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Cell-mediated immune responses to Neisserria gonorrhoeae infection measured with gonococcal ribosomes

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CELL-MEDIATED IMMUNE RESPONSES TO *Neisserria gonorrhoeae* INFECTION MEASURED WITH GONOCOCCAL RIBOSOMES

by

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Cell-mediated immune responses of Neisseria gonorrhoeae infection measured with gonococcal ribosomes. (64 pp.)

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Bacterial ribosomes have been shown to be specific antigens in immunoassays. However, N. gonorrhoeae ribosomes have not been tested for use in a reliable immunological test for the detection of gonorrhea. In the present study, in vitro macrophage migration inhibition (MIF) tests were employed to characterize the cell-mediated immune (CMI) response of gonorrhea patients to a crude gonococcal ribosome preparation. It was ascertained that lymphocytes from patients had become sensitized to homologous ribosomes within 10 days of contact of the disease and lose sensitization 8 to 16 weeks post-therapy. A similar but non-specific response was observed with lymphocytes from patients and control subjects, when incubated with gonococcal endotoxin. Differential criteria for diagnosis were established according to lymphocyte responses to the two preparations, and were used in correct diagnosis of 7 of 9 culture-positive patients and 8 of 9 negative controls. Results from this research warrant the investigation of chimpanzee or human infection for in vivo responses to intradermal administration of gonococcal ribosomes.

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ABBREVIATIONS

ACEP . . . . . Ammonium Cloride EDTA Phosphate

$CELD_{50}$ . . . . . Chick Embryo LD_{50}

CMI . . . . . Cell-Mediated Immunity

FCS . . . . . Fetal Calf Serum

FI_{50} . . . . . Fever Index 40

GC . . . . . Gonococcus, Gonococci, or Gonococcal

LAL . . . . . Limulus Amebocyte Lysate

LPS . . . . . Lipopolysaccharide (endotoxin)

MEM . . . . . Minimal Essential Medium

MIF . . . . . Macrophage Migration Inhibition (Factor)

Pd . . . . . Dulbecco Phosphate Buffer Solution

T-M . . . . . Thayer-Martin

WBC . . . . . White Blood Cell (Leukocyte)

CHAPTER I

INTRODUCTION

Gonorrhea is currently the number one reported communicable disease in the United States. Yet, for every reported case it is estimated that there are three cases which go unreported (2). The primary reason for this is that gonorrhea is commonly without symptoms. Estimates of asymptomatic female infection range from 40% to 80%, and estimates of asymptomatic male infection range from 5% to 50% (32, 55). Without symptoms of infection, infected persons do not seek diagnosis, and therefore comprise a pool or reservoir of infection in the population which helps maintain the disease at epidemic proportions (12, 52). Despite adequate treatment procedures, reported cases of gonorrhea doubled in the six years from 1966-1972, with 624,000 cases reported in the U.S. in 1971 (10). The need is apparent for a means by which this epidemic may be controlled.

Researchers have studied the possibility of developing a protective vaccine against Neisseria gonorrhoeae infection. Arko (4, 5) was able to establish a protective immunity in chimpanzees with a formalinized whole-cell vaccine; protection lasted ten weeks. Others have stimulated increased

antibody levels by vaccination, without evident protective effects (30), although Drutz (24) has reported that patients with a history of gonococcal infection are more difficult to infect experimentally. Recent reports indicate that gonococcal ribosomes provide an effective vaccine, which stimulated inhibition of N. gonorrhoeae growth in subcutaneous chambers implanted in guinea pigs (16). Although development of a vaccine would be ideal in dealing with the epidemic, endeavors to date have revealed less than desirable results. The major barrier to developing a vaccine is lack of a gonococcal antigen which will elicit a protective response and will continue to elicit that response for long periods of time. Investigations to date have been able to do no better than mimic the natural infection which, because it is localized and somewhat isolated from the immune system, elicits an antibody response that lasts for only a few months post-therapy and fails to elicit a detectable protective immunity (38).

Therefore, investigators have focused on an approach to screen large populations in order to detect and treat this pool of infection. Diagnosis is presently based on the ability to culture N. gonorrhoeae organisms from the vagina or male urethra. In 1973 a gonococcal culture screening program was employed by the United States Public Health Service in an attempt to identify the reservoir of infection in the population (2). The program detected 242,276 positives from

nearly 5 million examined in 1973, or approximately 5 per cent. Though seemingly significant, this segment of the population examined represents a very small fraction of the total population at risk. The method of culturing is sufficient for laboratory diagnosis (70-90% reliable) (14, 55, 58, 72), but is time consuming and expensive for application in a large scale screening program.

Epidemiologically, a system of 5 to 8 minute interviews with culture-positive patients, in order to identify sexual partners, has detected many asymptomatic victims (10, 55). Such methods are expensive, time consuming, and inefficient at locating the partners. Therefore, this approach is impractical as a single screening tool, although useful as an adjunct.

In the search for reliable and easily administered diagnostic tests, researchers have turned to examination of patients' immune response. Studies have focused primarily on the humoral (circulating antibody) response, with various gonococcal preparations used as antigens. Complement fixation tests using N. gonorrhoeae protoplasm and protoplasm fractionated through Sephadex as antigens have detected higher serum antibody levels in patients than in controls (20, 38, 64, 68, 86). Gel immunodiffusion (13), microprecipitin (15, 67), and micro-flocculation with gonococcal extracts adhered to cholesterol-lecithin particles (66) have been studied. These tests lack specificity and sensitivity,

yielding a high percentage of false positive and false negative diagnoses. Radioimmunoassay with pilin and partially defined cellular preparations used as antigens, has been the most sensitive method examined to date (38, 53). These require simplification of technique and adaptation to equipment commonly available in clinical diagnostic laboratories, before being employed as a tool to screen for the pool of asymptomatic infections.

Cell-mediated immunity (CMI) or delayed hypersensitivity has been shown be David et al. (21, 22, 23) to be as specific as humoral immunity in non-gonococcal systems. With in vitro and in vivo methodologies of delayed hypersensitivity, he demonstrated no cross reactivity to antigens with animals sensitized to PPD, ovalbumin, and toxoid. Thor et al. (82) found similar specificity with PPD, histoplasmin, and coccidioidin. Such specificity of delayed hypersensitivity is the basis for tuberculosis skin test diagnosis.

Examination of CMI to gonococcal infection has received much less consideration than has humoral testing. In studies carried out early in this century, various gonococcal extracts were employed as antigens in skin tests on patients. Whole cells, autolysis extracts, and other N. gonorrhoeae culture filtrates revealed cutaneous induration 24 h following injection (17, 35, 40, 47). These tests were abandoned with the inception and widespread use of penicillin treatment.

Not until the 1970's did investigators resume study of CMI in gonococcal patients. Kraus et al. (49) used a lyophilized gonococcal sonicate as antigen and demonstrated CMI by in vitro blastogenesis of lymphocytes taken from men with repeated gonococcal urethritis. These studies failed to show significant differences in blastogenesis between controls and patients having had only one infection. Grimble and McIllmurray (31) in 1973 found 86% positive CMI response in vitro by patients' lymphocytes to a cell sonicate supernatant fluid (protoplasm). They found that lymphocytes became reactive within 4 to 5 days after contraction of infection. Lymphocytes from patients did not respond to similar antigen preparations from the closely related Neisseria meningitidis organism. Esquenazi and Streitfeld (26, 27) found in both the rabbit and human system that in vitro lymphocyte blastogenesis to various protoplasmic fractions disappeared 5 to 6 weeks after vaccination (rabbit) or treatment (human).

Most humoral and CMI immunological tests lack an antigen which is specific to N. gonorrhoeae. The antigen used must be one to which infected persons are sensitive, and which does not cross react with persons sensitive to closely related organisms. Present evidence indicates that ribosomes of each species of bacteria are antigenically specific for that organism (18, 59, 61). Otaka et al. (60) found the 30S ribosomal subunits of Escherichia coli strains K12 and B to

differ in one or more ribosomal proteins. Their work demonstrated that some ribosomal proteins from 2 strains of the same species are electrophoretically identical, yet some are different. Alberghina and Suskind (1) found that animals immunized with Neurospora crassa responded by making antibodies which precipitated N. crassa ribosomes. Sarkar and Som (69) immunized rabbits with ribosomal protein of plant, rat, E. coli, and yeast, and found reactions with immunodiffusion tests only with homologous ribosomal protein. Judd and Koostra (44, 48) using various cell wall, pilin, protoplasmic, and ribosomal preparations (crude ribosomes, 50S, 30S, ribosomal RNA, and ribosomal protein) demonstrated CMI in guinea pigs sensitized by whole cell N. gonorrhoeae vaccinations. They found that crude ribosomes were specific for guinea pigs sensitized to the homologous organisms, and minimal cross reactivity with ribosomes from closely related N. meningitidis organisms.

Other investigators have used ribosomes as immunogens to vaccinate animals against various microorganisms of tumors. These studies demonstrated that ribosomes are immunogenic. Zatti et al. (94) were able to protect rats from Yoshida ascites tumor by injection of homologous ribosomes. Similar protective immunity has been demonstrated in experimental animals against Streptococcus pyogenes (70), Staphylococcus aureus (87, 88), Diplococcus pneumoniae (79), N. gonorrhoeae (16), Mycobacterium tuberculosis (90, 91, 92,

93), and Salmonella typhimurium (39, 43, 63, 84). Johnson (43) found that the protection afforded mice against Salmonella typhimurium was specific; i.e., it did not provide protection against challenge with Salmonella enteritidis or Salmonella cholerae-suis.

Ribosomes may be immunogens, antigens to detect humoral immunity, and antigens involved in CMI. Baker et al. (6, 7) demonstrated CMI induced in rabbits to BCG crude ribosomes and a "30S-50S" pool of BCG ribosomes by skin testing, MIF testing, and skin reactive factor testing. Smith and Bigley (73, 74) demonstrated that ribosomal immunization would protect mice from 5000 LD_{50} of Salmonella typhimurium. This protection was transferred to normal mice by peritoneal exudate cells, lymph node cells, or spleen cells, but not by serum. Judd and Koostra (44, 48), as mentioned above, demonstrated CMI to N. gonorrhoeae ribosomes in sensitized guinea pigs, and showed a correlation between in vitro macrophage migration inhibition (MIF) tests of CMI and in vivo skin testing.

Lipopolysaccharide (LPS) from gram-negative bacteria has shown to cause lymphocytes to mimic CMI (33, 34, 62, 75). These investigators have demonstrated that LPS may inhibit cell migration in MIF tests, or stimulate blastogenesis in in vitro lymphocyte transformation tests. This has opened a major criticism of ribosomal immunology since ribosomal preparations from gram-negative bacteria almost certainly

are contaminated with LPS during their isolation. Therefore, LPS contained withing ribosomal preparations may be responsible for results obtained (63).

Many in vitro correlates of skin test CMI have been developed (9, 29, 45, 80, 81, 82, 89). Most of these measure lymphokine release from sensitive lymphocytes upon exposure to antigens. David et al. (22) discovered that guinea pig macrophages, when incubated with sensitive lymphoytes, were inhibited from migrating upon exposure to antigen. Peritoneal exudate (PE) cells from non-sensitive guinea pigs mixed in a ratio of 9:1 with PE cells from sensitive guinea pigs produced positive MIF test results. Lymphokines released from the sensitive lymphocytes when exposed to antigen, inhibited the non-sensitive PE cells from migrating. However, these non-sensitive PE cells, when incubated with sera from immunized animals, were not inhibited from migrating. Therefore, CMI is responsible for this phenomenon.

