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Gonococcal ribosomes as skin test antigens

Ralph Cutler Judd

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GONOCOCAL RIBOSOMES AS SKIN TEST ANTIGENS

By

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B.A., Whitman College, 1972

Presented in partial fulfillment of the requirements for the degree
of Master of Science

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I wish to thank Dr. Walter Koostra for his guidance and encouragement throughout this study.

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Gonorrhea is the most frequently reported communicable disease in the United States. It accounts for 60% of the specified diseases reported to the U.S. Public Health Service (68). There were 609,681 cases of gonorrhea reported in the United States in 1973. This represents a two-fold increase in the last five years. There is an urgent need for a method of diagnosing the disease which could be used in a large scale screening program (41,59).

At present, diagnosis depends on direct observation of the gonococcal cells in exudates and cultivation of the bacteria from swabs of infected areas. Observation is difficult and unreliable, while cultivation, which is the standard diagnostic procedure, is time consuming and less than 80% effective (48). The problems of diagnosis are compounded by a large reservoir of victims who are asymptomatic and therefore do not seek treatment. It has been reported that up to 80% of the females (27,41,48,68) and 40% of the males with the disease are asymptomatic (27). The number of reported cases in the United States represents only about one-third of the estimated 2,500,000 to 3,000,000 new cases of gonorrhea in 1973 (68). It is clear that present methods of control, including detection, are not effective in limiting the disease.

There has been a great deal of research aimed at developing a suitable diagnostic test for gonorrhea. A variety of sero-diagnostic tests have been employed with limited success. Complement fixation tests using gonococcal protoplasm, lipopolysaccharide, whole cells, and
"soluable" antigens were not releable (15,16,50,51,60,63,69). Flocculation tests using protoplasmic antigens and phonol extracted, acetone-precipitated antigen adsorbed onto lecithin-cholesterol and bentonite particles were not specific enough for wide-spread use (52,56,77). Microprecipitin and passive hemagglutination tests, using a variety of antigens, have been unable to detect known positives or avoid excessive false positive reactions (10,42,15,52).

Immunofluorescent tests, using heat labile surface antigens (11,24,56) and gonococcal pili (31,56) may prove to be of value. One firm, Clinical Sciences, Incorporated, plans to use Gaafar's immunofluorescent test (24) commercially in the near future. Clinical testing to determine the effectiveness of this test is now underway in Albany, New York (personal communication; Clinical Sciences, Inc.). Another diagnostic test recently offered was Organon Incorporated's "Gonosticcon Dri-Dot", a latex agglutination test using "soluable" antigen (48). This test was put on the market in late 1973 and removed three months later due to the number of false positive and false negative reactions yielded by the test (61).

To date, the majority of research done on the diagnosis and immunology of gonorrhea has been involved with the humoral immune response to infection. Canadian workers have attempted to produce protective antibodies by vaccination with whole cell lysates (25). Others have suggested the use of gonococcal pili (5,6,31) and type specific polysaccharide (8) as immunizing agents. As yet no success has been noted in the induction of protective immunoglobulins.

A relatively small amount of research has been done on the cell-mediated immune response in patients with gonorrhea. It was noted in the 1920's by Herrold (28) and Kohler (38) that persons with gonorrhea
responded to intradermal injections of gonococcal culture filtrates and whole cells of *Neisseria gonorrhoeae* with delayed hypersensitivity reactions. Further work in the 1930's and early 1940's by Corbus (13, 14), Casper (7), Rossett (57), and Conrad (12) confirmed the delayed hypersensitivity reaction to gonococcal whole cells, cell fractions, and culture filtrates in patients with the disease. Unlike the numerous criticisms of sero-diagnostic tests based on humoral immunity, all workers using skin tests reported favorably upon its specificity and usefulness.

With the advent of penicillin treatment of gonorrhea in the 1940's, basic research on gonorrhea largely ceased. The recognition of increased resistance of the gonococcus to antibiotics (1,9,41,63,64,73, 74) and the rapid increase in the number of cases of gonorrhea stimulated a new interest in both the humoral and cellular immunity of gonorrhea in the late 1960's (41,59). Kraus, et al. (39), demonstrated a true cell-mediated response in patients with gonorrhea in 1970 using lymphocyte transformation. Esquenazi and Strietfeld (21) and Grimble and McIlmurray (26) used a variety of antigens to demonstrate a cell-mediated response to gonococcal infection, again using the lymphocyte transformation technique.

In 1960, Kanai, Youmans, and Youmans demonstrated that a "ribosomal fraction" of *Mycobacterium tuberculosis* could stimulate a delayed hypersensitivity response in guinea pigs sensitized with *M. tuberculosis* (33). Further work by Youmans and Youmans (77) and Baker, et al. (2,3), has shown that the ribosomes and ribosomal subunits of mycobacteria are excellent antigens, possessing extreme sensitivity and specificity, when used to elicit delayed hypersensitivity in sensitized guinea pigs. The immunogenicity of ribosomes of other bacterial species has been...
demonstrated by several researchers who have used ribosomes from *Diploccocus pneumoniae* (66), *Streptococcus pyogenes* (32), *Staphlococcus aureus* (76), *Salmonella typhimurium* (31, 62, 70), *Pseudomonas aerogenosa* (75), and *Neisseria meningitidis* (65) as vaccines against infection in laboratory animals. The species specificity of the ribosome has been demonstrated by Sarker and Som (58), Otaka, et al. (49), and Bolton (4). The skin test specificity of ribosomes has been shown well with different mycobacterial species by Baker et al. (2).

Based on the immunogenicity of ribosomes and their ability to elicit a delayed hypersensitivity response, and the evidence of a cell-mediated immune response in individuals with gonorrhea, it is possible that gonococcal ribosomes could be of use as a skin test antigen. The demonstration of species specificity of the ribosome suggested that the ribosome might be a suitable antigen for use in a diagnostic test for gonorrhea.

It was proposed in the research reported here to isolate ribosomes from *Neisseria gonorrhoeae* and use these as skin test antigens in guinea pigs sensitized with homologous and heterologous organisms in order to determine their sensitivity and specificity in eliciting a cell-mediated delayed hypersensitivity response. An *in vitro* correlate of delayed hypersensitivity, the macrophage migration inhibition test was also to be employed.
CHAPTER II

MATERIALS AND METHODS

Animals

Hartley strain guinea pigs, male and female, weighing 300-500 g were used in this study. They were purchased from a local source in Hamilton, Montana.

Bacteria

Bacteria used included *Neisseria gonorrhoeae*, *Neisseria meningitidis*, *Neisseria lactamica*, *Neisseria flavescens*, and a *Moraxella* species. All were clinical isolates obtained from Dr. Walter Koosra, University of Montana. Each bacterium was grown separately in Thayer-Martin medium without inhibitors (64) for seven days at 37°C in candle jars.

Sensitization of Guinea Pigs

Incomplete Freund Adjuvant (IFA) was prepared by mixing 10 parts of the appropriate killed whole cells in 1.5 M saline, 9 parts of sterile mineral oil, and 1 part Arlacel A in a double syringe. The final concentration of antigen was 100μg/0.1 ml. Animals were inoculated with the appropriate antigen by injecting 0.1 ml of IFA into both hind foot pads (total inoculum, 200μg per guinea pig). The animals were skin tested every seven days by intradermal injection of 0.1 ml of a 200μg/ml preparation of whole cells in 1.5 M saline. Sterile 1.5 M saline was also injected as a control. Right angle diameters and le-
sion thickness (using a Kroplin Schnelltestor) were measured at 24 hours post injection. IFA stimulation was repeated until a delayed hypersensitivity lesion with a volume of 15mm³ or greater was observed. This volume was considered a positive skin test (74). The formula for lesion volume is:

\[ \text{Volume} = \text{Width} \times \frac{1}{2} \text{Height} \times \frac{3}{2} \]

\[ \text{Width} \times \text{Length} \times \frac{1}{2} \text{Height} \times \frac{3}{4} = \text{Volume} \]

PREPARATION OF SKIN TEST ANTIGENS

Crude Ribosome Preparation

A growth curve was done to determine when the organisms were in late log phase growth. At this point the cells are actively synthesizing protein in large quantities and therefore they have a maximum number of ribosomes. Ten flasks with 100 ml of Thayer-Martin medium without inhibitors were inoculated with 0.5 ml of a liquid gonococcal culture (approximately 1 x 10⁴ organisms per flask). The flasks were incubated in candle jars at 37°C. One flask was removed 24 hours after inoculation and an optical density reading was taken at 550nm. The culture was then centrifuged at 12,000×g for 20 minutes. The pellet was dried overnight at 80°C and the dry weight was determined. This was repeated each day for each of the flasks. The results appear in Figure 1. The organisms were in late log phase growth after 7 days of incubation.

