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Direct Evidence for the Presence of Lipopolysaccharide Components in a *Pseudomonas* Ribosomal Vaccine

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The presence of sugars specific to lipopolysaccharide, glucose, and rhamnose was demonstrated in a *Pseudomonas* ribosomal vaccine. The detection of these sugars was accomplished by radiological means after paper chromatography of the neutral fraction of acid-hydrolyzed vaccine.

The immunogenicity of ribosomal vaccines has been demonstrated for a large number of bacterial species (2, 3, 11, 14, 17, 20, 22, 23, 26–28). However, considerable controversy exists in the literature concerning the immunogenic principle in these vaccines. Some investigators believe that it is the ribonucleic acid (RNA) component of the ribosome that is the effective immunogen (25, 29). Others favor the ribosomal protein as the immunogen (12, 13, 21). Still others feel that both RNA and protein are required (15, 19), whereas a fourth group suggests that neither ribosomal RNA nor protein is the effective immunogen in these vaccines (4, 9, 10), but feels that an exogenous contaminant, probably lipopolysaccharide (LPS) or endotoxin, is the immunogenic principle (4, 9). Their conclusion is based on indirect evidence derived from the use of strains deficient in LPS synthesis (4) or biological assays of endotoxin activity (9). Direct evidence for the presence of LPS components in an effective *Pseudomonas* ribosomal vaccine is presented in this report. The presence of sugars specific to LPS is demonstrated by radiological detection. The immunogenicity of these vaccines will be detailed in subsequent publications.

Pseudomonas aeruginosa, Habs type 2 (7), was grown in 500 ml of brain heart infusion broth (Difco Laboratories) containing 250 μCi of D-[*U*-^{14}C]glucose (New England Nuclear Corp.) for 18 h at 37°C with aeration. The cells were harvested by centrifugation, washed three times with a mixture of 10 mM tris(hydroxymethyl)aminomethane and 10 mM $MgCl_2$ (pH 7.4), and subjected to ultrasonic disruption (with an Artek model 300 Dismembrator). Cell debris was removed by centrifugation, and the supernatant was treated with deoxyribonuclease (Sigma Chemical Co.) at 2 μg/ml for 30 min at 37°C. The ribosomes were then isolated by ammonium sulfate fractionation and ultracentrifugation as described by Fogel and Sypherd (6). The isolation procedure, using extensive dialysis, completely eliminates any carryover of [^{14}C]glucose from the culture medium into the ribosomal preparation. One-half of the purified ribosomes was then hydrolyzed in 1.0 N HCl at 120°C for 3 h in a sealed vial. The hydrolysate was neutralized (Cl^- removed) by batch treatment with IONAC A (strong-anion exchange beads, MCB Chemicals) in the CO_3^{2-} form, and the exchanger was removed by filtration. The neutral (sugar) fraction of the hydrolysate was then isolated by passage of the material through successive columns of IONAC A (Cl^- form) and IONAC C (strong-cation exchanger, MCB Chemicals) in the H^+ form. The effluent was then concentrated by roto-evaporation and subjected to paper chromatography in a solvent consisting of ethyl acetate–pyridine–water (8:2:1). Appropriate sugar standards were chromatographed, and the resultant spots were visualized by staining with $AgNO_3$ (24). The migration pattern of the sample was analyzed by cutting the chromatogram into 1-cm-wide strips and determining the amount of radioactivity per strip by liquid scintillation spectrometry with a cocktail consisting of 4 g of Omnifluor (New England Nuclear Corp.) per liter of toluene.

The chromatographic migration pattern of the neutral fraction of the ribosomal preparation is shown in Fig. 1. A large peak of radioactivity corresponding to glucose and a smaller peak corresponding to rhamnose are clearly evident. These two sugars are specific to LPS or "slime" polysaccharide (18). Furthermore, the ratio of the amounts of glucose to rhamnose found, about 5:1 as estimated from their respective peaks, falls within the range of from 3:1 to 8:1 reported for LPS from various types of *P. aeruginosa* (8). There is also some radioactivity remaining at the origin, as well as a small

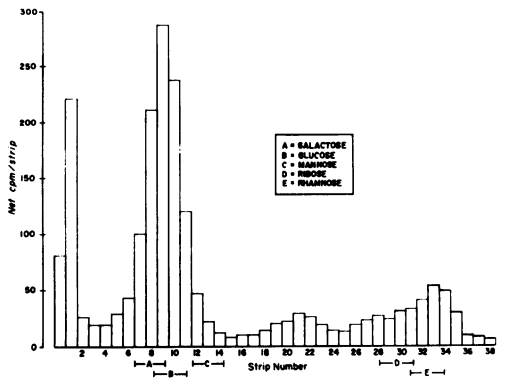

FIG. 1. *Paper chromatography of the neutral fraction of acid-hydrolyzed Pseudomonas ribosomal vaccine. The chromatogram was cut into 1-cm-wide strips, and the amount of the radioactivity in each strip was determined as described in the text. Sugar standards were chromatographed, and the resultant spots were stained with $AgNO_3$ (24). Radioactive peaks corresponding to glucose and rhamnose are clearly evident.*

unidentified peak migrating ahead of the hexoses but behind the pentose and methyl pentose. The radioactivity at the origin is probably a portion of incompletely hydrolyzed material that did not bind to the ion exchangers. Only a very small amount (<3%) of radioactivity was present in the ribose area of the chromatogram. Under the conditions of hydrolysis used, most of the ribose is apparently incompletely hydrolyzed (and therefore mostly bound to the ion exchangers). To confirm this point, a sample of the hydrolysate containing 150 μg of ribose, as determined by the orcinol method (1), was neutralized with Na_2CO_3 and adsorbed with 150 mg of acid-washed charcoal. The charcoal (which adsorbs purine- and pyrimidine-bound material, but not free sugars) was removed by filtration and the filtrate was assayed for ribose. The filtrate was found to contain only 8.8% of the ribose. Thus, most of the ribose remains bound in at least a nucleoside form.

It is difficult to quantitate the amount of LPS in the ribosomal vaccine from this experiment, since the extent of hydrolysis of the LPS is not determined. (If the hydrolysis conditions were made strong enough to ensure complete hydrolysis, the ribose liberated from the ribosomes by these conditions would obscure the rhamnose spot on chromatography, since these two sugars migrate in close proximity.) Assuming complete hydrolysis of LPS, however, and a glucose content in LPS of 9 to 15% (5, 16), the amount of contamination by LPS was estimated to be about 1 to 2% of the RNA in the ribosomal vaccine. Excellent protection with these vaccines against *Pseudomonas* has been achieved by vaccination of mice with doses as low as 1 μg of ribosomal RNA (data to be published). Thus, the amount of LPS in an immunizing dose of ribosomes would be in the order of 10 to 20 ng, seemingly well below the immunizing dose for LPS, even considering the possibility that the presence of ribosomal material functions as a highly effective adjuvant. Nevertheless, the results demonstrate the presence of the LPS components glucose and rhamnose, in the proper proportion, in a *Pseudomonas* ribosomal vaccine.

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