The work of David et al. (22) presented a method by which delayed hypersensitivity may be studied in vitro with human lymphocytes. Guinea pig macrophages may be mixed with human peripheral lymphocytes in order to perform MIF tests. If the human lymphocytes are sensitive to the antigen, they will release MIF to inhibit the guinea pig macrophages from migrating.

Since LPS within ribosomal preparations may cause lymphocytes to mimic CMI in vitro, it is necessary to compare

responses to LPS and ribosomes. This will determine whether results from ribosomal preparations as antigen are due to the ribosomes, and/or to LPS contained within.

Statement of Thesis

Gonorrhea patients have been shown to elicit CMI to various partially defined antigen preparations. Homologous bacterial ribosomes have been used as antigens to demonstrate CMI in many non-gonococcal infections. Previously, no studies had been carried out to test whether gonococcal patients develop CMI to N. gonorrhoeae ribosomes during the course of infection. Therefore, the purpose of this investigation was to: 1) determine whether patients, who are gonococcal culture positive, develop CMI to a N. gonorrhoeae ribosome preparation, and 2) characterize the moment of inception, and duration after drug therapy, of sensitization.

CHAPTER II

MATERIALS AND METHODS

Cultivation of Neisseria gonorrhoeae

GC Medium Base (76): In a 2 liter flat bottom flask (which provided large surface to volume ratio) the following substances were mixed with one liter of distilled water and the solution was sterilized by autoclaving:

Proteose peptone #3	15 gm
Corn starch	0.1 gm
K_2HPO_4	4 gm
NaH_2PO_4	1 gm
NaCl	5 gm

GC Supplement: The following substances were dissolved in 100 ml of distilled water and the solution was sterilized by filtration through a 0.45 µg Millipore filter:

Cocarboxylase	1 mg
Glutamine	0.5 gm
Glucose	20 gm

Modified Thayer-Martin (T-M) Broth: Twenty ml of GC Supplement were added to one liter of sterile GC Medium Base. The T-M Broth was incubated at 37°C for 48 h to insure sterility.

Modified T-M Chocolate Agar: Double strength CG Medium Base was prepared in 500 ml distilled water. Ten g of agar were added, and the mixture was sterilized by autoclaving.

Ten g of hemoglobin (Ditco) were mixed in 500 ml distilled water and the solution was sterilized by autoclaving.

When the sterilized solutions had cooled to 60°C, 20 ml of GC Supplement were added to CG Base and agar, and the mixture was poured into the hemoglobin solution. The Modified T-M Chocolate Agar was swirled to mix, poured into petri dishes, and allowed to solidify. When the Agar was solid, the petri dishes were sealed with tape to retain moisture, and refrigerated until needed.

Bacteria: Neisseria gonorrhoeae strain F62 was obtained from Dr. J. A. Rudbach, University of Montana. A stock culture was maintained on T-M Chocolate Agar at 37°C in a candle jar. The stock was transferred to new plates routinely every 48 h.

Twenty-four hour cultures of N. gonorrhoeae were washed from agar surfaces into sterile T-M broth. The broth was gassed with 5% CO_2 for 30 sec and incubated at 37°C on a wrist action shaker for 48 h (A growth curve was performed on the batch of cells, and revealed that late log was reached at 48 h). Flasks proven to be pure GC by subculturing onto T-M agar, oxidase reaction, and gram stain, were spun at 5900xg, 4°C for 7 min to pellet the cells. Yield from one

liter was approximately 5 g CG. The pellet was frozen at -70°C until 50 g of cells had been accumulated.

Ribosome Preparation

Buffers: "65S" (pH 7.4) 0.015 M $MgCl_2$
0.01 M Tris-HCl
0.5 M NH_4Cl

Crude Ribosome Isolation: All operations were carried out at 4°C. Fifty g of CG were suspended in 150 ml "65S" buffer. The suspension was poured over 100 ml ice and left at 4°C overnight to kill all GC. The killed cells were pelleted at 700 xg, resuspended in 100 ml "65S" buffer, and disrupted at 13,000psi in a pre-chilled Ribi Cell Fractionator (56). The disrupted cell suspension was centrifuged at 48,200xg for 45 min in a Sorvall RC1-B centrifuge to remove cell wall, cell membrane and other particulate matter. The supernatant fluid was centrifuged at 176,000xg for 3 h in a Beckman L2-65B ultracentrifuge with a Ti 60 rotor head. The pellet was resuspended in 30 ml "65S" buffer overnight. The suspension was centrifuged at 48,200xg to remove all particulate matter, and the ribosomes were pelleted again at 176,000xg for 3 h. This "washed" pellet was resuspended in 10 ml "65S" overnight and the concentration was adjusted to 5 mg/ml. The suspension was divided into 1 ml portions, and stored at -70°C until needed.

Purified Ribosomes: Crude ribosomes isolated as above were eluted through a Sephadex G100 column at 4°C. Fractions with peak absorbance at 254 nm were collected, sedimentation coefficient values were determined, and the purified ribosomes preparation was stored at -70°C.

Determination of Concentration: Using an extinction coefficient for ribosomes at 260 nm of 145 (36), the concentration was assayed with a Beckman DU-2 spectrophotometer.

$$\text{Concentration} = \frac{\text{Dilution} \times \text{A260}}{145} \times 10 \text{ mg/ml}$$

Determination of Protein and RNA Content: The ratio of absorbance at 260 nm and 280 nm is characteristically 1.0 for ribosomes (79). All preparations used in this study had ratios ± 4% of that figure.

Determination of Sedimentation Coefficient (S): A ribosomal preparation of 5 mg/ml was centrifuged at 260,000xg (60,000 RPM) in a Beckman Model E ultracentrifuge. Temperature of the An-D rotor was maintained at 4°C, and the density gradient in the centrifuge cell was monitored every 2 min with photographs of the schlieren optics. The distance of the schlieren peak from the reference hole was measured, analyzed in a Wang computer with a pre-written sedimentation coefficient program, and apparent sedimentation coefficients were determined for each peak. Any baseline perturbation and peaks other than 30S, 50S, and 70S were indicators of the impurity of the preparation.

Lipopolysaccharide (LPS) Preparation

Ten g of frozen GC cells were resuspended in 100 ml of distilled water. An equal volume of 88% phenol was added and the suspension was stirred at 65°C for 30 min. This suspension was poured into centrifuge tubes and stored at 4°C for 3 days. The tubes were centrifuged at 1600xg, 4°C for 1 h and the phenol phase was discarded. The aqueous phase was dialyzed against running tap water at room temperature for 7 days, dialyzed at 4°C against deionized distilled water through three changes of water (every 3 h), lyophilized, then stored at -70°C. The lyophilized LPS was resuspended to 5 mg/ml in "65S" buffer and stored at -70°C until needed.

Bioassays

Limulus Assay for LPS (25,65): Ten-fold dilutions of E. coli LPS, GC LPS, and GC crude ribosomes were made with triple-distilled water, in dilutions of 10^5 ng/ml through 0.1 ng/ml.

Limulus amebocyte lysate (LAL) was obtained from Rocky Mountain Laboratory, Hamilton, Montana. One-tenth ml limulus lysate was added to 0.1 ml of the dilution being tested, mixed with a Vortex mixer in 10 x 75 mm glass pyrogen-free test tubes, covered with aluminum foil, and incubated in a 37°C waterbath for 60 min. Tubes were removed, inverted, and interpreted as positive if the test sample plus LAL clotted and did not run down the side of the tube when it was inverted.

I assisted with the Chick Embryo LD_{50} and pyrogen assays, as they were performed at the Rocky Mountain Laboratory in Hamilton, Montana by Dr. K. C. Milner and Robert Pfeifer.

Chick Embryo LD_{50} Assay for LPS (28): GC LPS and GC crude ribosomes diluted in sterile physiological saline were injected in 0.1 ml volumes intravenously (i.v.) into 11-day old chick embryos which had been incubated in a humidified incubator at 37°C. They were observed daily by candling for 3 days post-inoculation. Intravenous inoculation was performed into a prominent allantoic vein through a 2 x 5 mm window in the shell. The window was made using a hand drill fitted with two abrasive discs separated by 2 mm; the shell flap was then lifted off with an 18 gauge needle. Inoculations were performed while candling, using a tuberculin syringe and 27 gauge needle.

Following inoculation, the eggs were returned to the incubator and examined as mentioned above for dead embryos. Ten eggs were used for each dilution. LD_{50} values were calculated by the method of Irwin and Cheeseman (41).

Pyrogen Tests for LPS (46): GC LPS and GC crude ribosomes diluted in sterile saline were injected in 2 ml volumes into the marginal ear vein of rabbits weighing 2 to 3 kg. Temperatures were taken by rectally implanted copper-constantan thermocouples and recorded automatically every 12 min with a Honeywell Multipoint Recorder for 6 h. Six rabbits used for

each dilution were acclimatized to the test area and rectal thermometers for 1 h before injection.

Febrile responses were plotted on 1 x 1 inch graph paper with 1°C and one hour equaling 1 inch. Area under the curve was measured in square centimeters, and Fever Index 40 (the minimal dose necessary to produce an area under the curve of 40 cm^2) was calculated by the method of Keene et al. (46).

In Vitro Studies of CMI

Patients: Ten ml peripheral blood samples were drawn from persons diagnosed as positive by GC cultivation. The blood was transferred to sterile heparinized (30 units/ml blood) test tubes, and kept at 4°C until transported to the laboratory. All samples were collected from the University of Montana Student Health Service and the Missoula City-County Health Department by laboratory technicians employed there. A coded system was employed to assure anonymity of subjects beyond confines of the Health Service or Health Department. Medical histories, where relevant, were provided under the coding system.

Controls: Graduate students, professors, and employees of the Microbiology Department at the University of Montana were assumed gonococcal negative and with no history of infection based on their word (An assumption which many investigators may consider invalid). Ten ml peripheral blood samples were

drawn by Dr. W. L. Koostra or Bonna Graham, and treated the same as was positive blood.

BCG+: Persons vaccinated with BCG at any time in their life were bled and the blood was treated as above.

Media and Reagents: Physiological Saline - 0.85% NaCl was made in distilled water, sterilized by autoclaving and stored at 4°C.

Methylcellulose - Methylcellulose (Mallinckrodt, St. Louis; 1% = 15 centipoise) was added to distilled water and stored at 4°C.

ACEP (Ammonium Cloride, EDTA, Phosphate) -

NH_4Cl	0.8%
EDTA	0.1%
KH_2PO_4	0.01%

was mixed in distilled water and the mixture was stored at 4°C.

MEM (Minimal Essential Medium) -

MEM (Gibco)	0.96%
$NaHCO_3$	0.15%

was made in distilled water and filter sterilized. One ml of Penicillin (100 units/ml) plus Streptomycin (100 µg/ml) was added to each liter of MEM. The solution was stored at 4°C.

Antigen dilution medium - MEM + 10% FCS (Fetal Calf Serum - Difco) + 0.01 M $MgCl_2$.

Pd (Dulbecco Phosphate Buffer Solution) -

NaCl	0.8%
KCl	0.2%
$Na_2HPO_4 \cdot 7H_2O$	0.216%
KH_2PO_4	0.02%

was made in distilled water. The mixture was sterilized by autoclaving, and stored at 4°C.