Ribosomes were extracted from seven day cultures of N. gonorrhoeae, and N. meningitidis using methods described by Hill, et al. (30). All preparations were kept below 5°C at all times.
Buffers:

70S buffer - 0.01 M MgCl₂; 0.01 M Tris-HCl (pH 7.4);
   0.07 M KCl

50S buffer - 0.001 M MgCl₂; 0.01 M Tris-HCl (pH 7.4);
   0.07 M KCl

65S (wash) buffer - 0.01 M MgCl₂; 0.01 M Tris-HCl (pH 7.4)
   0.5 M NH₄Cl₂

A typical procedure for the isolation of ribosomes is as follows:

Approximately 15 g of a cell paste was washed twice in 10 volumes of 70S buffer and centrifuged at 12,000xg for 10 minutes. The pellet was resuspended at a concentration of 175 mg/ml wet weight in 70S buffer. This slurry was then loaded into a prechilled Sorvall-Ribi Cell Fractionator and the cells were disrupted at 13,000 pounds per square inch.

Whole cells, cell walls, and cellular debris were removed by two centrifugations at 20,200xg for 30 minutes in a Sorvall RC-2B refrigerated centrifuge. The supernatant was then centrifuged at 254,000xg for 2.5 hours in a Type 60 Ti rotor using a Beckman model L2-65B ultracentrifuge. The pellet was resuspended in 200 ml wash buffer and stirred overnight at 4°C. The preparation was again centrifuged at 20,200xg for 30 minutes to remove particulate matter and then centrifuged at 254,000xg for 2.5 hours to repellet the ribosomes. The resultant pellet was resuspended in a minimal amount of wash buffer (or 50S buffer if the preparation was to be used for subunit isolation) and stored at -30°C until needed.
Isolation of Ribosomal Subunits

Crude ribosomes were stirred for 3 hours in 50S buffer at 4°C. The decreased magnesium concentration caused the ribosomes to become unstable and dissociate into their 50S and 30S subunits (67). This suspension was layered on a 10-30% continuous sucrose density gradient and centrifuged at 81,000xg in an SW-27 rotor for 8.5 hours. The preparation was then collected by puncturing the bottom of the centrifuge tubes and collecting 1.0 ml samples. The samples were analyzed in a Gilford-modified Beckman DU spectrophotometer at 260nm and 280nm. The 260nm readings were plotted against volume number (Figure 2) and the samples under the 50S and 30S peaks were combined to form the 50S and 30S subunit pools.

50S-30S Pool

The 50S-30S pool was prepared by resuspending a crude ribosome preparation in 50S buffer. No attempt was made to isolate specific subunits from this preparation.

Ribosome concentration in all preparations was assayed spectrophotometrically at 260nm using the extinction coefficient $E_{260nm} = 145$ (Hill, et al., 29) in the equation:

\[
\text{Concentration (in grams %)} = \frac{\text{Dilution} \times A_{260nm}}{145}
\]

Purity of the ribosomal preparations was assayed using the ratio of absorbance at 260nm and at 280nm. This is the ratio of nucleic acid to protein. In a pure ribosome preparation the ratio should be approximately 1.9 (66). All ribosome preparations used had a 260nm/280nm ratio between 1.85 and 1.95.
Crude Ribosome Protein

Protein from the crude ribosome preparation was isolated using two methods. The first method was described by Spitnik-Elson (65): A solution of 6.0 M LiCl and 3.0 M urea was added to an equal volume of crude ribosomes in 70S buffer. The solution was mixed and left overnight at 4°C. The resultant precipitate was separated by centrifugation at 12,000xg for 10 minutes. The pellet was washed twice in 3.0 M LiCl-4.0 M urea solution and the washings were added to the original supernatant. To this solution, which contains all of the ribosomal protein, was added an equal volume of 20% trichloroacetic acid. This was left at 4°C for 21/4 hours. The precipitated protein was centrifuged out, washed twice with ether, suspended in 0.01 M HCl, and dialysed against the same solvent for 21/4 hours. This was then centrifuged at 20,000xg for 30 minutes at room temperature. Protein concentration was assayed using the method described by Lowry, et al. (43).

The second method of protein extraction was described by Fogel, et al. (22). Five volumes of cold 2-chloroethanol were added to 1 volume of crude ribosomes in wash buffer. Hydrochloric acid was added to a final concentration of 0.06 N. The mixture was maintained at 4°C for two hours. The precipitated RNA was removed by centrifugation at 5,000xg for 10 minutes. The supernatant, containing protein, was transferred to a dialysis sac and dialysed at 4°C against distilled water. The precipitated RNA was similarly dialysed. Protein concentration was determined using the method described by Lowry, et al. (43)
Crude Ribosome RNA

Ribosomal RNA was extracted in two ways. The first method, described above, utilized the RNA precipitated by 2-chloroethanol treatment.

The second method of ribosomal RNA extraction was described by Kirby (37). Equal volumes of gonococcal ribosomes and 90% phenol were combined and allowed to stand at 25°C for 40 minutes. This was then centrifuged at 2,000xg for 1 hour at 4°C. The aqueous layer was separated from the phenol layer and potassium acetate was added to the aqueous phase to a concentration of 2.0%. The RNA was precipitated by the addition of 2 volumes of 95% ethanol, collected by centrifugation, and washed with 75% ethanol. The residue was allowed to sit at 25°C overnight in an open container. It was then resuspended in a minimal amount of 1.5 M saline and stored at 4°C until needed.

Protoplasrn

Protoplasrn was extracted as described by Martin, et al. (47). Cold killed gonococci were harvested by centrifugation and washed twice in a sterile saline. The cells were then disrupted as previously described in a Sorvall-Ribi Cell Fractionator. The effluent was centrifuged at 27,000xg for 1 hour. The final supernatant was removed and subjected to two additional centrifugations at 27,000xg for 1 hour. The final supernatant was removed and lyophilized in a Virtis freeze dryer and stored at -80°C until needed.
Cell Walls

The pellets from the protoplasm isolation centrifugations were combined. These were washed twice in sterile saline, then lyophilized and stored at -80 C until needed.

Pilin

Pilin, the protein of gonococcal pili, was prepared as described by Buchanan, et al. (6). Cells from T1 and T2 colony types (34) were grown in 15 liter quantities in a Microferm fermentor (New Brunswick Scientific Co., New Brunswick, N.J.) in Thayer-Martin medium without inhibitors. The cells were harvested using a Sharples continuous flow centrifuge (Sharples Div., Penwalt Corp., Philadelphia, Pa.). Pili, which were sheared from the gonococci during centrifugation, were precipitated from the supernatant by adjusting the pH to 4.0 with HCl. Following storage at 4 C for 12 hours, the precipitate was collected by Sharples centrifugation and then redissolved by stirring at 4 C in 0.01 M Tris (pH 7.5) and 0.01 M NaN3. This suspension was centrifuged at 12,000xg and the supernatant was dialysed overnight at 4 C against distilled water. MgCl2 was added to the dialysed supernatant to a concentration of 0.1 M and the mixture was maintained at 4 C overnight. The MgCl2 treated preparation was then centrifuged at 12,000xg for 10 minutes. The pellet was suspended in 0.01 M Tris (pH 8.0), 0.01 M NaN3 and dialysed for three days against the same buffer. The suspension was then centrifuged at 12,000xg for 10 minutes and the supernatant was dialysed overnight against distilled water. The dialysed supernatant was subjected to three cycles of precipitation with MgCl2 and the dissolution in 0.01 M Tris (pH 8.0), 0.01 M NaN3. Protein concentration was determined using the method of Lowry, et al. (43)
The preparation was frozen until needed.

The identity of the product was assayed by electrophoresing the product, which was boiled for 1 minute in 1% SDS and 1% 2-mercapto-ethanol, along with known molecular weight proteins, in 10% polyacrylamide gel with 0.3% bisacrylamide and 1% SDS.

