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CELL MEDIATED IMMUNE RESPONSE IN GONOCOCCAL INFECTION

by

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## ABSTRACT

A protein component of Neisseria gonorrhoeae (B<sup>-t</sup>) has been used in a peripheral leukocyte migration inhibition test to delineate the cellular immune response following gonococcal infection.

A specific cellular response was demonstrated in approximately fifty per cent of the patients following gonococcal infection. Maximum inhibition occurred seven to ten days following the onset of symptoms and the duration of that response varied from fourteen to more than forty days. There was a statistically significant positive correlation between the number of previous infections and migration inhibition. In addition, the phenomenon of "early" migration inhibition has been shown to occur following B<sup>-t</sup> stimulation of lymphocytes of patients with gonococcal infection.

Delayed type hypersensitivity to gonococcal cytoplasm developed in guinea pigs following immunization with gonococcal cytoplasmic material in complete Freund's adjuvant. The dermal response could be suppressed by treating the animals with 100 mg of niridazole. As well as suppression of delayed type hypersensitivity, niridazole was shown to have an antibacterial effect on N. gonorrhoeae. The minimum inhibitory concentration of niridazole for

N. gonorrhoeae was 0.008 ug/ml to 8.0 ug/ml.

Suppression of the cellular immune response of guinea pigs by treatment with niridazole increased the infection rate in the subcutaneous chambers when they were subsequently challenged with N. gonorrhoeae. These results suggest that cell mediated immunity is one of the components in the protection mechanism as observed in the guinea pig chamber model.



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## CHAPTER I

### INTRODUCTION

The current epidemic of gonococcal infection presents a challenge to society as a whole as well as to the medical practitioner and to the scientist. Undoubtedly it will require a concerted effort if the control of gonorrhoea is to be achieved.

The problems are many. A vast reservoir of asymptomatic infection in both males (47,96) and females (71,92) remains for the most part undetected. Serological screening test to date lack the sensitivity and specificity required to justify mass screening programs (106). Current efforts directed at epidemiological control are hampered by incomplete reporting of diagnosed cases (104) and by the short incubation period of the infection.

Chemotherapy, which until recently has been effective, is now being seriously challenged by the introduction of beta-lactamase producing strains of gonococci (4,93). Research, which was abandoned in the late 1930's with the advent of penicillin, aimed at the development of a vaccine, has now been renewed. The need for such a vaccine was recognized by the WHO Expert Committee on Gonococcal Infections as early as 1963 (133).

A thorough knowledge of the immunological response elicited by the gonococci and the implications of that response in the protection and pathogenesis of gonococcal disease will have to be the first and essential step in the development of vaccine prophylaxis. Recent studies on the

development of a gonococcal vaccine (45) will have to be reevaluated in the light of further developments in the understanding of the pathogenesis of Neisseria gonorrhoeae. Reinfection occurs with alarming frequency in spite of the existence of a variety of potential effectors that may eliminate the organism.

The role of the cellular immune response in gonococcal disease has received very little attention. In view of the apparent failure of infection to confer immunity, a better understanding of the total immune response is essential for future vaccine evaluation.

The objective of the present investigation was to clearly delineate the cellular immune response following natural infection, and to determine the role of such a response in protection in the animal model.

CHAPTER II

LITERATURE REVIEW

Although the body of this thesis is concerned with cell mediated immunity (CMI) in gonorrhoea, it is necessary to view the immunology of the gonococcus in a broader sense. The complexity of the interaction of host and parasite will be reviewed so that the CMI component may be examined as a part of the whole system.

#### COLONIAL MORPHOLOGY OF NEISSERIA GONORRHOEAE

The observation that the gonococci exhibit several colony types (61) and the subsequent demonstration that only certain of these colony types are virulent (60) have been major contributions in the study of the pathogenicity of the gonococci.

Kellogg et al (61) described four morphologically distinct colony types of Neisseria gonorrhoeae, using a stereomicroscope with oblique substage lighting, and designated them T1, T2, T3, and T4. The delineating characteristics of the four colony types observed included: the colony size of T1 and T2 is smaller than T3 and T4; the colony density ranges from dark gold for T1 and T2 to light brown and colorless for T3 and T4; T1 and T2 colonies have greater convexity; T3 have a granular structure and T2 have a more friable consistency than the other three colony types. More recently, two additional colony types have been

described, T1' (18) and type 5 (55) which resembles T3 and T4. T1 is the predominant colony type found in primary culture of mucosal exudate from patients with gonococcal infection, while non-selective in vitro subculture of the organism will yield predominantly type 4 (61).