Separation of Human White Blood Cells (WBC) (Adapted from Bøyum as cited in 19): Blood was diluted 3-fold with physiological saline in sterile test tubes which had been treated with Siliclade (Clay Adams) ("siliconized"). Two ml methylcellulose was added and mixed with each 10 ml of diluted blood. The tube was tilted to a 45 degree angle and the red blood cells were allowed to settle at room temperature for 30 min. The WBC-rich supernatant portion was pipetted into chilled siliconized centrifuge tubes and centrifuged at 230xg, 4°C for 10 min. The supernatant fluid was discarded and each cell pellet was resuspended in 2 ml ACEP. Five ml more ACEP was added to each tube, mixed, and incubated at 37°C for 5 min to lyse those red blood cells remaining.

Tubes were centrifuged again at 230xg, 4°C for 10 min to pellet the WBC's. The supernatant fluid was discarded and cells were resuspended in 1 ml MEM. Cell viability was ascertained on the basis of uptake of 0.4% Trypan Blue. Five drops of diluted cells were mixed with one drop of Trypan Blue and were enumerated in a hemocytometer. Cell numbers

were adjusted to 2 - 4 x 10^7 WBC/ml in 1 ml. The WBC's were kept in an ice bath until needed.

Collection of Guinea Pig Macrophages: Normal guinea pigs were stimulated by intraperitoneal injection of 20 ml sterile mineral oil to induce increased numbers of macrophages in the peritoneal cavity.

Whenever macrophages for MIF testing were needed, animals were sacrificed and exudate cells were washed from the peritoneum with 50 ml Pd into a chilled beaker. All operations from this point on were carried out on ice or at 4°C. The Pd was poured into a separatory funnel and the oil phase was discarded. The macrophage-rich Pd was centrifuged at 230xg for 10 min and the supernatant fluid was discarded. The cells were resuspended and "washed" twice in MEM. After the final washing the cells were resuspended in 0.4 ml MEM, and viability and cell counts were performed with Trypan Blue.

Antigen Dilutions: On the day of testing, a sample of ribosomes and a sample of LPS were allowed to thaw at room temperature. These were diluted to 10 µg and 100 µg per ml in antigen dilution medium. These were prepared just prior to assembling the Mackaness chambers.

Macrophage Migration Inhibition Factor (MIF) Tests: Guinea pig macrophages were added to the human leukocytes so that the ratio of guinea pig cells to human leukocytes was

approximately 3 to 2. Micro-hematocrit capillary tubes were filled two-thirds with the suspension of human and guinea pig cells (approximately 4 x 10^6 total cells per tube). The tubes were sealed at one end by melting over a micro-burner, and then centrifuged at 55xg, 4°C for 10 min to pack the cells. Tubes were cut at the interface between the packed cells and supernatant portion. The packed tubes were attached by the sealed end with grease to the bottom slide in five sterile mackaness chambers. The chambers were closed, each filled with one of the antigen dilutions and one with the dilution media without antigen. They were then incubated at 37°C, and read after 20 h. If possible, four hemotocrit tubes were used at each dilution.

Reading MIF Tests: After incubation for 20 h, the pattern of cell migration from the tubes was enlarged on a Simmon Omega Model D-2 enlarger with Wollensak 50 mm enlarging raptur, traced on Artesian Bond paper, and the pattern was cut out and weighed on a Mettler H20T balance. The area (weight) of cell migration, when incubated with no antigen, was designated as 1.00. Areas of migration, for cells incubated with antigens, were expressed as a decimal fraction of migration when incubated with no antigen. Such decimal fraction was thereafter designated as an index of migration. Therefore, if cells migrated 25% less when incubated with antigen, the index of migration would be expressed as 0.75.

Statistical Analysis

The Wilcoxon T-test (42) and 2-sample t-test (8) were used to determine whether the sample of patients and sample of negative controls had been drawn from different populations (with regards to cell migration indices, or with regards to ratios of indices). Both statistical tests were performed, as a double check. Results of the analysis indicated similar levels of significance, so only one value of significance will be reported.

The 2-sample t-test may be used to compute a value of t by the following equation:

$$t = \frac{P - C}{\sqrt{\frac{s_p^2}{n_p} + \frac{s_c^2}{n_c}}}$$

where $\bar{P}$ and $\bar{C}$ are the mean values of patients and controls respectively; s_p^2 and s_c^2 are variance values of patients and controls, and; n_p and n_c are the number of individuals in the sample of patients and controls. The computed t may be interpreted for levels of significance from table a of t values.

The Wilcoxon T-test was used in the case that there may not be a normal distribution of data values (an assumption made when using the above 2-sample t-test). Data from patients and controls were randomly paired, the differences between data values of paired subjects calculated, and the differences ranked according to be absolute value of the

difference. The ranks were assigned + or - values according to whether the difference between paired subjects was + or -. The value of T was the summation of the + or - ranks (whichever was least), and significance levels interpreted from an appropriate T table.

CHAPTER III

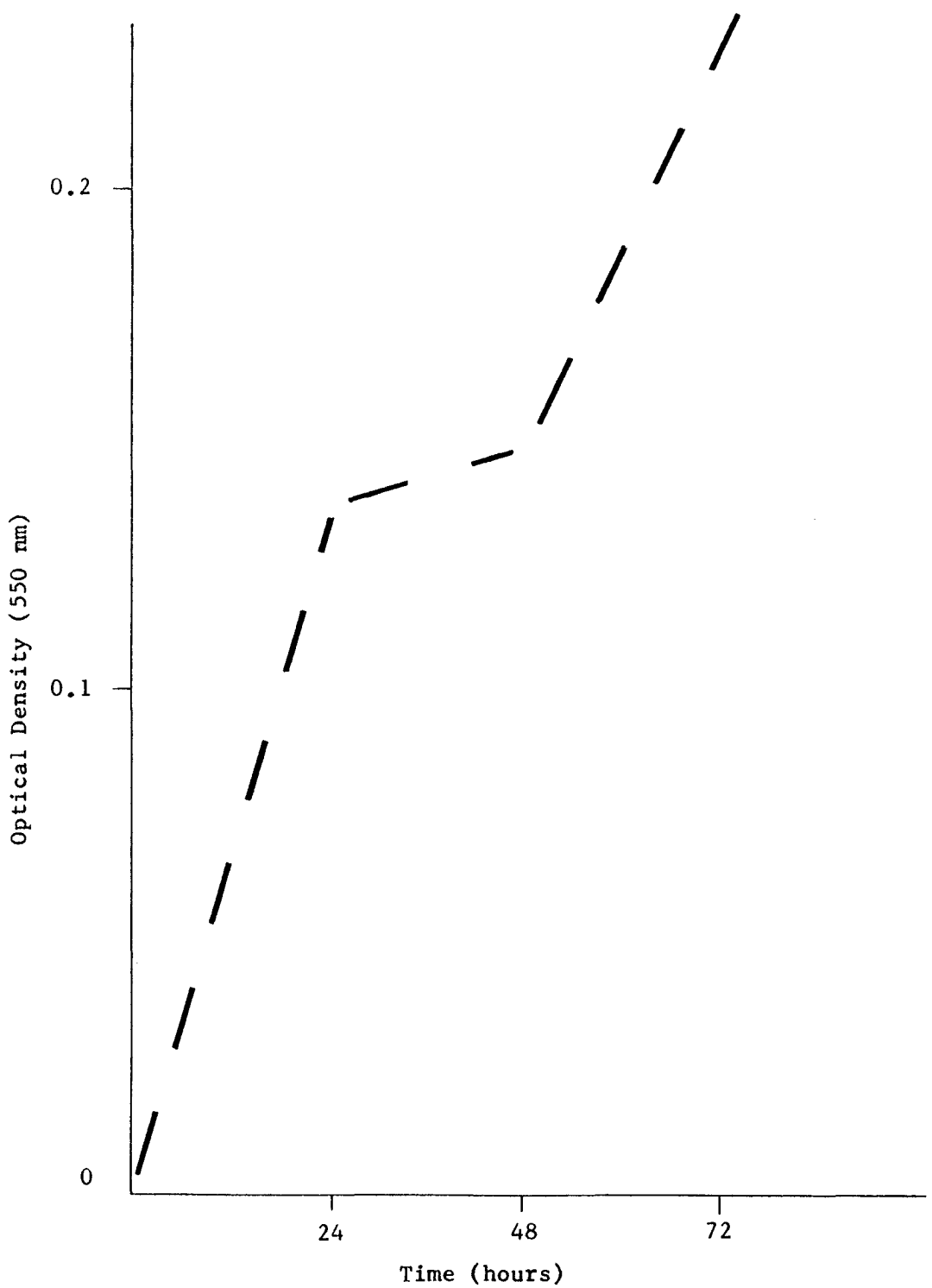
RESULTS

GC Growth Curve: In order to obtain the optimal ribosome yield from each culture of bacteria, it was necessary to determine when they would be in the middle or late exponential phase of growth. Cultures were therefore harvested at 24 h intervals, and an approximate growth curve was plotted (Fig. 1). Results from the growth curve, confirming tradition, indicated 48 h cultures to be at log phase of growth and the optimal time to harvest. During log phase, the bacteria are synthesizing protein rapidly and will have high yields of ribosomes.

It was observed that 72 h cultures were often difficult to subculture, indicating late log or death phase. During such phases, autolysis and ribonuclease destruction of the ribosomes would be maximal, and ribosomes isolated from those cultures would be damaged. Therefore, all cultures were harvested at 48 h.

Sedimentation Velocity Determinations: Subsequent to crude ribosome isolation it was necessary to examine the preparation so that I could discover the nature of its constituents. Without such examination it cannot be defined as a "ribosome"

Fig. 1. Growth curve of N. gonorrhoeae in modified T-M broth.



preparation. Therefore, analytical sedimentation velocity ultrocentrifugation was performed to determine the purity and sedimentation coefficient (5) of constituents present (Fig. 2).

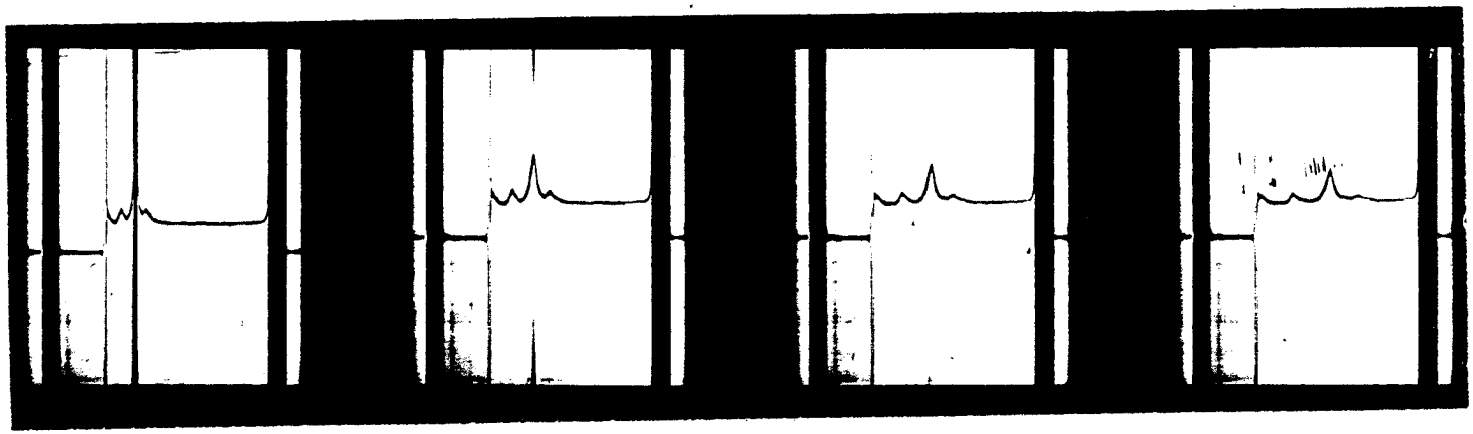
Schlieren optics provided an indication of the sample present within the centrifuge cell. Reading the photographs from left to right, one notices three distinct peaks emerging in the first frame. The sedimentation coefficient for each peak was determined by the rate that each peak moves down the cell. The first peak (left) had a sedimentation coefficient of 24.3, second 48.2, and third 66.3. These values can be extrapolated to 30S, 50S, and 70S at zero concentration, revealing that the preparation does contain ribosomes without any other cellular constituents at major concentration.