Whole cells

Whole cells of gonococci were obtained by harvesting a cold killed (see below) seven day culture by centrifugation, washing the cells twice in sterile saline, and resuspending the cells in saline. Whole cells of heat killed (see below) meningococci were obtained using the same method.

SKIN TESTING PROCEDURES

Primary Testing

The first series of skin tests was designed to determine if the ribosomal fraction was capable of producing a delayed hypersensitivity response in sensitized animals. Also, an attempt was made to determine which method of killing the cells prior to animal immunization produced the best sensitizing antigen. Finally, several closely related Neisseria and Moraxella species were used to sensitize animals to determine if the gonococcal ribosome preparations possessed the specificity necessary for an effective skin test.

Four groups of guinea pigs were sensitized as previously described using the following organisms:

Group I consisted of six animals sensitized with \textit{N. gonorrhoeae}
which were killed by heating at 100 °C for 1 hour (heat killed). The
cells were harvested by centrifugation at 12,000xg for 15 minutes,
washed twice in distilled water and weighed. They were then suspend-
ed in sterile saline and used to make the IFA preparation as previously
described.

Group II consisted of six animals sensitized with were killed
by pouring the culture over ice and holding at 4 °C for 24 hours (cold
killed). These were harvested and used to immunize the guinea pigs
as previously described.

Group III consisted of six animals sensitized with heat killed
*N. meningitidis*.

Group IV consisted of six animals sensitized with heat killed
*N. lactamicus, N. flavescens*, and a Moraxella species. The cultures
were harvested separately and equal amounts of each were combined.

Whole gonococcal cells and a gonococcal crude ribosome prepara-
tion from seven day cultures of cold killed *N. gonorrhoeae* were used
to skin test four animals in each group. The preparations were dilut-
ed to 10.0, 5.0, 1.0, 0.5, and 0.25 µg/0.1 ml in saline and wash buffer
respectively. The guinea pig were injected intradermally with 0.1 ml
of each of the cellular and ribosomal dilutions as well as saline and
wash buffer controls. The reactions were observed at 24 hours post
injection. Lesions were measured and volumes determined.

Skin test dose 50's (STD^50) were determined using the method of
Reed and Meunch for determining 50% endpoints (19). Total positives
for each dilution were obtained by placing the accumulated positives
in the numerator and the accumulated positives and negatives in the
denominator. The STD^50 was then found using the equations:
Proportional Distance (PD) = \( \frac{14 \text{ positives above 50\%-50\%}}{\text{positives above 50\%-negatives below 50\%}} \)

Dilution with over 50% positives - PD \( \times \) (dilution with \( \text{STD}_{50} \) over 50% positives) - (dilution with less than 50% positive)

Results are tabulated in Table 1.

Six animals from each of the above groups were skin tested with dilutions of gonococcal crude ribosomes, 50S subunits, 30S subunits, and sucrose controls three months after the final immunization. All animals showed a decreased reactivity with as many as four animals in some groups failing to yield a positive reaction. Results of this test appear in Table 2. One boosting dose with cold killed gonococcal cells resensitized the guinea pigs in Group II. This group was then skin tested with the above antigens. Results appear in Table 3.

Skin testing to determine sensitivity of the crude ribosome

This series of skin tests was designed to determine the sensitivity of the crude ribosome preparation. Several other gonococcal antigens were used to compare their reactivity with that of the gonococcal crude ribosome.

Nineteen guinea pigs were sensitized as described with cold killed \( \text{N. gonorrhoeae} \). Gonococcal cell walls, protoplasm, crude ribosomes, and a 50S-30S pool were diluted to the following concentrations: 10.0, 5.0, 1.0, 0.5, and 0.125ug/0.1 ml. One-tenth of a milliliter of each dilution was injected intradermally. The reactions were observed at 4 hour post injection for an immediate (Arthus) reaction and at 24 hours post injection for the delayed hypersensitivity reaction. Results of these skin test appear in Table 4. Average areas of the Arthus lesions are recorded. Also included are photomicrographs of histological sec-
Skin Testing to Determine Specificity of the Crude Ribosome

This series of skin tests was designed to determine the specificity of the crude ribosome preparation. Crude ribosomes from both gonococci and meningococci, as well as gonococcal whole cells, cell parts, and ribosomal components were used to skin test animals sensitized with either *N. gonorrhoeae* or *N. meningitidis*.

Ten animals were sensitized with cold killed *N. gonorrhoeae* and ten with heat killed *N. meningitidis*. All twenty animals were skin tested with dilutions of meningococcal and gonococcal crude ribosome preparations. Eight animals in each group were then skin tested with dilutions of meningococcal and gonococcal crude ribosomes, gonococcal crude ribosome protein, and gonococcal protoplasm. Seven animals were then retested with meningococcal and gonococcal crude ribosome dilutions. Five animals from each group were then skin tested with meningococcal and gonococcal whole cells, and gonococcal crude ribosome RNA, crude ribosome protein, and pilin. Results appear in Tables 5-10.

**In vitro correlate of Delayed Hypersensitivity**

**Macrophage Migration Inhibition (MIF)**

In order to determine if the crude ribosome could be used in an *in vitro* test of delayed hypersensitivity, the method described by George and Vaughn (24) and David, *et al.* (16, 17) to test for macrophage migration inhibition was employed.

Gonococcal and meningococcal sensitized guinea pigs and unsenitized-
ed guinea pigs were injected intraperitoneally with 30 ml of sterile mineral oil. Seventy-two hours later the animals were sacrificed, and 50 ml of cold Hank's Balanced Salt Solution (GIBCO) with 6 units of heparin per ml was injected intraperitoneally. The abdomen was gently kneaded, then opened with a micline incision through the peritoneal wall, and the fluid was removed. The fluid was placed in a cold, sterile separatory funnel and the layers were allowed to separate. The aqueous phase was drawn off into siliconized centrifuge tubes. These were centrifuged at 1,000xg for 10 minutes. The supernatant was discarded, and the cells were washed twice in Hank's BSS without heparin. After the final wash the cells were resuspended in to a final concentration of 10% in Eagle's Modified Minimal Essential Medium (EMEM) (Gibco) with 10% fetal calf's serum (FCS). Sterile capillary tubes were filled 2/3 full with the cell suspension and one end was sealed by gentle heating. The tubes were centrifuged at 1,000xg for 10 minutes. The tubes were cut at the cell-supernatant interface and the fragments with the peritoneal exudate cells (PEC) were placed in sterile Sykes-Moore chambers (2 Tubes, 1 chamber; each in triplicate). They were sealed and filled with the following:

Control:
One third of the chambers: EMEM containing 10% FCS plus 100ug streptomycin and 100 units penicillin/ml plus MgCl₂ (0.01 M)

Gonococcal Ribosomes:
One third of the chambers: EMEM containing 10% FCS plus 100ug streptomycin and 100 units penicillin/ml plus MgCl₂ (0.01 M) plus 0.5ug gonococcal crude ribosomes

Meningococcal Ribosomes:
One third of the chambers: EMEM containing 10% FCS plus 100ug streptomycin and 100ug penicillin/ml plus MgCl₂ (0.01 M) plus 0.5 ug meningococcal crude ribosomes
The chambers were incubated at 37 C for 24 hours. Areas of migration were determined by projecting the images of migration onto high quality bond paper using an Omega Photographic Enlarger. The outline of the images were drawn on the paper, and the outlined regions were cut out. These were then weighed and compared to weights of known areas to determine the area of migration. Results appear in Table 12 and Figure 4.

This test was repeated using 0.3ug of the gonococcal or meningococcal crude ribosome preparations as test antigens. Results of this test appear in Table 13 and are displayed graphically in Figure 5.
CHAPTER III

RESULTS

Ribosomes are prevalent in cells that are actively synthesizing protein. Cells in the late logarithmic phase of growth should contain a maximum number of ribosomes. In order to determine when gonococcal cells reach logarithmic growth in Thayer-Martin medium without inhibitors at 37°C in candle jars, a growth curve was prepared. The results of a 10 day growth curve appear in Figure 1. Both optical density at 550nm and dry weights were determined for each 100ml sample used. The results indicated the gonococcal cells suffered a lag phase of about 4 days, followed by a logarithmic growth phase of about 4 more days. The cells were in late logarithmic phase growth at 7 days after the medium was inoculated with N. gonorrhoeae cells.

All cultures from which ribosomes were isolated were harvested after 7 days of incubation at 37°C in candle jars.