It was later demonstrated that T1 and T2 colonies were the virulent forms of N. gonorrhoeae and were capable of producing disease when inoculated into the urethra of human volunteers, and T3 and T4 were non virulent by the same criteria. This virulence is a heritable characteristic demonstrable after numerous in vitro passages (60). The association of T1' and T5 with virulence in humans has not been reported. However,  $10^{10}$  colony forming units (CFU) of strain K<sub>2</sub>43562, colony type 5 are avirulent in the chimpanzee (64).

The significant point to be gained from these studies was that, for the first time, it was possible to study the virulent form of the gonococci. Since non-selective serial subcultures yield predominantly T4 organisms, material published prior to 1963 will, in all likelihood, reflect results obtained with the non virulent colony type and should be interpreted in that context.

We will now consider those factors which may be significant to the virulence of the gonococcus.



## PROPOSED MECHANISMS OF PATHOGENESIS

Pili

One of the prime considerations in the establishment of infection of the urethra, cervical, or pharyngeal mucosa is the mechanism whereby the organisms adhere to the tissue, preventing mechanical removal by the natural secretions. Pili, the proteinaceous projections on the bacterial cell surface, could function in this capacity.

The first report of the presence of pili (fimbriae) in the genus Neisseria was by Wistreich and Baker (137) who, using electron microscopy, observed surface-appendages on strains of N. perflava, N. subflava and N. catarrhalis. It was later shown that pili were present on the surfaces of all N. gonorrhoeae cells from type 1 and type 2 colonies but absent from organisms from type 3 and type 4 colonies (114,54). In addition, it has been shown that pili promote adhesion to the surfaces of various mammalian cells (112,98, 113,54). The results of these studies suggest a correlation between virulence and the presence of pili.

In some instances, the ability of a bacterial species to attach to certain mucosal surfaces and not to others may be responsible for the selective tissue tropism. Virulent, M protein containing, strains of Streptococcus pyogenes, which normally colonize the pharynx, adhere readily to pharyngeal epithelial cells; whereas, Escherichia

coli, which does not normally colonize the pharynx, adheres poorly to the epithelial cells from the oropharyngeal area. When the same organisms are tested for their ability to adhere to the surface of the urinary bladder, their affinities are reversed (34). Similarly, the ability of enterotoxigenic E. coli strains to adhere to the small intestinal wall appears to be associated with their virulence (56).

When pilated and non-pilated strains of gonococci are compared, the ability of virulent, pilated strains to adhere to human epithelial cells is significantly greater than the non-pilated gonococci. However, the pilated strains adhere equally well to endocervical and ectocervical tissue even though the ectocervical area is not infected in vivo. Both pilated and non-pilated strains will adhere to guinea pig urethral and cervical tissue, while again, in vivo infection of the urethra or cervix does not occur in this species (117).

The sheer possession of pili does not dictate virulence since species of Neisseria not normally associated with pathogenesis are pilated (136). It would seem that the adherence of the gonococci is critical in the establishment of infection, while the development, or final outcome of such an infection is dictated by other factors.

#### Resistance to phagocytosis

A second mechanism important in the establishment of

infection is resistance to phagocytosis, classically demonstrated by the M protein of streptococci, another possible role of gonococcal pili.

Studies to date, however, have been rather contradictory in their findings and three differing points of view are presented. One group of investigators (97,119) has indicated a significant difference in the ability of human polymorphs to phagocytize pilated and non pilated gonococci. Type 1, which were found to resist phagocytosis following mechanical or enzymatic (trypsin) depilation, were as susceptible to phagocytosis as type 4.

Swanson and Zeligs (116), using critical-point-dried specimens, were able to visualize phagocytosis of both pilated and non pilated organisms, and demonstrated that some non pilated organisms (T4\*) showed levels of association with leukocytes higher than those for pilated gonococci (114,115). They postulated that a surface factor in addition to pili was involved in these interactions. Novotny et al (85), although they were not studying phagocytosis at the time, postulated that pili are components of cell walls of all gonococci, which, under certain conditions, take on the familiar appearance of pili. They were able to produce antibody by hyper-immunization with T3 cells which reacted strongly with pili from the T2 homologous strain.