The amplitudes of the peaks are a reflection of the concentrations of each constituent. Apparently most of the 70S ribosomes were dissociated into constituent subunits within the buffer, because the 30S and 50S peaks represent the major portion of the ribosomal concentration. This sample was the only sample used for MIF tests performed in which crude ribosomes were tested with antigen.

Bioassays: LPS contamination of ribosome preparations is an inevitable result of ribosome isolation from gram-negative bacteria. It was therefore necessary to assay for the endotoxin level within the crude ribosome preparation. Such determination would aid in interpreting whether ribosomes or

Schlieren Optics of GC Crude Ribosomes
used in CMI studies
(4.8 mg/ml)

Bar angle 70°



0 min 2 min 4 min 6 min

Figure 2

LPS were the antigen within the crude ribosome preparation responsible for results obtained in the CMI studies.

Limulus amebocyte lysate (LAL) tests were performed with the crude ribosome and LPS preparations. The clotting endpoint for GC LPS and GC crude ribosomes was 10^3 ng/ml (Table 1). Although both LPS and crude ribosomes had equivalent endpoints in this test, it was not conclusive that they had equivalent levels of endotoxin. Since the decrement of each dilution tested was by 10-fold, the endpoint dilution of one preparation may contain as much as nine times more endotoxin than the other preparation.

Another possible limitation of the LAL test is its specificity. Researchers have demonstrated in studies with various blood plasma factors that the LAL test will react positively and clot with nucleoprotein (25). This suggested that the LAL may have been reacting with the ribonucleoprotein of the ribosomes. Because the endotoxin level in the crude ribosomes was to be so important in interpretation of the MIF results, we employed other tests of endotoxic activity to further quantify the endotoxin present.

Chick Embryo LD_{50} tests were performed as a measure of endotoxic activity for both the LPS and crude ribosome preparations (Table 2). $CELD_{50}$ doses calculated by the method of Irwin and Cheeseman (41) yielded $CELD_{50}$ of 5.3 µg for the GC LPS and a $CELD_{50}$ of 2.0 µg for the GC crude ribosomes. Five-fold dilutions were used in performing this test, so

Table 1. Clotting reactions to limulus amebocyte lysale (LAL) of bacterial preparations.

Concentration (ng/ml)	Bacterial Preparation		
	E. coli LPS	GC LPS	GC crude Ribosomes
10^5	ND	+	+
10^4	+	+	+
10^3	ND	+	+
10^2	+	-	-
10	ND	-	-
1	-	-	-
0.1	-	-	-

+ = clotting after incubation at 37°C

- = no clotting after incubation at 37°C

ND = not done

Table 2. Chick embryo responses to injection of GC LPS or GC crude ribosomes, and determination of Chick Embryo LD_{50} ($CELD_{50}$).

Dose (μg)	Number inoculated	GC LPS			GC Crude Ribosomes		
		Live	Hem	Dead	Live	Hem	Dead
0.0032	10	10		0	10		0
0.016	10	10		0	10		0
0.08	10	9	1		10		0
0.4	10	10		0	10		0
2.0	10	9		1	5		5
10.0	10	2		8	0		10
		$CELD_{50}$* 5.3 μg			$CELD_{50}$* 2.0 μg		

Hem = hemorrhage
* = as determined by the method of Irwin and Cheeseman (41).

that there is room for error. The test results indicate the crude ribosomes to contain more than twice the endotoxic activity of the LPS preparation.

Rabbit pyrogen tests were employed as the third test of the preparations for endotoxin. Milner and Finkelstein (57) have presented evidence that $CELD_{50}$ and pyrogen FI_{40} are measures of the same biological substance, therefore in interpretation of rabbit pytogen tests similar to $CELD_{50}$ is applicable. There is no significant difference between the FI_{40} of the LPS and crude ribosomes, (Table 3) that for LPS being 17 µg and that for crude ribosomes being 21 µg (Fig. 3). Again, 5-fold dilutions were utilized in execution of the test, so that either preparation may contain four times as much endotoxin as the other. As with the $CELD_{50}$ tests, endotoxin pyrogenicity of the crude ribosome preparation may have been amplified by ribosome presence in the mixture.

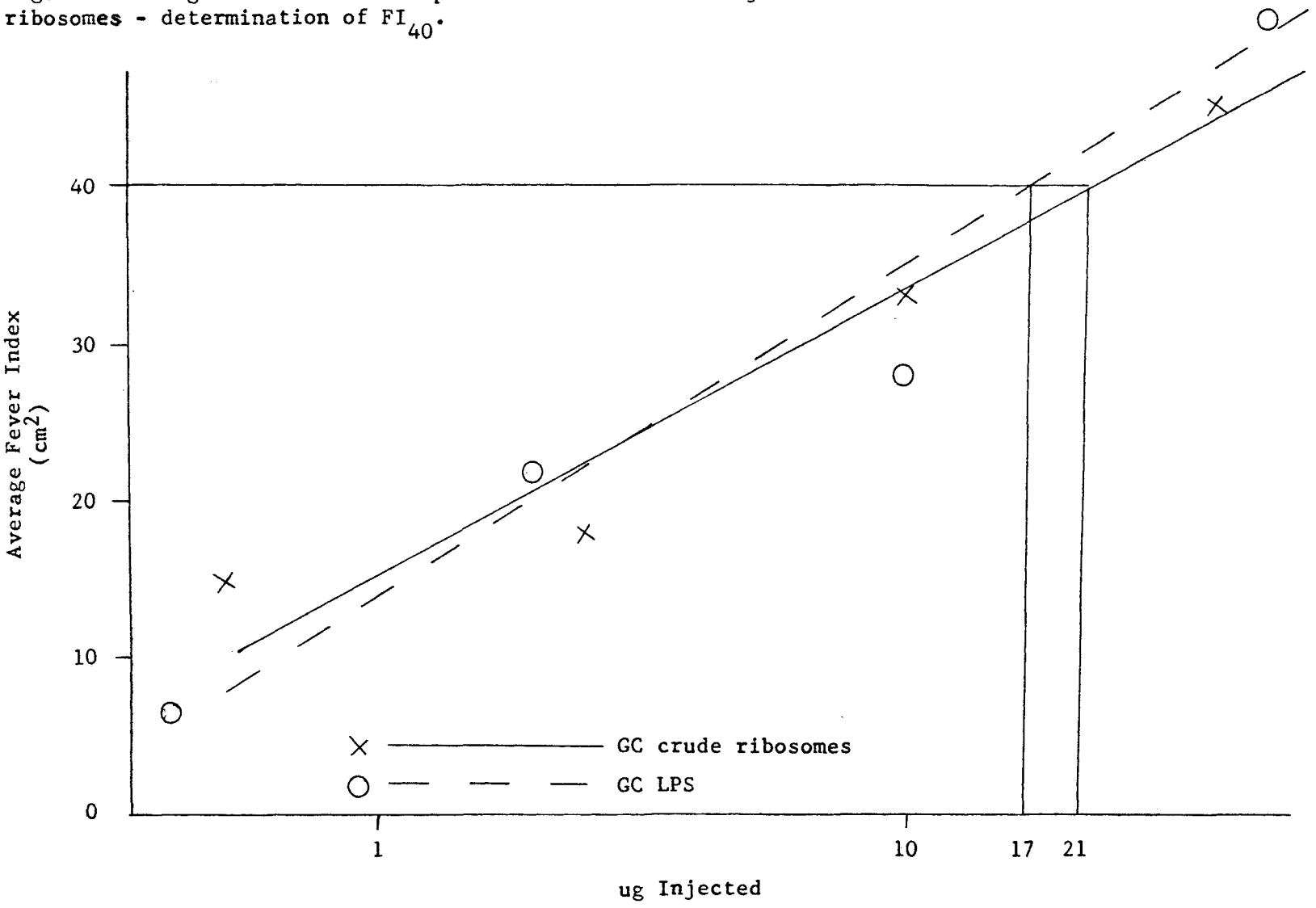
The quality or form of febrile response in the pyrogen tests contributes additional information about the preparations. Classically, endotoxin will induce a biphasic response in rabbits (46). That is, there will be an early febrile response followed by a drop, then another rise in temperature. This type of response occurs only with endotoxin injection, and can be used to determine whether the febrile response is induced by endotoxin or other substances. The febrile responses induced by our GC LPS and GC crude ribosomes were biphasic. Therefore, endotoxin in the

Table 3. Average rabbit fever response indices to i.v. injection of GC LSP or GC crude ribosomes.

GC LPS		GC Crude ribosomes	
Dose (μg)	Average Fever Index (cm^2)	Dose (μg)	Average Fever Index (cm^2)
0.4	6.4	0.625	15.2
2.0	21.8	2.5	18.0
10.0	28.1	10.0	33.2
50.0	50.8	40.0	45.6
	FI_{40}* 17 μg		FI_{40}* 21 μg

* = as determined by the method of Keene et al. (46).

Fig. 3. Average rabbit fever response indices to i.v. injection of GC LPS or GC crude ribosomes - determination of FI_{40}.