Sucrose density gradient centrifugation of crude ribosome preparation

To determine the constituents of the crude ribosome preparation a crude ribosome preparation was layered on a 10-30% continuous sucrose density gradient and centrifuged at 81,000xg for 8.5 hours. Results appear in Figure 2a. The results indicated the crude ribosome preparation had an abundance of 70S ribosomes with some dissociation of ribosomes into 50S and 30S subunits. The small peaks in the 0-5ml region represents macromolecular cellular debris. The small peak in the 30ml region represents smaller molecules of cell debris. This graph in toto
Figure 1. Growth curve of *N. gonorrhoeae* grown in Thayer-Martin medium without inhibitors. 100 ml samples were grown at 37°C in candle jars. One sample was removed daily. Optical density was read at 550nm and dry weights determined by centrifuging the sample and drying the pellet overnight at 80°C.

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Figure 2a. Centrifugation of gonococcal crude ribosome preparation on a 10-30% sucrose density gradient. Preparation centrifuged at 81,000xg for 8.5 hours in 70S buffer at 4 °C. The absorbance was measured at 260nm.
represents a crude ribosome preparation.

**Isolation of 50S and 30S ribosomal subunits**

Crude ribosomes were isolated from *N. gonorrhoeae*. These were suspended in 50S buffer which, due to the decreased magnesium ion concentration, caused the intact ribosomes to dissociate into 50S and 30S subunits. This preparation was layered on a 10-30% continuous sucrose density gradient, centrifuged, and fractionated. The results of a typical isolation procedure appear in Figure 2b. Fractions collected in the shaded regions were combined to form the 50S pool and the 30S pool.

The location of the 50S and 30S peaks compares favorably with data presented by Baker, et al. (2), for the isolation of ribosomal subunits of *M. bovis* (B99). The large area under the "30S" region indicated there was considerable contamination of the 30S subunits, possibly by decomposed 50S particles. The 260/280nm ratio was 2.0 for this preparation. Since the preparation failed to display greater activity than the crude ribosomes, further purification was curtailed. The "50S" and "30S" preparations are hereafter placed in quotes to indicate the preparations were not purified.

The method employed to isolate subunits was the same as that described by Hill, et al. (29), except that a 0.001 M magnesium ion concentration was used to isolate the subunits. This concentration is 2.5 times that used in the standard isolation of *Escherichia coli* ribosomal subunits.
Figure 2b. Isolation of "50S" and "30S" ribosomal fractions from H. gonorrhoeae on 10-30% sucrose density gradient. Crude ribosome preparation centrifuged at 81,000xg for 8.5 hours in 50S buffer at 4°C. The absorbance was measured at 260nm. Shaded areas represent combined fractions to form 50S and 30S pools.
Delayed hypersensitivity reactions provoked by gonococcal crude ribosomes and whole cells

Groups of guinea pigs were sensitized with whole cells of *N. gonorrhoeae*, *N. meningitidis*, and *N. lactamica*, *N. flavescens*, and a Moraxella species. These were used to determine if crude ribosomes from *N. gonorrhoeae* could provoke a delayed hypersensitivity reaction, and whether the crude ribosomes possessed the specificity necessary to be useful as a skin test antigen. Also, two methods of killing the gonococcal cells were employed to determine which method produced the best sensitization.

The animal's immunity was boosted with the appropriate cells in IFA until skin testing with 20ug of homologous organisms produced a positive delayed hypersensitivity lesion. The animals were then skin tested with titrations of whole gonococcal cells and gonococcal crude ribosomes. The results, recorded in Table 1, showed a marked difference in the volumes of lesions provoked by crude ribosomes in animals sensitized with homologous organisms when compared to those animals sensitized with heterologous organisms. Saline and wash buffer produced no detectible reactions in any animal.

The ribosomes were about 10 times as potent, based on skin test dose 50's, than the whole cells when used as skin test antigens. The similar results using cold killed and heat killed gonococci to sensitize the animals showed both methods to produce satisfactory sensitization. Based on these results, crude ribosomes show the specificity necessary to differentiate animals sensitized with closely related organisms.
Table 1. Delayed hypersensitivity reactions* elicited in guinea pigs sensitized with cold killed _N. gonorrhoeae_, heat killed _N. gonorrhoeae_, heat killed _N. meningitidis_, and heat killed _N. lactamica_, _N. flavescens_, and a Neoraxella species and skin tested with gonococcal crude ribosomes and whole cells.

<table>
<thead>
<tr>
<th>STD50 (µg)</th>
<th>Sensitizing antigen</th>
<th>Skin test antigens:</th>
<th>Amount of antigen provoking reaction (µg):</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>10.0</td>
</tr>
<tr>
<td>3.00</td>
<td>Heat killed <em>N. gonorrhoeae</em></td>
<td>Whole cells</td>
<td>4/4**</td>
</tr>
<tr>
<td>0.34</td>
<td></td>
<td>Crude ribosomes</td>
<td>4/4</td>
</tr>
<tr>
<td>4.53</td>
<td>Cold killed <em>N. gonorrhoeae</em></td>
<td>Whole cells</td>
<td>4/4</td>
</tr>
<tr>
<td>0.31</td>
<td></td>
<td>Crude ribosomes</td>
<td>4/4</td>
</tr>
<tr>
<td>5.00</td>
<td>Heat killed <em>N. meningitidis</em></td>
<td>Whole cells</td>
<td>4/4</td>
</tr>
<tr>
<td>3.68</td>
<td>Heat killed <em>N. lactamica</em>, <em>N. flavescens</em>, &amp; Neoraxella sp.</td>
<td>Crude ribosomes</td>
<td>3/4</td>
</tr>
<tr>
<td>1.92</td>
<td></td>
<td>Whole cells</td>
<td>2/4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Crude ribosomes</td>
<td>4/4</td>
</tr>
</tbody>
</table>

* 24 hour reaction greater than $10 \times 10 \times 0.4$ mm ($15 \text{ mm}^3$)

** Numerator - no. of animals positive. Denominator - no. of animals tested
Delayed hypersensitivity reactions provoked by gonococcal crude ribosomes, "50S", and 30S ribosomal fractions

Since the crude ribosome preparation was found to be both specific and sensitive enough to be useful as a skin test antigen, the next step was to determine if ribosomal subunits were capable of producing the same, or better, results.

Results of skin testing gonococcal sensitive guinea pigs three months after the final sensitizing injection with cold killed gonococci are recorded in Table 2. The results indicated the animals had lost their sensitivity. Four of the six animals tested failed to give a positive response to any of the antigens. The guinea pigs apparently had lost their sensitivity in a relatively short period of time.

These animals were then injected with 200μg of gonococcal whole cells in IFA and skin tested one week later. Results of this test appear in Table 3. The booster injection stimulated an anamnestic response and subsequent return of skin test reactivity in the animals.

The results showed that the ribosomal subunit fractions are less potent as skin test antigens than is the crude ribosome preparation. Of the two fractions, the "30S" fraction seems to be a better antigen than does the "50S" fraction. Sucrose produced no detectible reactions in any animal.

Immediate and delayed hypersensitivity reactions provoked by gonococcal crude ribosomes, ribosomal subunit fractions, protoplasm, and cell walls

Workers in the area of diagnosing gonorrhea have reported on the use of a variety of cellular antigens. These fall into two main classes,
Table 2. Delayed hypersensitivity reactions* elicited in guinea pigs three months after final sensitizing injection with cold killed *N. gonorrhoeae* and skin tested with gonococcal crude ribosomes, "50S" fraction, "30S" fraction, and sucrose.

<table>
<thead>
<tr>
<th>STS50 (ug)</th>
<th>Antigens used to skin test:</th>
<th>Amount of antigen provoking a delayed reaction (ug):</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>10.0       5.0       1.0       0.5       0.25</td>
</tr>
<tr>
<td>-</td>
<td>Crude ribosomes</td>
<td>2/6**      2/6       1/6       0/6       0/6</td>
</tr>
<tr>
<td>-</td>
<td>&quot;50S&quot; fraction</td>
<td>2/6        2/6       2/6       0/6       0/6</td>
</tr>
<tr>
<td>-</td>
<td>&quot;30S&quot; fraction</td>
<td>2/6        1/6       1/6       0/6       0/6</td>
</tr>
<tr>
<td>-</td>
<td>Sucrose</td>
<td>0/6        0/6       0/6       0/6       0/6</td>
</tr>
</tbody>
</table>

* 24 hour reaction greater than 10 x 10 x 0.4 mm (15 mm³)
** Numerator - no. of animals positive. Denominator - no. of animals tested
Table 3. Delayed hypersensitivity reactions* elicited in guinea pigs given one boosting injection of cold killed *N. gonorrhoeae* three months after the final original sensitizing injection and skin testing with gonococcal crude ribosomes, 50S subunits, 30S subunits, and sucrose one week after boosting.