A third proposal (129,118) was that T4 are phagocytized by leukocytes and thus exposed to internal digestion.

T1, on the other hand, adhere to or remain positioned with respect to the cell membrane in such a way that they cannot be digested but remain susceptible to penicillin or bactericidal serum.

These studies, even though they express somewhat different interpretations of the data, were in agreement that human polymorphs are not as efficient as mouse or guinea pig polymorphs (animals not susceptible to infection with the gonococcus) at phagocytizing the gonococcus. Further, we can conclude that there is a relationship between phagocytosis and the colony type which may be pilus determined or which may be multifactorial as suggested by Swanson. There are no recent data to support the findings of Smith (112) who reported that the gonococci can survive and multiply within the leukocyte.

Attachment followed by the ability to resist phagocytosis are conceivably the first steps in the establishment of gonococcal infection.

#### Strain variability

The clinical experience of recurrent infections of the otherwise immunologically competent host suggests that reinfection may be attributable to strain variability of the gonococci. Tramont has shown (122) that the antigen(s) responsible for epithelial cell adhesion is antigenically distinct for specific strains of gonococci. Presumably this

antigen is pili or some other cell wall component which promotes cell attachment. Further evidence for heterogeneity of gonococcal pili was reported by Novotny (85). The reaction between pili from 31 strains and specific IgG raised against 3 strains was followed by direct visual observation with an electron microscope indicating only a few cross reactions.

An in vitro bactericidal assay that utilized 20 antisera to N. meningitidis (122) showed a considerable variability in the patterns of killing of different strains of gonococci, adding further evidence for strain variation. In this case, the structural changes in the organism, which account for the difference in bactericidal effect, are unknown.

Another area of current interest in the study of strain variability is the structure of the cell envelope. N. gonorrhoeae has an outer membrane outside the peptidoglycan layer consistent with other gram-negative bacteria. The greater permeability of the outer membrane of the gonococcus to antibiotics and basic gentian violet dye than E. coli suggests that there are fundamental differences in structure. Gas chromatography of the polysaccharide component of the LPS indicates the presence of glucose, galactose, heptose, KDO, and glucosamine, which is not dissimilar to E. coli (140). The concentration of heptose, however, is reported to be a tenth of the amount found in E. coli (139). Heptose

deficient mutants of E. coli have been reported to be highly permeable (6).

Perry et al (93) have reported a difference in the LPS composition of T1 and T4 colony type cells. T4 cells were found to produce a common type of LPS consistent with that found in typical R-type LPS of gram-negative bacteria. T1 cells were reported to produce additional high molecular weight polysaccharide which the author has suggested is consistent with the O polysaccharide in S-type LPS of gram-negative bacteria. In addition, T1 cells showed considerable differences in glycose composition for each strain examined.

If among the gonococci there are a large number of serologically different types, as is suggested by the results of Perry et al (93), this could offer an explanation for the apparent lack of immunity following infection. Further studies of the epidemiology of the proposed serotypes, as well as further studies of the protection offered by immunization with the O polysaccharide, will be necessary to establish the importance of this interesting proposal.

The findings of another group of investigators have not been in agreement with the results of Perry et al (112). Stead et al (112) were unable to detect significant differences between the lipopolysaccharides of T1 and T2 colony type cells and concluded that the gonococcal LPS lacked O-antigen side chains.

The lipid A portion of the LPS contains B-hydroxy myristic acid, lauric acid, and stearic acid, the latter of which is not present in the lipid A of E. coli. Myristic acid, which is present in E. coli, appears to be absent from N. gonorrhoeae. The phospholipids isolated from the lipid A include phosphatidylethanolamine, phosphatidylglycerol and diphosphatidylglycerol (139).

The main chemical difference between E. coli outer membrane and that of N. gonorrhoeae appears to be in the protein component with the gonococcus containing many more hydrophilic proteins. Hydrophilic pores in the membrane may be the reason for the greater permeability to charged molecules (e.g. penicillin) in N. gonorrhoeae. Changes in the cell wall affecting this permeability could produce the low level drug resistance that has been noted in recent years (139).