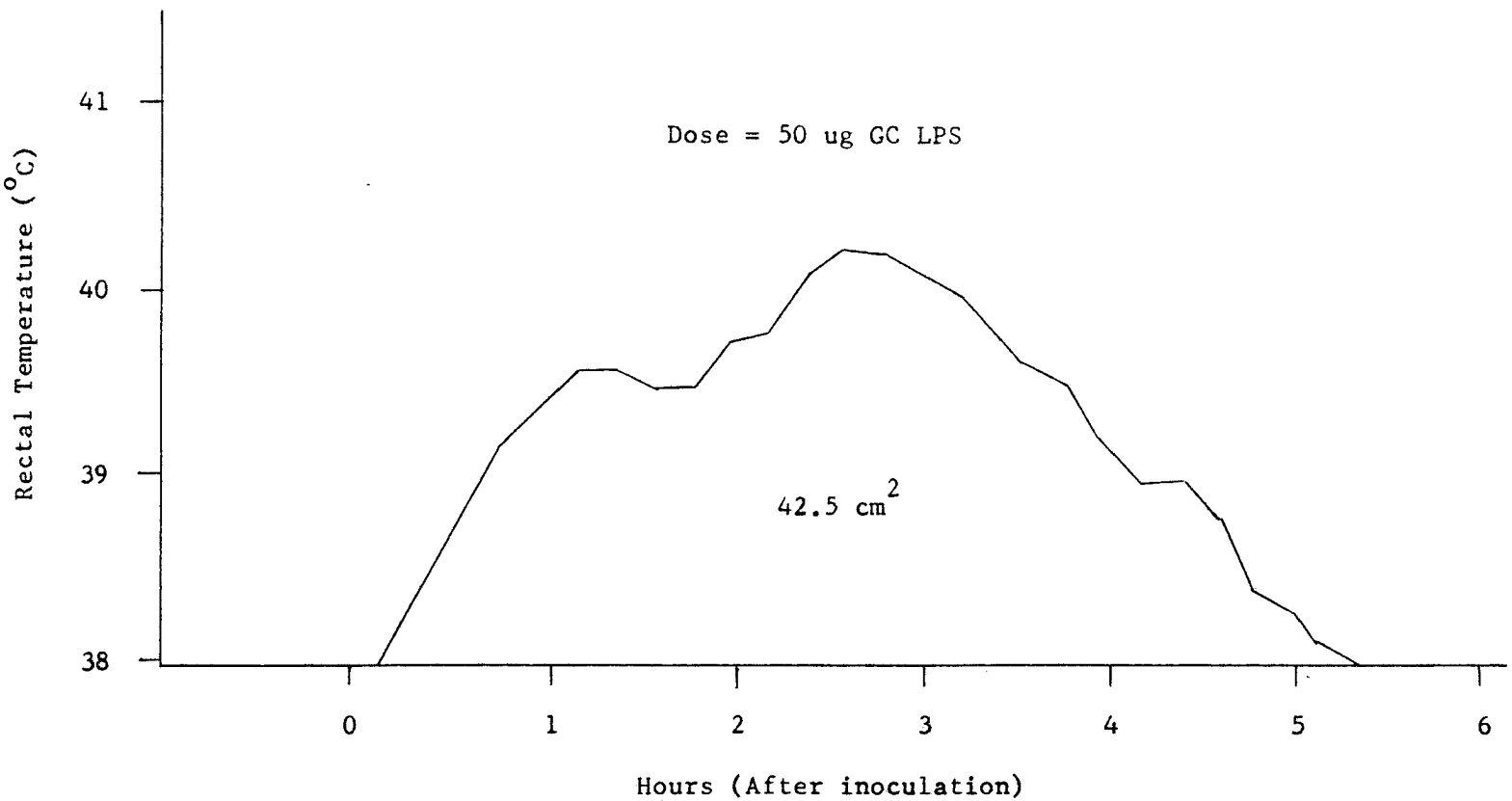


preparations was the moiety responsible for inducing the fever (Fig. 4).

All three endotoxin tests gave the same results. This demonstrates similar levels of endotoxic activity for both preparations. It was therefore necessary to compare MIF tests responses to equal concentrations of each preparation. The reason for a comparison of responses to both preparations is to determine whether it is endotoxin or ribosomes within the crude ribosomes that is the agent responsible for results obtained. If the crude ribosome preparation (in concentration equal to the LPS preparation) causes a greater reaction (more migration inhibition) in the MIF system than does the LPS preparation, then a moiety other than endotoxin in the crude ribosome preparation is the responsible antigen.

In Vitro CMI Studies: The area of migration of cells without antigen was designated as 1.00. Areas of migration with GC crude ribosomes or GC LPS as antigen were expressed as an index in relation to the area of migration without antigen. An index of migration of 0.60 indicates cell migration 60% of that without antigen; or, in other words, 40% inhibition of migration. The area of migration designated as 1.00 was determined for each subject due to difference in cell migration between tests by using macrophages from different individual guinea pigs. Such differences were usually less than 20% between tests.

Fig. 4. Representative biphasic rabbit febrile response to i.v. injection of endotoxin.



It was necessary to compare responses of lymphocytes from patients and control persons to GC LPS and GC crude ribosomes in establishing criteria by which samples would be diagnosed as positive or negative. That is, the index of migration using GC crude ribosomes as antigen was compared to the index using GC LPS as antigen.

Concentrations of antigen ranging from 1 to 200 μg were used at various times in search of a reliable concentration. Responses to 10 μg crude ribosomes were inconsistent (Table 4). Of nine patients sampled, four did not respond with inhibition of more than 20%. Therefore, 10 μg crude ribosomes was rejected as a reliable concentration for diagnosis. One hundred μg of antigen elicited consistent responses by cells from patients and from control subjects (Table 4).

Tradition dictates 20% inhibition, or 0.80 index of migration as the threshold response for a positive diagnosis (37). Samples with areas of migration less than 0.80 would therefore be diagnosed as positive. Such a straightforward diagnosis scheme would be applicable to this model if the inhibition of cell migration were entirely due to cell-mediated immunity. But, as mentioned earlier, the crude ribosome preparation may be contaminated with homologous LPS during isolation, and monocytes may respond in peculiar ways to the presence of endotoxin (62). Therefore the endotoxin contamination may mimic a CMI response in the MIF test. This can be witnessed by observing indices of migration for both

Table 4. MIF test responses of culture positive persons tested on the first day of drug therapy.

Patient	Migration Index **** GC Crude Ribosomes 10 µg	GC Crude Ribosomes 100 µg	GC LPS 10 µg	GC LPS 100 µg	** Index Ratio	*** Interpretation
6	0.40*	0.34	ND	ND	-	Pos
7	0.52*	0.59	ND	ND	-	Pos
9	0.87*	0.79	ND	ND	-	Pos
10	0.78	0.75	1.20	0.94	0.80	Pos
12	1.31	0.63	0.75	0.64	0.99	?
13	0.90	0.60	0.82	1.00	0.60	Pos
14	0.53	0.57	0.95	0.84	0.68	Pos
15	0.71	0.86	0.76	0.69	1.24	Neg
16	0.80	0.65	0.77	0.88	0.76	Pos

ND = not done.

* = 20 g instead of 10 g were used.

** = ratio of indices with 100 µg crude ribosomes to 100 µg LPS.

*** = migration <0.80 with 100 µg crude ribosomes and ratio of indices <1.00 as criteria for positive.

**** = migration index defined as the area of migration with antigen expressed as a decimal fraction of the area of migration without antigen.

? - no interpretation made, because values were not definitely positive or negative.

patients and controls when their cells were incubated with 100 µg GC crude ribosomes. If we use only the 0.80 criteria, 8 of 9 patients responded with positive diagnosis, but 4 out of 9 controls also responded with migration inhibited by more than 20% (Table 5) -- i.e., there were 44% false positive diagnosis.

It was therefore necessary to establish an extra criterion which would take into account the presence of LPS contamination. As demonstrated in the above bioassays, 100 µg crude ribosomes and 100 µg LPS exhibited approximately equal endotoxicity. Cells which are inhibited from migrating more by the crude ribosomes than the LPS can be said to be sensitive to a factor other than, or in addition to, LPS in the crude ribosomes preparation -- i.e., ribosomes. We therefore established the second criterion, that the migration index of cells incubated with crude ribosomes be less than the index of the cells with LPS.

Five of the 6 GC patient cells tested with both 100 µg LPS and 100 µg crude ribosomes responded more to the crude ribosome preparation than to the LPS. Even more significant was the demonstration that, of the four false positive by the 0.80 criterion alone, three were interpreted as negative by this additional criterion. In other words, only 1 of the 9 controls (11%) remained a false positive when both criteria were employed -- the only false positive being patient 7.

Table 5. MIF test responses of gonococcal negative control subjects.

Control	Migration Index *** GC Crude Ribosomes 10 µg	100 µg	GC LPS 10 µg	100 µg	** Index Ratio	*** Interpretation
1	0.71*	1.40	ND	ND	-	Neg
2	ND	1.00	ND	ND	-	Neg
3	0.79	1.23	0.60	0.61	2.00	Neg
4	0.80	0.69	0.62	0.41	1.68	Neg
5	1.05	0.91	1.08	0.79	1.15	Neg
6	0.74	0.65	0.82	0.58	1.12	Neg
7	1.50	0.79	0.93	1.16	0.68	Pos
8	1.91	1.51	1.93	1.71	0.88	Neg
9	0.90	0.74	0.74	0.56	1.20	Neg

ND = not done.

* - 20 µg instead of 10 µg were used.

** = ratio of indices with 100 µg crude ribosomes to 100 µg LPS.

*** = migration <0.80 with 100 µg crude ribosomes and ratio of indices <1.00 as criteria for positive.

**** = migration index defined as the area of migration with antigen expressed as a decimal fraction of the area of migration without antigen.

Diagnosis of all subjects was therefore made on the basis of two criteria for positive interpretation: 1) migration with 100 µg ribosomes must be less than 0.80, and 2) the ratio of the indices for 100 µg crude ribosomes to 100 µg LPS be less than 1.00. When these two criteria were used, there was 78% correct diagnosis of GC culture positive individuals, and an 11% rate of false positivity. Diagnosis of GC culture positive persons was actually 89% correct, when we adhere strictly to the criteria. But, because data from patient 12 was on the threshold of positivity, we interpreted him as neither positive nor negative.

Comparison and statistical analysis of differences between patient and control indices of migration to 100 µg GC crude ribosomes (Tables 4 and 5) indicates a level of significance of $p < 0.03$. A similar analysis of the differences between the index ratios of patients and controls (Tables 4 and 5) indicates a level of significance of $p < 0.13$.

Persons with stimulated cell-mediated immune systems were examined to determine what cross reactivity may occur (Table 6). Because mycobacterial infection and vaccination stimulates CMI, persons vaccinated with M. bovis strain BCG were examined. Despite a small sampling, various observations can be made First of all, lymphocytes from these persons responded vigorously to both the LPS and crude ribosomal preparations. This is evidenced by migration indices well below 0.80. Secondly, because the ratio of the indices

Table 6. MIF test responses of subjects immunized with BCG.

Person	Migration Index **** GC Crude Ribosomes 10 µg	100 µg	GC LPS 10 µg	100 µg	** Index Ratio	*** Interpretation
1	0.65	0.29	ND	ND	--	Pos
2	0.54	0.62	0.59	0.30	2.05	Neg
3	0.56	0.28	0.41	0.26	1.08	Neg

ND = not done.

* = 20 µg instead of 10 µg were used.

** = ratio of indices with 100 µg crude ribosomes to 100 µg LPS.

*** = migration <0.80 with 100 µg crude ribosomes and ratio of indices <1.00 as criteria for positive.

**** = migration index defined as the area of migration with antigan expressed as a decimal fraction of the area of migration without antigen.

was above 1.00, 2 of the 3 persons samples were diagnosed as negative. The other was not tested against LPS, so that a ratio of indices was not established.

Characterization of CMI to GC infection entails examination of both inception and termination of the response. Therefore, this investigation monitored patient responses to the MIF test at various intervals post-therapy (Table 7). Again, because of differences in degree of sensitization, we expected and found varied lengths of sensitization. The only patients tested at one week post-therapy, patients 2 and 3, were still sensitive by our established criteria, and similarly, at six weeks, patient 5 had retained sensitivity to the antigen.

Eight weeks appears to be somewhat of a threshold for retention of sensitivity; patient 1 had converted to negative. Other negative diagnoses were found for patient 1 tested 10 weeks post-therapy and patients 4, 8, and 10 tested at 16 weeks post-therapy. Patient 5 remained strongly positive even 20 weeks post-therapy. It appears that 8 to 16 weeks post-therapy may be the threshold for reversion to negative.

Based upon statements concerning duration of infection prior to treatment and MIF testing (Table 8), it was observed that even patients who contacted the disease 7-10 days before testing, responded positively (patients 2, 3, and 6). It may therefore be inferred that within 10 days after

Table 7. MIF test responses of culture positive persons tested on the first day of drug therapy, and at various intervals post-therapy.