<table>
<thead>
<tr>
<th>Antigens used to skin test</th>
<th>Amount of antigen provoking a delayed reaction (ug):</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>10.0</td>
</tr>
<tr>
<td>Crude ribosomes</td>
<td>6/6**</td>
</tr>
<tr>
<td>50S subunits</td>
<td>6/6</td>
</tr>
<tr>
<td>30S subunits</td>
<td>6/6</td>
</tr>
<tr>
<td>Sucrose</td>
<td>0/6</td>
</tr>
</tbody>
</table>

* 24 hour reaction greater than 10 x 10 x 0.4 mm (15 mm³)

** Numerator - no. of animals positive. Denominator - no. of animals tested
those isolated from cellular protoplasm, and those isolated from cell walls. The delayed hypersensitivity reactions provoked by preparation of gonococcal protoplasm and cell walls were compared to those provoked by gonococcal crude ribosomes and 50S-30S subunit pool. Reactions were observed at 4 hours after antigen injection to determine if an immediate (Arthus) reaction was elicited as well as the 24 hour delayed hypersensitivity reaction.

Results of these tests appear in Table 4. The results showed that an immediate reaction does occur, indicating a humoral response to the antigens, and that this response correlates well with the delayed reaction. Crude ribosomes again showed the greatest potency, while cell walls and protoplasm gave relatively weak reactions. The 50S-30S subunits showed decreased activity compared to the crude ribosome preparation. It should be noted that while the immediate reactions occurred with all antigens at the lowest (0.25ug) dilution, no attempt was made to define a positive Arthus reaction. The delayed reactions also occurred at very low concentrations of antigen, but these were not great enough to be considered positive (15mm$^3$).

Histological sections of the immediate and delayed lesions appear in Figure 2. The skin site was excised 4 hours and 24 hours after antigen injection, fixed, processed for histological preparation, and paraffin sections were made.

The Arthus reaction, which is maximal 3 to 5 hours after injection, in characterized by infiltration of polymorphonuclear cells (PMN's) in and around the dermal blood vessels. Vascular damage allows erythrocytes and serum to escape into the tissue, causing erythema and edema at the injection site.

The delayed hypersensitivity lesion arises later, being maximal
Table 4: Immediate* and delayed hypersensitivity reactions* elicited in guinea pigs sensitized with *N. gonorrhoeae* and skin tested with gonococcal crude ribosomes, cell walls, protoplasm, and 50S-30S ribosomal pool.

<table>
<thead>
<tr>
<th>STD&lt;sub&gt;50&lt;/sub&gt; (ug)</th>
<th>Antigen used to skin test:</th>
<th>Amount of antigen provoking the reactions (ug):</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Crude ribosomes</td>
<td>10.0</td>
</tr>
<tr>
<td>0.32</td>
<td>Immediate</td>
<td>233.8††</td>
</tr>
<tr>
<td></td>
<td>Delayed</td>
<td>19/19**</td>
</tr>
<tr>
<td>8.20</td>
<td>Cell walls</td>
<td>Immediate</td>
</tr>
<tr>
<td></td>
<td>Delayed</td>
<td>13/19</td>
</tr>
<tr>
<td>7.50</td>
<td>Protoplasm</td>
<td>Immediate</td>
</tr>
<tr>
<td></td>
<td>Delayed</td>
<td>12/19</td>
</tr>
<tr>
<td>0.98</td>
<td>50S-30S pool</td>
<td>Immediate</td>
</tr>
<tr>
<td></td>
<td>Delayed</td>
<td>13/19</td>
</tr>
</tbody>
</table>

* Reaction measured at 4 hours after antigen injection (Arthus reaction). †† Area in mm<sup>2</sup>
* 24 hour reaction greater than 10 x 10 x 0.4 mm (15 mm<sup>3</sup>)
** Numerator - no. of positives. Denominator - no. of animals tested
Figure 3. Four and twenty-four hour skin reactions to gonococcal crude ribosomes in *N. gonorrhoeae* sensitized guinea pigs. C and D are from the same lesion.

A. Normal skin; normal tissue (x280)

B. Four hour immediate (Arthus) reaction provoked by gonococcal crude ribosomes showing thickening of vascular endothelium in upper dermis (x280)

C. Twenty-four hour delayed hypersensitivity reaction provoked by gonococcal crude ribosomes showing mononuclear cell invasion of upper dermis (x280)

D. Twenty-four hour delayed hypersensitivity reaction provoked by gonococcal crude ribosomes showing mononuclear cell invasion of deep dermis (x280)
at 24 to 48 hours after antigen injection. There is a more persistent and intense infiltration of cells than in the Arthus reaction. The cells are generally mononuclear (lymphocytes and histiocytes) cells. The reaction lacks the general necrosis, PMN infiltration and hemorrhage present in the immediate reaction.

Based on the histological sections taken from 4 and 24 hour lesions the reactions observed were indeed immediate (Arthus) and delayed hypersensitivity responses.

Delayed hypersensitivity reactions provoked by gonococcal whole cells, protoplasm, pilin, crude ribosomes, crude ribosomal protein, crude ribosomal RNA, and meningococcal whole cells and crude ribosomes in animals sensitized with N. gonorrhoeae and N. meningitidis

N. meningitidis has been shown, using DNA recombination studies (36), to be very closely related to N. gonorrhoeae. Also, N. meningitidis occurs endemically and could cross react in any tests designed to demonstrate gonorrhea. For these reasons guinea pigs were sensitized with gonococci and meningococci and skin tested with various antigens to determine their specificity.

Table 5 displays the results of skin testing gonococcal sensitive animals with gonococcal and meningococcal crude ribosomes. Table 6 shows the results of skin testing meningococcal sensitive animals with gonococcal and meningococcal crude ribosomes.

As in the first series of skin tests, the gonococcal crude ribosomes showed excellent specificity due to their sensitivity. The meningococcal crude ribosomes showed similar specificity and sensitivity in animals sensitized with homologous organisms. The skin test dose
Table 5. Delayed hypersensitivity reactions* elicited in guinea pigs sensitized with * epididymis and skin tested with gonococcal and meningococcal crude ribosomes

(115,310),(826,504)

<table>
<thead>
<tr>
<th>STDyn (µg)</th>
<th>Antigen used to skin test</th>
<th>Amount of antigen provoking the reaction (µg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>10.0</td>
</tr>
<tr>
<td>0.34</td>
<td>Gonococcal crude ribosomes</td>
<td>10/10**</td>
</tr>
<tr>
<td>0.25</td>
<td></td>
<td>-</td>
</tr>
<tr>
<td>0.29</td>
<td></td>
<td>-</td>
</tr>
<tr>
<td>4.60</td>
<td>Meningococcal crude ribosomes</td>
<td>8/10</td>
</tr>
<tr>
<td>2.60</td>
<td></td>
<td>-</td>
</tr>
<tr>
<td>4.53</td>
<td></td>
<td>-</td>
</tr>
</tbody>
</table>

Table 6. Delayed hypersensitivity reactions* elicited in guinea pigs sensitized with N. meningitidis and skin tested with gonococcal and meningococcal crude ribosomes.