A second mechanism to account for strain variability was proposed by Davis and Salton (31). Envelope preparations of N. gonorrhoeae were shown to contain a D-alanine carboxypeptidase which releases terminal alanine residue from uridine-5-diphosphate-N-acetyl muramylpentapeptide substrate. The activity of this enzyme was shown in vitro to be inhibited by B-lactam antibiotics. A possible physiological role in vivo could be to decrease the amount of cross linkage of peptidoglycan or to function in transpeptidation reactions, a step known to be penicillin sensitive. Gonococci known to be sensitive to penicillin bind ten times as much substrate

as the strains shown to be moderately resistant. A decrease in the binding capacity of the D-alanine carboxypeptidase with B-lactam antibiotics was proposed as the possible mechanism in the development of low-level penicillin resistance in the gonococci.

Recent documentation of the presence of plasmid mediated B-lactamase producing strains of gonococci, whose penicillin MIC may be greater than 128 mg/l, is now the major area of concern, at least in clinical management of the patient (94,4).

A difference in virulence was reported to be associated with the auxotype of the gonococci (62). Strains requiring arginine, hypoxanthine and uracil for growth accounted for 89 per cent of the cases of disseminated gonococcal infections and a disproportionate 38 per cent of the uncomplicated cases in the same area. In addition, none of the strains isolated in the Philippines and Taiwan, a region where disseminated infections rarely occur, were Arg<sup>-</sup>Hyx<sup>-</sup>Urc<sup>-</sup>. This was the first report of strains possessing increased capacity to produce DGI. In addition to the characteristic auxotype, the strains were highly sensitive to penicillin with MIC's of 0.015 ug/ml.

#### Virulence factors

A number of studies have suggested that the gonococci possess "virulence associated" factors. Ward et al (129) found that gonococci isolated from exudates were resistant to the bactericidal effect of serum and complement but that this



resistance was lost on subculture. They proposed that the resistance to lysis may be due to the presence of a protective surface component comparable to the K antigen of E. coli.

Another hypothesis of virulence was put forward by Kwapinski and Cheng (67) stating that the immunogenicity of the gonococcus was adversely affected by a component of the cytoplasm of the virulent T1 and T2 cells. This toxic component, upon release during phagocytosis or "processing" of gonococci by the macrophages, depressed the normal immune response of the lymphocytes. It was further postulated that if the immunogenic properties could be separated from the toxic properties it would be an important agent in the protection mechanism. Using a combination of gel filtration and isoelectric focusing the cytoplasm of T1 was fractionated and compared with similar fractions from T4 cells. A protein fraction was isolated which was reported to be absent from T4 colony type cells, and to be highly toxic for the 11-day-old chick embryo. When this toxic material was subjected to polyacrylamide gel electrophoresis a single protein fraction was obtained which consisted of only 6 or 7 amino acids with an estimated molecular weight of 28,000 daltons. This fraction (B<sup>-t</sup>) was found to be non-toxic for the chick embryo (19). Later observations were reported to indicate that B<sup>-t</sup> was an immunogenic agent which produced a humoral and cell mediated immune response. It was also tentatively concluded that it was capable of eliciting an immune protection against gono-

coccal infection in rabbits (67).

An antigen described by Pierce (95) was isolated from T1 colonies of strain F62 which was absent from T3 and T4 cells of the same strain. This was distinguishable from LPS, pili, and the cytoplasmic antigen described by Cheng et al (19). The "virulence associated" antigen was only partially purified by the authors and the role, if any, of this antigen in virulence per se has not been evaluated.

An extracellular enzyme capable of cleaving IgA<sub>1</sub> is another potential virulence factor that has been identified (96). Enzyme production is detectable with all four colony types with a protease activity selective for IgA<sub>1</sub>, secretory IgA<sub>2</sub> subclass being resistant. The relative concentration of the resistant subclass in the urogenital tract is not known. Assuming the enzyme is active at the mucosal surface and that IgA<sub>2</sub> is not the dominant subclass, we may speculate on the significance of this enzyme in the establishment of infection.

#### HOST DEFENCE MECHANISMS

Having discussed the mechanisms of pathogenesis of the gonococcus we will now consider the nature of the response of the host to infection with this organism.