Patient	Time after therapy when tested	Migration Index **** GC Crude Ribosomes 10 µg	GC Crude Ribosomes 100 µg	GC LPS 10 µg	GC LPS 100 µg	** Index Ratio	*** Interpretation
1	0	0.53*	0.20	ND	ND	-	Pos
	8 wk	0.69	0.65	0.75	0.52	1.25	Neg
	20 wk	0.78	0.82	0.59	0.89	0.92	Neg
2	0	0.96*	0.41	ND	ND	-	Pos
	1 wk	0.58*	0.34	ND	ND	-	Pos
3	0	0.13*	0.08	ND	ND	-	Pos
	1 wk	0.75*	0.33	ND	ND	-	Pos
4	0	0.93*	0.60	ND	ND	-	Pos
	16 wk	1.12	1.30	1.37	1.18	1.10	Neg
5	0	0.71*	0.50	ND	ND	-	Pos
	6 wk	1.05	0.56	0.80	0.96	0.58	Pos
	20 wk	0.72	0.58	0.50	0.61	0.95	Pos
8	0	0.58*	0.72	ND	ND	-	Pos
	16 wk	0.57	1.29	0.94	1.20	1.07	Neg
10	0	0.78	0.75	1.20	0.94	0.80	Pos
	16 wk	0.92	0.81	0.78	0.76	1.06	Neg

wk = weeks.
ND = not done.
* = 20 µg instead of 10 µg were used.
** = ratio of indices with 100 µg crude ribosomes to 100 µg LPS.
*** = migration <0.80 with 100 µg crude ribosomes and ratio of indices <1.00 as criteria for positive.
**** = Migration index defined as the area of migration with antigen expressed as a decimal fraction of the area of migration without antigen.

Table 8. Medical histories of all patients tested.

Patient	Sex	Age	Estimated Duration Prior to Testing	Clinical	Venereal Disease History
1	F	19	2 months	Symp	None
2	F	19	10 days	Asymp	None
3	M	20	10 days	Symp	June 75(Trichuria)
4	F	21	6 weeks	Asymp	None
5	F	24	NA	Symp	NA
6	M	23	1 week	NA	NA
7	M	20	NA	Symp	July 75(GC)
8	F	19	5 months	Asymp	None
9	F	20	1 month	NA	None
10	F	20	NA	Symp	None
12	F	19	12 months	Asymp	None
13	M	27	NA	Symp	NA
14	M	17	NA	Symp	None
15	M	20	2 weeks	Asymp	None
16	M	24	NA	Symp	NA

Symp = symptomatic

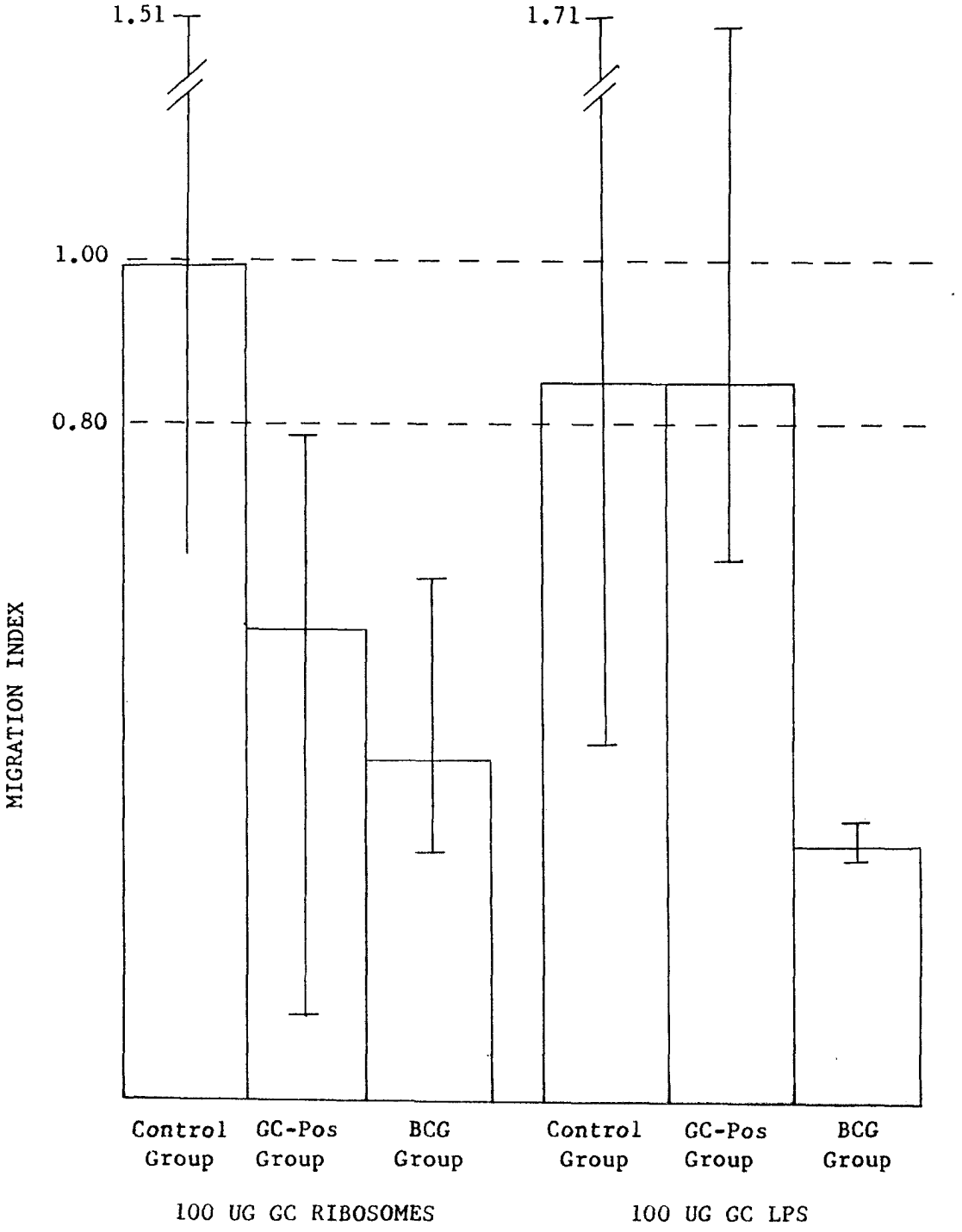
Asymp = asymptomatic

NA = not available

gonococcal contact, sensitization takes place. Patient 15 failed to respond with positive diagnosis with MIF testing two weeks post-contact. Therefore, the period of time before sensitization takes place, as well as the period of time before sensitization ceases, is a variable of individual differences of infection.

Cumulative MIF Test Response Results: The control group had an average migration index of 0.99 when incubated with 100 µg crude ribosomes (Fig. 5). The GC culture-positive group, on the other hand, responded to 100 µg crude ribosomes with an average migration index of 0.55 -- i.e., 45% inhibition of migration. Although there was a distinct difference between the two groups of subjects, when cells were incubated with 100 µg crude ribosomes, they responded equally to 100 µg LPS -- i.e., an average index of 0.83 for each group. The BCG immunized group responded significantly to both crude ribosomes and LPS. However, unlike the GC culture-positives, the BCG group responded more to 100 µg LPS than to 100 µg crude ribosomes.

Fig. 5. Cumulative averages of MIF test responses to 100 ug GC crude ribosomes or 100 ug GC LPS. Migration index is the area of migration with antigen expressed as a decimal fraction of migration without antigen. Bars represent the ranges of responses.



CHAPTER IV

DISCUSSION

In the present study, CMI of gonorrhea patients to a gonococcal crude ribosome preparation was demonstrated *in vitro*. Statistical analysis of the data indicated a significant ($p < 0.02$) difference in the level of responses between lymphocytes from patients and control subjects. Previous to the present investigation, studies of lymphocyte transformation *in vitro* had been used to demonstrate CMI against *N. gonorrhoeae* protoplasm preparations (27, 31, 49). Grimble and McIllmurray (31) observed positive responses with lymphocytes from 10 of 12 patients studied, but they also noted that lymphocytes from 4 of 10 control subjects gave false positive responses.

In the present study, 20% inhibition of migration was used as the threshold for a positive response, and 8 of 9 patients gave positive responses. However, 4 of 9 uninfected controls also had such responses. It was considered that the positive responders were sensitized to some moiety within the crude ribosome preparation. Alternatively, something within the preparation may have caused lymphocytes to mimic CMI when incubated with it. Because crude ribosomes

might have been contaminated with LPS during their isolation, it was decided to test an LPS preparation (with endotoxicity equal to that of the crude ribosomes) in parallel with the crude ribosome preparation. When incubated with GC LPS, lymphocytes from infected patients and from uninfected control subjects nonspecifically inhibited migration of guinea pig macrophages. Others have reported that LPS might inhibit migration in MIF tests (34). This raised the question of whether or not LPS within the crude ribosome preparation was responsible for the migration inhibition observed. It was noted that lymphocytes from infected patients responded with more migration inhibition to the crude ribosome preparation than they did to the LPS preparation, whereas lymphocytes from uninfected controls did not respond more to the crude ribosome preparation than to the LPS preparation ($p < 0.13$). Because the antigenic preparations of Grimble and McIllmurray (31), and of others (27, 49) were ill-defined, moieties acting as nonspecific mitogens may have been present within their preparations, and were responsible for eliciting the high rate of false positives.

There appears to be some question as to the nature of the antigen, within the crude ribosome preparation, which was indicating the patient's sensitivity; i.e., what moiety was the agent to which lymphocytes from GC patients had become sensitized? Several possibilities were considered:

1) In the present study, incubation of lymphocytes with the LPS preparation and with the crude ribosomes preparation did not give similar responses, therefore LPS could not have been the antigen. Although LPS was not the specific antigen, it might have played a role as a molecular carrier of the antigenic determinant.

2) Was nascent protein, which was being synthesized on the ribosomes, the specific antigen within the crude ribosome preparation? Most of the nascent protein was probably removed during ribosome isolation. Photographs of Schlieren patterns of the crude ribosome preparation illustrated that the ribosomes had dissociated into 30S and 50S subunits, and therefore would have released any nascent protein (36). When the ribosomes were centrifuged, the nascent protein would have remained in the supernatant fluid and would have been discarded. Any homogeneous nascent protein which did remain would have been in such low concentration that it is very unlikely to have been the specific antigen in the crude ribosome preparation.

3) Buchanan et al. (13) have demonstrated that pilin or pili could be used as a specific antigan for seo-testing. Is it possible that this gonococcal protein was contained within the crude ribosome preparation and was responsible for CMI results obtained? Individual pilin molecules, as well as pili, were probably eliminated in the same manner as was nascent protein, since their sedimentation coefficient would

be very low. This is verified by lack of perturbation in the baseline of the Schlieren photographs of the GC crude ribosomes. Therefore, pilin and pili may be eliminated from consideration as the antigen responsible for specific migration inhibition observed.

4) Esquenazi and Streitfeld (27), Grimle and McIllmurray (31), and Kraus et al. (49) prepared an antigen of sedimented protoplasm extracts of the gonococcus. Entities with sedimentation properties similar to those of the crude ribosomes must have been contained in their antigenic preparations. The ribosomal material which I employed was a better prepared and defined preparation than a simple protoplasmic extract. Methods used for preparation of the ribosomes eliminated many soluble cytoplasmic factors contained in the other preparations.

5) Since patients with gonorrhea do mount an immunological response to several GC antigens (3, 11, 38, 500, 51, 71, 83), it was necessary to utilize an antigen in CMI or sero-testing which will best demonstrate specific sensitivity. Ribosomes make up 25% of the total cellular mass of some kinds of bacterial cells (85), and therefore ribosome structural proteins are immunogens which would be generally plentiful, and which might elicit sensitization during infection. Therefore, ribosome structural proteins provide a high level of homogeneous protein to which immunocompetent cells may become sensitized. Although ribosome structural

proteins have not been proven to contain the antigenic determinant responsible for the MIF test results, they are implicated as the antigen.