<table>
<thead>
<tr>
<th>STDyn (µg)</th>
<th>Antigen used to skin test</th>
<th>Amount of antigen provoking the reaction (µg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>10.0</td>
</tr>
<tr>
<td>3.36</td>
<td>Gonococcal crude ribosomes</td>
<td>6/10**</td>
</tr>
<tr>
<td>2.60</td>
<td></td>
<td>-</td>
</tr>
<tr>
<td>1.96</td>
<td></td>
<td>-</td>
</tr>
<tr>
<td>0.43</td>
<td>Meningococcal crude ribosomes</td>
<td>10/10</td>
</tr>
<tr>
<td>0.32</td>
<td></td>
<td>-</td>
</tr>
</tbody>
</table>

* 24 hour reaction greater than 10 x 10 x 0.4 mm (15 mm³)

* * Numerator - no. of animals positive. Denominator - no. of animals tested

† This dilution not tested

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50's showed that the results were consistent from test to test and that the crude ribosomes were from 6 to 12 times as potent in animals sensitized homologous organisms than in animals sensitized with heterologous organisms. Due to the difficulty of isolating meningococcal ribosomes, meningococcal ribosomal subunits could not be prepared for use as skin test antigens. Tables 7 and 8 display the results of skin testing in animals sensitized with either N. meningitidis or N. gonorrhoeae using two preparations of ribosomal protein and two preparations of ribosomal RNA. In all cases the protein and RNA elicited stronger reactions in the gonococcal sensitive animals than in the meningococcal sensitive guinea pigs. The skin test dose 50's showed the preparations to be less than one-tenth as potent as the crude ribosome preparations. The protein preparations were slightly more reactive than the RNA preparations.

If either crude ribosomal protein or RNA is responsible for the immunogenicity of the crude ribosome preparation, the immunogenicity is lost when the fractions are separated.

Table 9 presents the results of skin testing with gonococcal whole cells, protoplasm, pilin, and meningococcal whole cells using gonococcal sensitized guinea pigs. Table 10 presents results of skin testing with the same antigens using meningococcal sensitive animals. The results show that none of these fractions are as sensitive or specific as the crude ribosome preparation. Pilin displayed greater potency than any of the other antigens displayed in these tables, but it was still less sensitive and not as specific as the crude ribosome preparation.

Table 11 is a summary table showing all results of skin testing in gonococcal and meningococcal sensitive animals with gonococcal and meningococcal crude ribosomes. This table shows the consistency of the crude ribosome skin tests. The meningococcal crude ribosomes ap-
Table 7. Delayed hypersensitivity reactions* elicited in guinea pigs sensitized with *N. gonorrhoeae* and skin tested with two preparations of gonococcal crude ribosome protein and two preparations of crude ribosome RNA.

<table>
<thead>
<tr>
<th>STD&lt;sub&gt;50&lt;/sub&gt; (ug)</th>
<th>Antigens used to skin test:</th>
<th>Amount of antigen provoking the reaction (ug):</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>10.0  5.0  1.0  0.5  0.25  0.125</td>
</tr>
<tr>
<td>3.48</td>
<td>Gonococcal crude ribosome protein (preparation #1)</td>
<td>--   5/8&lt;sup&gt;++&lt;/sup&gt; 2/8 0/8 0/8 0/8</td>
</tr>
<tr>
<td>3.92</td>
<td>Gonococcal crude ribosome protein (preparation #2)</td>
<td>5/5  3/5  1/5 0/5 0/5 --</td>
</tr>
<tr>
<td>&gt;5.0</td>
<td>Gonococcal crude ribosome RNA (preparation #1)</td>
<td>2/5  0/5  0/5 0/5 0/5 --</td>
</tr>
<tr>
<td></td>
<td>Gonococcal crude ribosome RNA (preparation #2)</td>
<td>0/2  0/2  0/2 0/2 0/2 --</td>
</tr>
</tbody>
</table>

Table 8. Delayed hypersensitivity reactions* elicited in guinea pigs sensitized with *N. meningitidis* and skin tested with two preparations of gonococcal crude ribosome protein and two preparations of crude ribosome RNA.

<table>
<thead>
<tr>
<th>STD&lt;sub&gt;50&lt;/sub&gt; (ug)</th>
<th>Antigens used to skin test:</th>
<th>Amount of antigen provoking the reaction (ug):</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>10.0  5.0  1.0  0.5  0.25  0.125</td>
</tr>
<tr>
<td></td>
<td>Gonococcal crude ribosome protein (preparation #1)</td>
<td>--   0/8&lt;sup&gt;++&lt;/sup&gt; 0/8 0/8 0/8 0/8</td>
</tr>
<tr>
<td></td>
<td>Gonococcal crude ribosome protein (preparation #2)</td>
<td>1/5  0/5  0/5 0/5 0/5 --</td>
</tr>
<tr>
<td></td>
<td>Gonococcal crude ribosome RNA (preparation #1)</td>
<td>0/5  0/5  0/5 0/5 0/5 --</td>
</tr>
<tr>
<td></td>
<td>Gonococcal crude ribosome RNA (preparation #2)</td>
<td>0/1  0/1  0/1 0/1 0/1 --</td>
</tr>
</tbody>
</table>

* 24 hour reaction greater than 10 x 10 x 0.4 mm (15mm<sup>3</sup>)

<sup>++</sup> Numerator - no. of animals positive. Denominator - no. of animals tested

<sup>↑</sup> This dilution not tested
### Table 9. Delayed hypersensitivity reactions* elicited in guinea pigs sensitized with *H. hominis* and skin tested with gonococcal whole cells, protoplasm, and pilin, and meningococcal whole cells.

<table>
<thead>
<tr>
<th>STD₅₀ (µg)</th>
<th>Antigens used to skin test:</th>
<th>Amount of antigen provoking the reaction (µg):</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>10.0</td>
</tr>
<tr>
<td>6.60</td>
<td>Gonococcal whole cells</td>
<td>4/5**</td>
</tr>
<tr>
<td>&gt;5.00</td>
<td>Gonococcal protoplasm</td>
<td>---</td>
</tr>
<tr>
<td>0.83</td>
<td>Gonococcal pilin</td>
<td>---</td>
</tr>
<tr>
<td>8.50</td>
<td>Meningococcal whole cells</td>
<td>3/5</td>
</tr>
</tbody>
</table>

Table 10. Delayed hypersensitivity reactions* elicited in guinea pigs sensitized with *N. meningitidis* and skin tested with gonococcal whole cells, protoplasm, and pilin, and meningococcal whole cells.

<table>
<thead>
<tr>
<th>STD₅₀ (µg)</th>
<th>Antigens used to skin test:</th>
<th>Amount of antigen provoking the reaction (µg):</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>10.0</td>
</tr>
<tr>
<td>&gt;10.0</td>
<td>Gonococcal whole cells</td>
<td>2/5**</td>
</tr>
<tr>
<td></td>
<td>Gonococcal protoplasm</td>
<td>---</td>
</tr>
<tr>
<td>1.64</td>
<td>Gonococcal pilin</td>
<td>---</td>
</tr>
<tr>
<td>7.50</td>
<td>Meningococcal whole cells</td>
<td>4/5</td>
</tr>
</tbody>
</table>

* 24 hour reaction greater than 10 x 10 x 0.4 mm (1.5 mm³)

** Numerator - no. of animals positive, Denominator - no. of animals tested

† This dilution not tested
Table 11. Summary of delayed hypersensitivity reactions* elicited in guinea pigs sensitized with *N. gonorrhoeae and *N. meningitidis and skin tested with gonococcal and meningococcal crude ribosomes.

<table>
<thead>
<tr>
<th>Gonococcal sensitive guinea pigs</th>
<th>Meningococcal sensitive guinea pigs</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>STDno.</strong></td>
<td><strong>ST Antigen:</strong></td>
</tr>
<tr>
<td>(ug)</td>
<td></td>
</tr>
<tr>
<td>0.32</td>
<td>G.C.C.R. a</td>
</tr>
<tr>
<td>0.34</td>
<td>G.C.C.R.</td>
</tr>
<tr>
<td>0.25</td>
<td>G.C.C.R.</td>
</tr>
<tr>
<td>0.29</td>
<td>G.C.C.R.</td>
</tr>
<tr>
<td>0.31</td>
<td>G.C.C.R.</td>
</tr>
<tr>
<td>4.53</td>
<td>X.C.R.</td>
</tr>
<tr>
<td>2.60</td>
<td>X.C.R.</td>
</tr>
</tbody>
</table>

* 24 hour reaction greater than 10 x 10 x 0.4 mm (15 mm³)
* Number - no. of animals positive. Denominator - no. of animals tested
a Gonococcal crude ribosomes
b Meningococcal crude ribosomes
r This dilution not tested
peared to possess the same sensitivity and specificity in animals sensitized with homologous organisms as did the gonococcal crude ribosomes. The crude ribosome preparation possessed the sensitivity and the specificity, based on the preparation's sensitivity, necessary for a diagnostic test in the guinea pig system.

