in saline and distilled water and stained for 15 min with Trichrom, a mixture of 0.2% light green, 0.2% ponceau red, and 0.1% amido black (6).

Immunization. Young rabbits were immunized by weekly injections of envelope fractions or supernatant fluid in amounts corresponding to 2 to 3 mg of protein. The antigens were mixed thoroughly with 0.5 ml of complete Freund adjuvant (Difco). After a period of 3 to 5 months, the animals were bled and serum was prepared.

Immunoadsorption. Adsorbed antisera were prepared by precipitation with appropriate antigens as described (13).

RESULTS

All of the results reported here were obtained with the Ouchterlony double immunodiffusion technique. Antisera prepared against both extracts and the envelope fractions of Tc^S strains of *S. typhi* and *E. coli* gave identical results with antigens prepared from Tc^S and Tc^R strains. Antiserum against supernatant fluid of *S. typhi* Tc^S interacted with the same fraction of *E. coli* to give several lines of precipitation, but the corresponding fraction of *P. aeruginosa* gave only one weak precipitation band. An antiserum against supernatant fluid of *E. coli* Tc^S gave no precipitation with this fraction of *P. aeruginosa*, but several were found with the *S. typhi* supernatant. In no case did envelope fractions give a precipitation band with antiserum against any fractions of heterologous Tc^S strains.

When antiserum prepared against supernatant fluid of *E. coli* Tc^R was adsorbed with the corresponding antigen from *E. coli* Tc^S it reacted only weakly with fractions from Tc^R strains; the same was the case with antiserum against envelopes of *S. typhi* Tc^R which was adsorbed with *S. typhi* Tc^S envelopes. Most remarkable were the results obtained with sera against supernatant fluid of *S. typhi* Tc^R adsorbed with this fluid from *S. typhi* Tc^S and serum against envelope fractions of *E. coli* Tc^R adsorbed with *E. coli* Tc^S envelopes. These sera

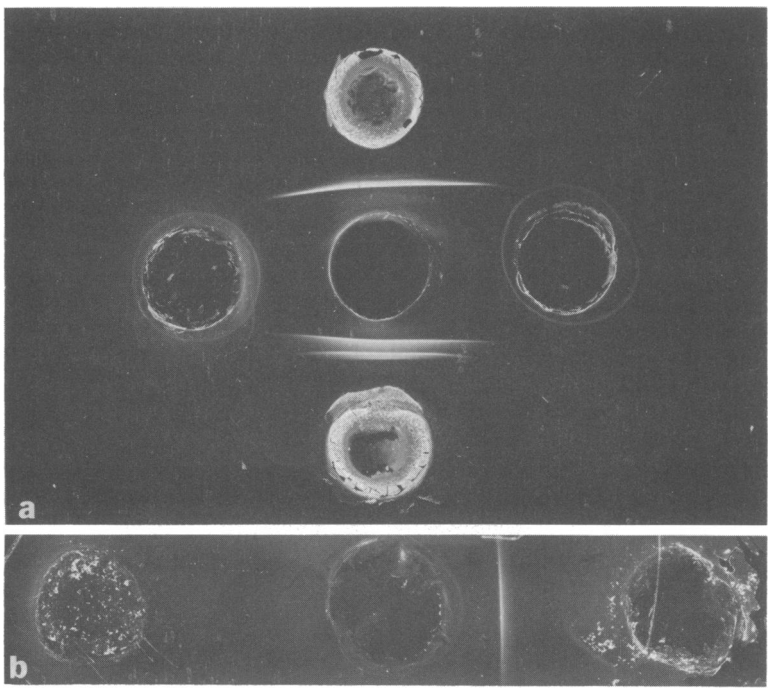

FIG. 1. *(a) Immunodiffusion with antiserum against an envelope fraction of E. coli Tc^R (central well). The antigens were envelope fractions of E. coli Tc^S (top), envelope fractions of E. coli Tc^R (bottom), supernatant fluid of E. coli Tc^S (left), and supernatant fluid of E. coli Tc^R (right). (b) Immunodiffusion with antiserum against an envelope fraction of E. coli Tc^R adsorbed with an envelope fraction of E. coli Tc^S (central well). The antigens were envelope fractions of E. coli Tc^S (left) and E. coli Tc^R (right).*

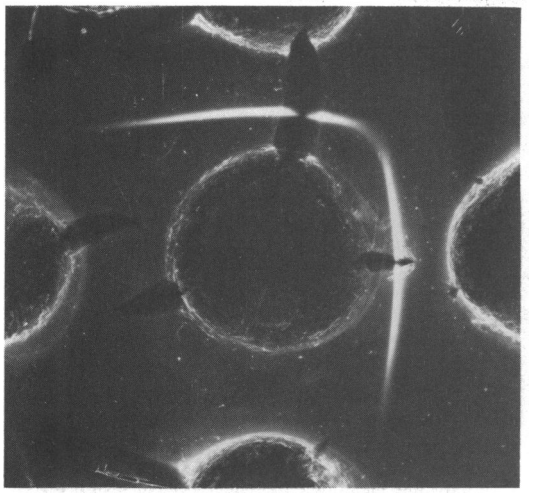

FIG. 2. *Immunodiffusion with antiserum against supernatant fluid of S. typhi Tc^R adsorbed with the same fraction of S. typhi Tc^S. Antigens were: supernatant fluid of Pseudomonas aeruginosa Tc^R (top), envelope fraction of P. aeruginosa Tc^R (right), supernatant fluid of P. aeruginosa Tc^S (bottom), and envelope fraction of P. aeruginosa Tc^S (left).*