In this investigation, a preparation which contained GC ribosomes was used as an antigen. Although only a pilot study, the study did demonstrate that gonorrhea patients can become specifically sensitized to a moiety within a gonococcal crude ribosome preparation.

Attempts to develop an immuno-diagnostic test for detection of gonorrhea have been confronted with difficulties in detecting sensitization, either humoral or cell-mediated. This project encountered similar problems. In order to detect consistently CMI against the crude ribosome preparation, it was necessary to use ribosomal concentrations of 100 µg/ml. There are various possible explanations for the need of a high antigen concentration in detecting sensitization. One problem may have been the possible degradation of the antigen used in the test. The GC were killed by pouring fresh cells over ice and holding them at 4°C overnight. Although the cold would retard enzymatic autolysis, it would not stop it completely. Many of the antigens might have been degraded, therefore, although 100 µg/ml of the final preparation was used, only a fraction of it could have been of specific, intact antigen.

Another possible explanation for difficulty in detecting sensitization might have been that there was a very low level

of sensitization in the infected patients. There are several possible mechanisms for the failure of the patients to develop sensitization. First, the immunogens could have been autolyzed during infection before immunocompetent cells were able to process them. Second, since gonorrhea is a superficial infection, relatively isolated from the immune system, immunocompetent cells might have only infrequently reached the immunogens for sensitization.

The time factor involved in sensitization is worthy of note. Esquenazi and Streitfeld (27) have reported that lymphocytes from gonococcal patients responded to various gonococcal sonicates with blastogenesis in vitro. They demonstrated sensitization 5 days after gonococcal contact and cessation of sensitivity 5 weeks after therapy was begun. Within the limitations set by sampling, results herein paralleled theirs; i.e., sensitization to the gonococcal crude ribosome preparation was demonstrated to begin approximately 7 days after contact, and sensitivity was observed to cease 8 to 16 weeks post-therapy. Data obtained from a single patient (#5) indicated that delayed hypersensitivity was present as long as 20 weeks post-therapy. Since GC cannot be cultured from urogenital areas of patients 2 days after successful antibiotic treatment (54), viable GC were probably cleared from the system. If patient 5 had been cured, the immunogens should have been cleared and the patient would have returned to negative diagnosis. Because of the high

level of sensitivity remaining at 20 weeks in patient 5, this suggests active infection. Though generally effective, treatment does not result in cures in all cases. Most researchers define as successful a 95% cure rate (depending on the program and type of drug therapy used) (55, 77). Since subjects were tested on a voluntary basis, the laboratory was unable to examine patient 5 to see whether she had been cured of the infection.

The amount of stimulus necessary to elicit demonstrable sensitization requires mention. Kraus et al. (50) have reported that a sequence of two infections was required before CMI could be detected. The present studies were able to detect a CMI response in most of the patients after a single infection. Two possible reasons are evident for this difference. Either, 1) MIF testing is a more sensitive test than is blastogenesis for detecting CMI to N. gonorrhoeae infection, or 2) The antigenic preparation contained an antigenic determinant which detects sensitization better than did the preparation used by Kraus.

The MIF test employed to demonstrate CMI is a very tedious procedure. MIF testing, therefore, is not suggested as a diagnostic methodology to screen populations for GC infection. However, results of this in vitro study demonstrate the potential fruitfulness of studying various moieties from the GC crude ribosome preparation as in vivo skin test antigens. Such a test, if developed, would provide an ideal

diagnostic aid in detecting and treating the reservoir of infections, thereby reducing and maintianing gonococcal infection at reasonable levels.

CHAPTER V

SUMMARY

The purpose of this investigation was to determine whether cell-mediated immunity (CMI) could be demonstrated to gonococcal crude ribosomes. As the investigation evolved, it was necessary to consider factors within the crude ribosomes, which had been isolated by differential ultracentrifugation, that may lead to nonspecific responses to endotoxin present.

In MIF tests lymphocytes from patients with gonorrhea were found to respond significantly more than did lymphocytes from negative control subjects when incubated with GC crude ribosomes. This indicated that persons infected with GC were sensitized to a moiety within the crude ribosome preparation. Bioassays failed to distinguish crude ribosomes from LPS in terms of endotoxicity. To correct the results for effects of LPS within the crude ribosomes, lymphocytes were also incubated in MIF tests with concentrations of LPS equal to that present in the crude ribosomes. It was observed that GC LPS nonspecifically induces lymphocytes from patients, controls, and BCG vaccinated persons to inhibit guinea pig macrophage migration. Crude ribosomes, on the other hand, specifically inhibit guinea pig macrophages

incubated with lymphocytes of patients with gonorrhea. Based upon lymphocyte responses to the two gonococcal preparations, correct diagnosis of 7 of 9 infected patients and 8 of 9 negative controls was made.

Using criteria established from lymphocyte responses to the two preparations, several patients were tested at various intervals post-therapy. Based upon these follow-up studies and medical histories it was determined that sensitization was established within 2 weeks of the contraction of infection, and it subsided 8 to 16 weeks post-therapy.

REFERENCES CITED

1. Alberghina, F.A.M., and S.R. Suskind. 1967. Ribosomes and ribosomal protein from Neurospora crassa I. Physical, chemical, and immunochemical properties. J. Bacteriol. 94:630-649.

2. American Social Health Association. 1974. Today's V.D. Problem 1974. N. Y.

3. Apiccella, M.A., and J.C Allen. 1973. Isolation and characterization of the B antigen of Neisseria gonorrhoeae. Infect. Immun. 7:315-321.

4. Arko, R.J., W.P. Duncan, W.J. Brown, W.L. Peacock, and T. Tomizawain. 1976. Immunity in infection with Neisseria gonorrhoeae: Duration and serological response in the chimpanzee. J. Infect. Dis. 133:441-447.

5. Arko, R.J., S. J. Kraus, W. J. Brown, T.M. Buchanan, and U.S.G. Kuhn. 1974. Neisseria gonorrhoeae: Effects of systemic immunization on resistance of chimpanzees to urethral infection. J. Infect. Dis. 130:160-164.

6. Baker, R.E., W.E. Hill, C.L. Larson. 1972. Delayed hypersensitivity reactions provoked by ribosomes from acid-fast bacilli. Infect. Immun. 6:258-265.

7. Baker, R.E., W.E. Hill, and C.L. Larson. 1973. Ribosomes of acid-fast bacilli: Immunogenicity, serology, and in vitro correlates of delayed hypersensitivity. Infect. Immun. 8:236-244.

8. Batson, H.C. 1960. An introduction to statistics in the medical sciences. p. 19. Burgess Publishing Co. Minneapolis.

9. Bloom, B.R., and P.R. Glade (Editors). 1971. In vitro methods in cell mediated immunity. Academic Press Inc. New York and London.

10. Blountin, J.H. 1972. A new approach for gonorrhea epidemiology. Am. J. Pub Health. 62:710-712.

11. Boor, A.K., and C.P. Miller. 1944. A carbohydrate-lipid fraction of gonococcus and meningococcus. J. Infect. Dis. 75:47-57.

12. Brown. L., B.D. Brown, M.J. Walsh, C.I. Pirkle. 1963. Urethritis in males produced by Neisseria gonorrhoeae from asymptomatic females. J. Am. Med. Assoc. 86:153.

13. Buchanan, T.M., J. Swanson, K.K. Holmes, S.J. Kraus, E.C. Gotschlich. 1973. Quantitative determination of antibody to gonococcal pili. J. Clin. Invest. 52: 2896-2909.

14. Caldwell, J.G., E.V. Prince, G.J. Pazin, and C.E. Cornelius. 1971. Sensitivity and reproductibility of the Thayer-Martin culture medium in diagnosing gonorrhea in women. Am. J. Obstet. Gyn. 109:463-468.

15. Chacko, W.W., and G.M. Nain. 1969. Sero-diagnosis of gonorrhea with a micro precipitin test using a lipo-polysaccharide antigen from N. gonorrhoeae. Brit. J. Ven. Dis. 45:33-39.

16. Cooper, M.D., R.P. Tewari, and D.V. Bowser. 1976. Immunogenicity of ribosomes from Neisseria gonorrhoeae. (Abstr.) Annu. Mtg. ASM 1976:69.

17. Corbus, B.C., and B.C. Corbus, Jr. 1941. The cutaneous diagnosis of gonococcic infections. J. Am. Med. Assoc. 116:113-115.

18. Craven, G.R., P. Voynow, S.J.S. Hardy, and C.G. Kurland. 1969. The ribosomal proteins of E. coli II. Chemical and physical characterization of the 30S ribosomal protein. Biochem. 8:2906.

19. Cutts, J.H. (Editor). 1970. Cell separation - Methods in hematology. p. 66. Academic Press Inc., New York and London.

20. Danielsson, D.G., J.D. Schmale, W.L. Peacock, Jr., and J.D. Thayer. 1969. Antigens of Neisseria gonorrhoeae: Characterization by gel filtration, complement fixation, and agar-gel diffusion of antigens of gonococcal protoplasm. J. Bacteriol. 97:1012-1017.

21. David, J.R., S. Al-Askari, H.S. Lawrence, and L. Thomas. 1964. Delayed hypersensitivity in vitro; I. The specificity of cell migration by antigens. J. Immunol. 93:264-273.

22. David, J.R., H.S. Lawrence, and L. Thomas. 1964. Delayed hypersensitivity in vitro: II. Effect of sensitive cells on normal cells in the presence of antigen. J. Immunol. 93:274-278.

23. David, J.R., H.S. Lawrence, and L. Thomas. 1964. Delayed hypersensitivity in vitro: III. Specificity of hapten-protein conjugates in the inhibition of cell migration. J. Immunol. 93:279-282.

24. Drutz, D.J. 1974. immunity to reinfection in patients with gonorrhea. Western J. Med. 120:485-587.

25. Elin, R.J., and S.M. Wolff, 1973. Nonspecificity of the limulus amebocyte lysate test. J. Infec. Dis. 128: 349-352.

26. Esquenazi, V., and M.M. Streitfeld. 1973. Lymphocyte transformation and serum agar gel diffusion of rabbits immunized against Neisseria gonorrhoeae and Neisseria catarrhalis. Can. J. Microbiol. 19:1099-1102.

27. Esquenazi, V., and M.M. Streitfeld. 1973. Transformation of lymphocytes in gonorrhea before and after therapy. Infect. Immunity. 8:503-509.

28. Finkelstein, R.A. 1964. Observations on mode of action of endotoxin in chick embryos. Proc. Soc. Exp. Biol. Med. 115:702-707.

29. George, M., and J.H. Vaugn. 1962. In vitro cell migration as a model for delayed hypersensitivity. Proc. Soc. Exp. Biol. Med. 111:514-521.

30. Greenberg. L., B.B. Diena, C.P. Kenny, and R. Znamirowski. 1971. Preliminary studies on the development of a gonococcal vaccine. Bull., World Health Org. 45:531-535.

31. Grimble, A.S., and M.B. McIllmurray. 1973. Cell-mediated immune response in gonorrhea. Brit. J. Ven. Dis. 49:446-449.

32. Handsfield, H.H., T.O. Lipman, J.P. Harnisch. E. Tronca, K.K. Holmes. 1974. Asymptomatic honorrhea in men. New Engl. J. Med. 290:117-123.

33. Heilman, D.H., and H.W. Bernton. 1961. The effects of endotoxin on tissue cultures on spleen of normal and tuberculin-sensitive animals. Am. Rev. Resp. Dis. 84: 862-871.