In vitro correlate of delayed hypersensitivity: The macrophage migration inhibition test

To determine if the crude ribosome preparation could be used in vitro demonstration of delayed hypersensitivity, the macrophage migration inhibition test was performed. Peritoneal exudate cells (PEC's) were taken from gonococcal-sensitive, meningococcal-sensitive and non-sensitized guinea pigs. These were incubated in triplicate in Eagle's Modified Minimal Essential Medium (Gibco) with either gonococcal crude ribosomes, meningococcal crude ribosomes, or without antigen. The results of this test appear in Table 12 and are displayed graphically in Figure 4.

Results showed the gonococcal crude ribosomes caused an 85% inhibition of macrophage migration in the gonococcal-sensitized PEC's compared to non-sensitized and meningococcal-sensitized PEC's. The meningococcal crude ribosomes showed a 60% inhibition of macrophage migration in meningococcal sensitive PEC's compared to non-sensitized PEC's, while inhibiting the gonococcal sensitive PEC's less than 11%.

The ribosomes were toxic to normal PEC's at this concentration. Results of Johnson and Bigley (personal communication) have shown that ribosomes from Streptococcus pyogenes and Salmonella typhimurium are highly toxic for both sensitized and non-sensitized PEC's. The gonococcal
Table 12. Comparison of macrophage migration inhibition of normal, *N. gonorrhoeae*, and *N. meningitidis* sensitized peritoneal exudate cells cultured in the absence of antigen or in the presence of either gonococcal or meningococcal crude ribosomes.

<table>
<thead>
<tr>
<th>Antigen added (0.5ug)</th>
<th>Average area of migration* of:</th>
<th>% Migration** of:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Normal cells</td>
<td>Gonococcal sensitive cells</td>
</tr>
<tr>
<td>None</td>
<td>5.2</td>
<td>1.8</td>
</tr>
<tr>
<td>Gonococcal crude ribosomes</td>
<td>2.4</td>
<td>0.3</td>
</tr>
<tr>
<td>Meningococcal crude ribosomes</td>
<td>3.8</td>
<td>2.0</td>
</tr>
</tbody>
</table>

* Area of migration in mm²
** Percent of migration equals the area of migration of cells with antigen divided by the area of migration of cells without antigen times 100
Figure 4. Results of macrophage migration inhibition test. Average area of spreading of macrophages of non-sensitized and N. gonorrhoeae and N. meningitidis sensitized guinea pigs exposed to 0.5 ug of either gonococcal or meningococcal crude ribosomes.

<table>
<thead>
<tr>
<th>Antigen Added to System</th>
<th>Normal PEC</th>
<th>Gonococcal Sens. PEC</th>
<th>Meningococcal Sens. PEC</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gonococcal crude ribosome</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Meningococcal crude ribosome</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
and menigococcal crude ribosomes appeared to be a great deal more toxic for non-sensitized PEC's than for sensitized PEC's, allowing this test to be useful as an in vitro test for delayed hypersensitivity in guinea pigs sensitized to gonococci and meningococci.

The macrophage migration inhibition test was repeated using 0.3μg of gonococcal or menigococcal crude ribosomes as test antigens. Results appear in Table 13 and Figure 5. The decreased ribosome concentration diminished the toxicity of the preparation drastically. Normal PEC's migration was inhibited by only 3-4% by the crude ribosome preparations.

At this concentration the ribosome preparations specifically inhibited macrophage migration when the PEC's were from animals sensitized with homologous organisms. The gonococcal crude ribosomes inhibited macrophage migration by greater than 90% in the homologous system while cross reacting with menigococcal sensitized cells only slightly, inhibiting their migration by less than 15%. The menigococcal crude ribosomes inhibited migration by greater than 80% in the homologous system. Cross reacting inhibition of cells sensitized with gonococci was around 30%.

These results confirm the suitability of the gonococcal crude ribosome preparation as antigen for in vitro testing. The crude ribosome is specific in this system for cells taken from animals sensitized with homologous organisms, and they are not toxic for non-sensitized cells at this concentration.
Table 13. Comparison of macrophage migration inhibition of normal, *H. gonorrhoeae*, and *N. meningitidis* sensitized peritoneal exudate cells cultured in the absence of antigen or in the presence of either gonococcal or meningococcal crude ribosomes.

<table>
<thead>
<tr>
<th>Antigen added (0.3μg)</th>
<th>Average area of migration * of:</th>
<th>% Migration ** of:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Normal cells</td>
<td>Gonococcal sensitive cells</td>
</tr>
<tr>
<td>None</td>
<td>9.6</td>
<td>8.3</td>
</tr>
<tr>
<td>Gonococcal crude ribosomes</td>
<td>9.3</td>
<td>0.6</td>
</tr>
<tr>
<td>Meningococcal crude ribosomes</td>
<td>9.0</td>
<td>5.7</td>
</tr>
</tbody>
</table>

* Area of migration in mm²
** Percent of migration equals the area of migration of cells with antigen divided by the area of migration of cells without antigen times 100.
Figure 5. Results of macrophage migration inhibition test. Average area of spreading of macrophages of non-sensitized and N. gonorrhoeae and N. meningitidis sensitized guinea pigs exposed to 0.3ug of either gonococcal or meningococcal crude ribosomes.

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The development of a diagnostic test for gonorrhea is of major importance. The disease has reached epidemic proportions in the United States (68). The study of gonorrhea was long slowed by the "social stigma" attached to any venereal disease and the assumption that drugs, especially penicillin, would control the disease. Research on gonorrhea has lagged behind that of other diseases, consequently our knowledge of the gonococcus, and gonococcal infection, is still quite limited.

Laboratory study of any disease requires the use of a suitable model system. Gonorrhea lacks an "ideal" model system. Other than man, only certain primates, which are expensive to maintain and difficult to work with, are susceptible to gonococcal infection (41). Researchers are forced to use artificial systems, in which laboratory animals are artificially sensitized to gonococcal antigens. The guinea pig has long been an excellent model for delayed hypersensitivity studies. While the guinea pig is not susceptible to gonococcal infection, the animal is easily sensitized and reacts with a strong cell-mediated delayed hypersensitivity response.

Though the guinea pig model cannot be directly related to the human infection, the work presented here is of more than academic value. Many researchers have demonstrated a cell-mediated immune response to gonococcal infection in the human. The transfer of results of skin testing in the guinea pig cannot be made to humans, but it is hoped that other researchers with proper facilities will continue this work and try to determine the validity of skin testing in the human infection.
The results indicated that the gonococcal crude ribosome was sensitive and specific for animals sensitized with homologous organisms. It was shown that crude ribosome preparations failed to react in either the closely related *N. meningitidis* sensitive animals or in animals sensitized with the less closely related saprophytic species at dilutions of less than 1.0μg. The gonococcal whole cell preparations, cell walls, protoplasm, pilin, "50S" and "30S" ribosomal fractions, crude ribosomal protein, and crude ribosomal RNA all failed to demonstrate the specificity and sensitivity displayed by the crude ribosome preparation.

Testing with crude ribosomes and ribosomal subunits demonstrated that the crude ribosome preparation possessed greater antigenicity than either the "50S" or "30S" fraction. This differs from the results reported by Baker, *et al.* (2), which showed the 30S particle to be 3-4 times as potent a skin test antigen in the mycobacterial system. Apparently the antigenicity of the gonococcal crude ribosome differs from that of the mycobacterial ribosome. Attempts to isolate the antigenic fraction of the crude ribosome preparation yielded negative results. Further studies using purified subunits might clarify this situation.

Delayed hypersensitivity reactions are generally associated with larger molecules, specifically proteins, as antigens compared to the smaller haptenic regions capable of stimulating specific humoral antibodies (17). For this reason, proteins in the crude ribosome preparation could be responsible for the preparation's antigenicity in a skin test. There are four sources of protein in the crude ribosome preparation.

The first source is protein associated with the ribosome. Ribosomal protein constitutes approximately 40% of the material in the ribosome (11). These proteins, which are bound to the ribosome in intimate contact with ribosomal RNA, are responsible for the enzymatic processes...
of polypeptide synthesis by the ribosome.

A second source of protein are the initiation factors responsible for the binding of the 50S and 30S subunits during protein synthesis (20,55). These factors could be liberated when the subunits dissociate and thus could account for the decreased activity of the 50S and 30S particles.