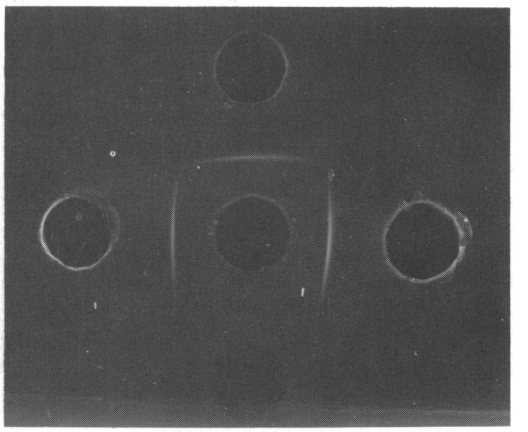

FIG. 3. *Immunodiffusion with antiserum against an envelope fraction of E. coli Tc^R adsorbed with an analogous fraction of E. coli Tc^S. The antigens were: supernatant fluid of S. typhi Tc^R (top); E. coli Tc^R, deoxycholate extract of envelopes (left); Pseudomonas aeruginosa Tc^R, deoxycholate extract of envelopes (right); and E. coli Tc^S, deoxycholate extract of envelopes (bottom).*

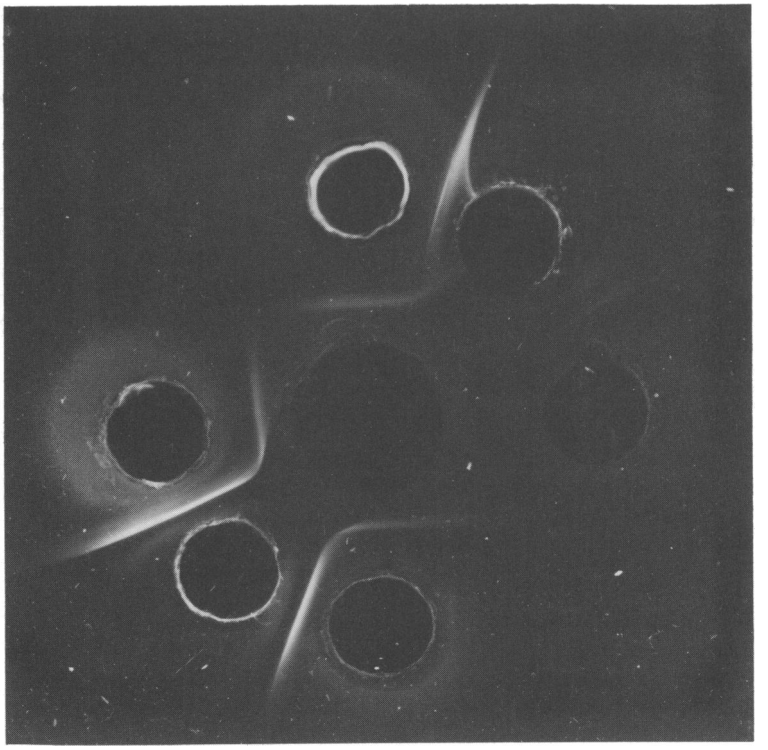

FIG. 4. *Immunodiffusion with antiserum against an envelope fraction of E. coli Tc^R adsorbed with an analogous fraction of E. coli Tc^S (center) and the same antiserum nonadsorbed (lower-left and upper-right wells). The antigens were: envelope fraction of E. coli Tc^R, first, second, and third extraction with 1.6% sodium deoxycholate (left, bottom and right wells, respectively). In the upper well: suspension of envelopes in 1.6% deoxycholate.*

reacted only with fractions from resistant bacteria (Fig. 1) and gave identical results. With envelopes of *E. coli* Tc^R, supernatant fluid of *S. typhi* Tc^R, and both envelopes and supernatant fluid of *P. aeruginosa* Tc^R (Fig. 2), a single distinct precipitation band was obtained. When these antigens were placed in the same gel against adsorbed resistance serum, the precipitation bands merged (Fig. 3), indicating serological cross-reaction between them. If *E. coli* Tc^R envelopes were extracted repeatedly with 1.6% deoxycholate, the reacting resistance antigen was obtained in the two first extractions (Fig. 4).

DISCUSSION

This study shows that Tc^R strains of *S. typhi*, *E. coli*, and *P. aeruginosa* contain an antigen which is absent from the corresponding susceptible strains. The serological cross-reaction between *S. typhi* Tc^R and *E. coli* Tc^R was expected since they contained the same R factor, but this was different from the *Pseudomonas* R factor. In *E. coli* Tc^R the antigen seemed to be attached to the envelope fraction but could be solubilized by sodium deoxycholate, whereas in *S. typhi* Tc^R it was found in the supernatant fluid (envelope-free cytoplasm), and in *P. aeruginosa* Tc^R it was found in both. It is possible that these differences are not too significant, but depend on the intensity of disintegration of the cells and the forces involved in the binding of the Tc-resistance antigen to the membrane.

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