34. Heilman, D.H., and R.C. Blast, Jr., 1967. In vitro assay of endotoxin by the inhibition of macrophage migration. J. Bacteriol. 93:15-20.

35. Herrold, R.D. 1925. Skin reactions with gonococcus filtrates (Toxin?). J. Am. Med. Assoc. 84:361.

36. Hill, W.E., G.P. Rossetti, and K.E. VanHolde. 1969. Physocal studies of ribosomes from E. coli. J. Mol. Biol. 44:263-277.

37. Hoffman, P.M., L.E. Spitler, and M. Hsu. 1976. Leukocyte-migration inhibition in guinea pigs: I. Correlation with skin test reactivity and macrophage migration inhibition. Cell. Immunol. 21:358-363.

38. Holmes, K.K. 1973. Gonococcal infection. Adv. Internal Med. 19:259-285.

39. Houchens, D.P., and G.L. Wright. 1973. Immunity to Salmonella typhimurium infection: Characterization of antigens in active protection by polyacrylamide gel electrophoresis. Infect. Immun. 7:507-511.

40. Irons, E.E. 1912. Cutaneous allergy in gonococcal infections. J. Infect Dis. 11:77-93.

41. Irwin, J.O., and E.A. Cheeseman. 1939. On an approximate method of determining the median effective dose and its error, in the case of a quantal response. J. Hyg. 39:574-580.

42. Johnson, P.O., and R.W.B. Jackson. 1959. Modern statistical methods: Descriptive and inductive. p. 264. Rand McNally and Co., Chicago.

43. Johnson, W. 1973. Ribosomal vaccines: II. Specificity of the immune response to ribosomal ribonucleic acid and protein isolated from Salmonella typhimurium. Infect. Immun. 8:395-400.

44. Judd, R.C., and W.L. Koostra. 1976. Gonococcal ribosomes as skin test antigens: II. Precision of the method, attempts to identify the ribosomal antigen, and correlation with the macrophage migration inhibition test. Brit. J. Ven. Dis. 52:28-32.

45. Kanai, K., G.P. Youmans, and S.S. Youmans. 1960. Allergenicity of intracellular particles, cell walls, and cytoplasmic fluid from Mycobacterium tuberculosis. J. Bacteriol. 80:615-621.

46. Keene, W.R., H.R. Silberman, and M. Landy. 1961. Observations on the pyrogenic response and its application to the bioassay of endotoxin. J. Clin. Invest. 40:295-301.

47. Kohler, H. 1931. The diagnosis of honorrhea through intracutaneous infection. J. Lab. Clin. Med. 17:497. (Abstract in English from Ztschr. f. urol. chir. 19:54).

48. Koostra, W.L., R.C. Judd, and R.E. Baker. 1976. Gonococcal ribosomes as skin test antigens: I. Preliminary tests of sensitivity and specificity. Brit. J. Ven. Dis. 52:24-27.

49. Kraus, S.J., G.H. Perkins, and R.C. Gellen. 1970. Lymphocyte transformation in repeated gonococcal urethritis. Infect. Immun 2:655-658.

50. Kwapinski, J.B.G., and A. Cheng. 1975. Preliminary observations on the protective action of a cytoplasmic immunogen of Neisseria gonorrhoeae. Can J. Microbiol. 21:332-337.

51. Lewin, R. 1973. The battle against gonorrhea. New Scientist. 58:355-357.

52. Lucas, J.B., E.V. Price, and J.D. Thayer. 1967. Diagnosis and treatment of honorhea in the female. New Engl. J. Med. 276:1454-1459.

53. Luoma, M.K., W.R. Cross, and J.A. Rudbach. 1975. Radioimmunoassay for quantifying antibody to N. gonorrhoeae in human sera. Brit. J. Ven. Dis. 51:387-391.

54. Mahony, J.D.H., J.S. McCann, and J.R.W. Harris. 1973. Comparison of trimethoprim-sulphamethoxazole and penicillin in the treatment of honorrhoeae. Brit. J. Ven. Dis. 49:517-520.

55. Marino, A.F., H. Pariser, and H. Wise. 1972. Gonorrhea epidemiology--is it worthwhile? Am. J. Pub. Health 62:713-714.

56. Martin, J.E., W.L. Peacock, G. Reising, D.S. Kellogg, E. Ribi, and J.D. Thayer. 1969. Preparation of cell walls and protoplasm of Neisseria with the Ribi Cell Fractionator. J. Bacteriol. 97:1009-1011.

57. Milner, K.C. and R.A. Finkelstein. 1966. Bioassay of endotoxin: Correlation between pyrogenicity for rabbits and lethality for chick embryos. J. Infect. Dis. 116: 529-536.

58. Norins, L.C. 1974. The case for gonococcal serology. J. Infect. Kis. 130:677-678.

59. O'Neill, D.M., L.S. Baron, P.S. Sypherd. 1969. Chromosomal location of ribosomal protein cistron determined by intergeneric bacterial nating. J. Vacteriol. 99: 242-247.

60. Otaka, E., T. Itoh, and S. Osawa. 1968. Ribosomal proteins of bacterial cells: Strain and species specificity. J. Mol. Biol. 33:93-107.

61. Panijel, J., G. Milanes, J.L. Van Etten, A. Perani, and O. Ciferri. 1967. Species specificity in protein synthesis. J. Mol. Biol. 28:295-309.

62. Peavy, D.L., W.H. Adler, and R.T. Smith. 1970. The mitogenic effects of endotocin and Staphylococcal enterotoxin B on mouse spleen cells and human peripheral lymphocytes. J. Immunol. 105:1453-1458.

63. Plescia. O.J., and W. Braun (Editors). 1968. Nucleic acids in immunology. Springer-Verlag New York, Inc , New York.

64. Ratnatunga, C.S. 1971. Evaluation of the gonococcal complement-fixation test. Brit. J. Ven. Dis. 47:279-288.

65. Reinhold, R.B., and J. Fine. 1971. A technique for quantitative measurement of endotoxin in human plasma. Proc. Soc. Exp. Biol. Med. 137:334-340.

66. Reising, G. 1971. Microflocculation assay for gonococcal antibody. Appl. Microbiol. 21.852-853.

67. Reising, G. and D.S. Kellogg, Jr. 1965. Detection of gonococcal antibody. Proc. Soc. Exp. Biol. Med. 120: 660-663.

68. Reising, G., J.D. Schmale, D.G. Danielsson, and J.D. Thayer. 1969. Reactivity of two selected antigens of Neisseria gonorrhoeae. Appl. Microbiol. 18:337-339.

69. Sarkar, P.K., and S. Som. 1969. Species specificity of ribosomal proteins in eucaryotic and procaryotic organisms. Biochem. Biophy. Res. Commun. 37:767-770.

70. Schalla, W.O., and W. Johnson. 1975. Immunogenicity of ribosomal vaccines isolated from group A, type 14 Streptococcus pyogenes. Infect. Immun. 11:1195-1202.

71. Schmale, J.D., D.G. Danielsson, J.F. Smith, L. Lee, and W.L. Peacock, Jr. 1969. Isolation of an antigen of Neisseria gonorrhoeae involved in the human immune response of gonococcal infection. J. Bacteriol. 99:469-471.

72. Schmale, J.D., J.E. Martin, Jr., and G. Domescik. 1969. Observations on the culture diagnosis of gonorrhea in women. J. Am. Med. Assoc. 210:312-314.

73. Smith, R.A., and N.J. Bigley. 1972. Detection of delayed hypersensitivity in mice injected with ribonucleic acid-protein fractions of Salmonella typhimurium. Infect. Immun. 6:384-389.

74. Smith, R.A., and N.J. Bigley. 1972. Ribonucleic acid-protein fractions of virulent Salmonella typhimurium as protective immunogens. Infect. Immun. 6:377-383.

75. Suter, E. 1964. Hyperreactivity to endotoxin after infection with BCG. J. Immunol. 92:49-54.

76. Thayer, J.D., and J.E. Martin. 1966. Improved medium selective for cultivation of N. gonorrhoeae and N. meningitidis. Publ Health Rep. 81:559-562.

77. Thin, R.N.T. 1973. Treatment of gonorrhea in Singapore using penicillin plus probenecid. Brit. J. Ven. Dis. 49:2160218.

78. Thomas, D.W., and E. Weiss. 1972. Response of mice to injection of ribosomal fraction from Group B Neisseria meningitidis. Infect. Immun. 6:355-363.

79. Thompson, H.C., and I.S. Snyder. 1971. Protection against Pneumococcal infection by a ribosomal preparation. Infect. Immun. 3:16-23.

80. Thor, D.E., and S. Dray. 1968. A correlate of human delayed hypersensitivity: Specific inhibition of capillary tube migration of sensitized human lymph node cells by Tuberculin and Histoplasmin. J. Immunol. 101:51-61.

81. Thor, E.D., and S. Dray. 1968. The cell migration inhibition correlate of delayed hypersensitivity: Conversion of human non-sensitive lymph node cells to sensitive cells with an RNA-extract. J. Immunol. 101: 469-480.

82. Thor, D.E., R.E. Jureziz, S.R. Veach, E. Miller, and S. Dray. 1963. Cell migration inhibition factor released by antigen from human peripheral lymphocytes. Nature. 219:755-757.

83. Tramont, E.C , and J.C. Sadoff. 1974. Cross reactivity of N. gonorrhoeae and N. meningitidis and the nature of antigens involved in bactericidal reactions J. Infect. Dis. 130:240-247.

84. Venneman, M.R., and L.J. Berry. 1971. Experimental Salmonellosis: Differential passive transfer of immunity with serum and cells obtained from ribosomal and ribonucleic acid-immunized mice. J. Reticuloendothelial Soc. 9:491-502.

85. Watson, J.D. 1976. Molecular biology of the gene. p. 316. W.A. Benjamine, Inc., Menlo Park, California.

86. Watt, J.J., M.E. Ward, and A.A. Glynn. 1971. A comparison of serological tests for the diagnosis of gonorrhea. Brit. J. Ven. Dis. 47:448-451.

87. Winston, S.H., and L. J. Berry. 1970. Antibacterial immunity induced by ribosomal vaccines. J. Reticuloendothelial Soc. 8:13-24.

88. Winston, S.H., and L.J. Berry. 1970. Immunity induced by ribosomal extracts from Staphylococcus aureus. J. Reticuloendothelial Soc. 8:66-73.

89. World Health Organization. Technical Report Series No. 519. 1973. Cell mediated immunity and resistance to infection. pp. 6-64.

90. Youmans, A.S., and G.P. Youmans. 1965. Immunogenic activity of ribosomal fraction obtained from M. tuberculosis. J. Bacteriol. 89:1291-1298.

91. Youmsna, A.S., and G.P. Youmans. 1966. Effect of trypsin and ribonuclease on the immunogenic activity of ribosomes and RNA isolated from Mycobacterium tuberculosis. J. Bacteriol. 91:2146-2153.

92. Youmans, A.S., and G.P. Youmans. 1966. Preparation of highly immunogenic ribosomal fractions of M. tuberculosis by use of sodium dodecyl sulfate. J. Bacteriol. 91:2139-2145.

93. Youmsna, A.S., and G.P. Youmans. 1970. Immunogenic Mycobacterial ribosomal and RNA preparations: Chemical and physical characteristics. Infect. Immun. 2:659-668.

94. Zatti, M., and R. Revoltella. 1966. Tumor immunity in rats following injection of homologous ribosomes. Experentia. 15:467.