The third source of protein is nascent protein. This is protein in the process of being synthesized. As long as the 50S and 30S particles remain bound to one another, and synthesis is not complete, varying amounts of nascent polypeptide could remain attached to the ribosome. When the subunits dissociate the nascent protein would be liberated, diluted extensively in the buffer, and reactivity lost.

The fourth source of protein in the crude ribosome preparation is from membraneous contamination. There must certainly be some membrane fragments in the crude ribosome preparation. If so, these fragments could contain a variety of proteins which could be responsible for the skin test activity seen in the crude ribosome preparation.

In order to determine if the antigenic fraction was protein in nature, regardless of source, proteins were isolated from the crude ribosome preparation by two methods. In both cases the protein preparations, which may contain protein from all four sources, showed a 10 fold decrease in activity when compared to the crude ribosome preparation in gonococcal sensitive animals. If any of the sources supplied the active protein, the removal of the ribosomal RNA caused a decrease in the activity of that protein. Since membraneous protein contaminants and nascent protein would suffer very little conformational change by removing the RNA, it is doubtful if these two sources supplied the active fraction of the crude ribosome preparation.
Ribosomal protein and initiation factors would suffer conformational changes when removed from the ribosomal RNA and therefore could, although showing decreased activity when removed from the RNA, supply the antigenicity when present in the intact ribosome.

None of the three sources of RNA, ribosomal RNA, constituting approximately 60% of the material in the ribosome (4), transfer RNA, and messenger RNA, appeared to be the active fraction. Two methods of extraction were employed to remove the RNA from the crude ribosome preparation. Regardless of the method of extraction, the RNA showed at least a 10-fold decrease in activity compared to the crude ribosome preparation. Again, if the RNA is the active fraction of the preparation, its configuration in the intact ribosome must be critical to its activity.

The immunology of the ribosome is just beginning to be studied extensively. At present, many researchers are using ribosomes and ribosomal fractions in many different studies involving a variety of organisms. Data obtained thus far are often contradictory. Smith and Bigley (62) have shown both ribosomal RNA and ribosomal protein from Salmonella typhimurium to be immunogenic. Venneman has reported that S. typhimurium ribosomal protein to be nonimmunogenic (70). Winston and Berry suggested the immunogenicity of Staphylococcus aureus ribosomes is due to the ribosomal protein, but the ribosomal RNA acts as an adjuvant (76). Thompson and Snyder (66) noted that in the Diplococcus pneumoniae system both ribosomal RNA and protein are required for immunogenicity but Youmans and Youmans (73) demonstrated that the removal of the ribosomal RNA decreased the immunogenicity of the mycobacterial ribosome while removal of the protein did not. Baker, et al. (2) has shown that the 30S ribosomal particle is 3-4 times more potent a skin test antigen than either the 70S ribosome or the 50S subunit using M. bovis (BBG).
Results of this study show this not to be the case when ribosomes from *N. gonorrhoeae* are used as skin test antigens. Until more testing is done with the gonococcal ribosome, as well as ribosomes from many other organisms, the exact nature of ribosomal immunogenicity will remain in question.

An interesting feature observed when the ribosomal subunits were being tested was the loss of sensitivity among the guinea pigs after only three months. While the model system is indeed artificial, the decrease in sensitivity may be important. Workers using the same model (Baker, personal communication) have demonstrated sensitization to mycobacterial antigens lasting over 18 months. It is possible that gonococcal antigens produce only a short period of sensitization, with a subsequent decrease in skin test reactivity. If this is true it would be of extreme importance in any diagnostic test for gonorrhea, where the possibility of repeated infection makes it difficult to differentiate between active infections and past infections which were successfully treated.

Most antigens used in a variety of diagnostic tests for gonorrhea fall into two main categories; those extracted from the cell wall, and those extracted from the protoplasm of the gonococcus. Both fractions failed to show the reactivity demonstrated by the crude ribosome preparation when used in skin testing. The protoplasmic fraction failed to react as well as expected since this fraction should contain intact ribosomes.

Several factors argue against the presence of ribosomes in the protoplasm. The gonococcus is extremely susceptible to autolysis. While care was taken to remove as many of the autolytic and RNA degrading enzymes as possible from the ribosome preparations, no attempt was
made to remove these from the protoplasm. Some ribosomal loss would occur by autolysis. Also, no magnesium was added to the system, so some dissociation of the 70S ribosomes had to occur as well. Finally, since there are many heterologous constituents of protoplasm, the concentration of ribosomes would be proportionally less in the protoplasm than in the crude ribosome preparation.

The immediate (Arthus) reactions observed 4 hours after antigen injection paralleled the delayed reactions observed at 24 hours after antigen injection. No attempt was made to define what constituted a positive Arthus reaction. The importance of these observations lies in the ability of the crude ribosome to react with humoral antibody. Based on the increased areas of the Arthus reactions provoked by the ribosome preparation as compared to those reactions provoked by the cell walls, protoplasm, and 50S-30S pool, the crude ribosome has greater sensitivity when reacting with humoral antibody than do any of the other antigens tested. Since the crude ribosome is capable of reacting with humoral antibody, it may be of value in a sero-diagnostic test as well as a skin test. Further research should be done in this regard.

The specificity of the gonococcal crude ribosomes was demonstrated using animals sensitized with either *N. gonorrhoeae* or *N. meningitidis*. Kingsbury, et al. (36), have demonstrated that the Neisseria genus can be divided into three distinct groups based on DNA homology. The three groups are the "pathogens", the "non-pathogens", and *N. catarrhalis*. In the pathogen group, *N. gonorrhoeae* and *N. meningitidis* have at least 80% of their nucleotide sequences similar. The non-pathogen group have from 8-15% similarity to the pathogens and *N. catarrhalis* shows no relation to any of the other species tested. Due to the similarity of the gonococcus and the meningococcus, *N. meningitidis* was used to son-
sitize animals to determine the specificity of the gonococcal crude ribosome preparation. It was shown that the crude ribosome was able to differentiate between guinea pigs sensitized with these two very closely related organisms.

The macrophage migration inhibition tests showed the skin test reactions to be true cell-mediated delayed hypersensitivity responses stimulated by crude ribosomes and mediated by lymphoid cells specifically sensitized to gonococcal antigens. The in vitro demonstration of cell-mediated immunity with the gonococcal and meningococcal crude ribosome preparations opens the way for the human cellular response with these antigens. It is difficult to obtain permission to use human subjects for in vitro testing, but the use of peripheral lymphocytes in an in vitro correlate of delayed hypersensitivity (51), such as the macrophage migration inhibition test, would allow the use of the gonococcal crude ribosome in testing the human cellular response to gonococcal infection.

This study has shown that the gonococcal crude ribosome preparation is both specific and sensitive when used as a skin test antigen in sensitized guinea pigs. Further studies should be carried out to ascertain whether the same holds true in the human infection with N. gonorrhoeae. Also observed during this study was the ability of the crude ribosome preparation to react with humoral antibody. This indicates the crude ribosome may be of value as an antigen in a serodiagnostic test for gonorrhea. This aspect should also be studied further. Finally, it was demonstrated that crude ribosome preparation was capable of being used in an in vitro correlate of delayed hypersensitivity, suggesting the human immune response in gonorrhea could be studied using the crude ribosome in in vitro experiments with human peripheral lymphocytes.
CHAPTER V

RESULTS OF THIS STUDY INDICATED THAT THE GONOCCOCAL CRUDE RIBOSOMES possessed the sensitivity and specificity required to differentiate between guinea pigs artificially sensitized with \textit{N. gonorrhoeae} and animals sensitized with the closely related organism, \textit{N. meningitidis} when used as a skin test antigen. The crude ribosome preparation displayed better sensitivity and specificity than any of the other gonococcal cell fractions utilized as skin test antigens.

It was noted that artificial sensitization of guinea pigs with whole gonococcal cells produced a transient sensitization lasting less than three months. It was also shown that the gonococcal crude ribosome was capable of reacting with humoral antibody indicating that the crude ribosome preparation may be useful in a sero-diagnostic test as well as a skin test.

The demonstration that the crude ribosome preparation could be used in an \textit{in vitro} correlate of delayed hypersensitivity opens the way for studying the human cell mediated response to gonococcal infection. Peripheral lymphocytes from humans could be used as an \textit{in vitro} test to determine the value of gonococcal ribosomes as a diagnostic test for the human infection with \textit{N. gonorrhoeae}.

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