##### Humoral antibody response

The gonococcal complement fixation test (GCFT),

introduced in 1906, was one of the earliest tests developed for the detection of gonococcal antibody. It was recently re-evaluated by Ratnatunga (99) using sera obtained from 1,873 patients. In uncomplicated gonorrhoea a positive reaction was found twice as frequently in females (34%) as in males (18%) and the seropositivity of the non-infected controls varied from 2.5 to 6.5 per cent. In complicated gonorrhoea 41% of the females had positive tests, which is not significantly different than females with uncomplicated infection. The GCFT uses a pool of gonococcal strains which as early as 1907 (99) was reported to yield more positive results than a single strain.

Since the introduction of the GCFT there has been a continuing development of new immunological assays for the detection of gonococcal antibody covering the entire spectrum of immunological procedures. These have included micro-precipitin (17), bentonite flocculation (126), indirect hemagglutination (75), radioimmunoassay (12), fluorescent antibody (134,123), latex slide agglutination test (32), microflocculation (102), sediment flocculation (69) and recently a method to quantitate serum antibody to gonococcal surface antigen, using radiolabelled protein A of Staphylococcus aureus, has been described (20).

The fact that numerous techniques have been introduced to measure the development of antibody in response to gonococcal infection is perhaps not as important as the need

to develop a better understanding of which bacterial components are involved in these reactions. This has been complicated by the use of complex and undefined gonococcal antigens. The specificity of the bacterial components is important in the development of diagnostic immunology, and their role in virulence is vital from the point of view of understanding the protective mechanism.

A protoplasmic antigen "A" of unknown chemical nature was prepared by gel filtration and ion exchange chromatography (107). The "A" antigen reacted with 72% of the sera from uninfected females, 20% of the infected males, and no normal sera. Lee and Schmale (69) have described another protoplasmic antigen "B". Using a slide flocculation test this antigen reacted with sera from 86% of female and 68% of male patients and 12% of the normal controls.

Ward and Glynn (128) used red cells coated with LPS from three different strains of N. gonorrhoeae. With strain G1, the most promising of the three, they were able to detect antibody in the sera of 84% of the females and 46% of the male patients, and found a 2% reactivity among the uninfected controls. It has also been shown that the antigens involved in the bactericidal reaction are lipopolysaccharides (43).

The immune response to purified pili has been documented (12). More females than males showed an elevated response to pili. This appears to be consistent with all other serological methods irrespective of the antigen used. Response

to pili was the weakest among the asymptomatic males. Significant antibody levels were detected in 50% of symptomatic and 33% of asymptomatic males, while 89% of the asymptomatic females with gonococcal infection showed elevated antibody to pili antigen .

#### The time sequence of antibody response

Elevated antibody to pili (12) in patients with gonococcal arthritis appears within one week of the onset of symptoms and persists for several months after treatment. It was reported that, if the initial serum antibody was very high, elevated serum levels persisted for a greater length of time.

The time sequence of humoral response has been reported (32) using a commercially prepared serological test - the Gonosticon Dri Dot Test. With this technique 11.4% of the males with a first episode of gonococcal infection had a positive test at the time of treatment. The mean time after the last sexual contact and the development of positive serology was 9 days. An additional 25% yielded a reactive response on follow-up. The mean number of days from sexual contact to reactive serology was 19.4 days. The remaining 63% failed to have detectable antibody. Those patients with positive serology remained positive for 8 - 102 days post-treatment. Earlier studies by the same author indicated that a patient may have reactive serology for up to one year following the initial

infection. Other procedures, measuring antibody to surface components of the cell or LPS (77,17,42), have found elevated antibody response 7 - 10 days after the onset of symptoms. Gonococcal antibody declined to normal levels in 1 month to 1 year in treated patients (77,49) and in 6 months to 2 years in untreated patients (42).

#### Immunoglobulin class in the humoral response

Using an indirect fluorescent antibody procedure Cohen (27) demonstrated the presence of IgG, IgM and IgA antibodies which were reactive with somatic antigens of virulent N. gonorrhoeae in the sera of normal persons with no history of a previous infection. Cohen et al later demonstrated a discernible increase in all three classes of antibody in the sera of male volunteers following experimental inoculation with N. gonorrhoeae (28).

IgA (secretory) is potentially the most important class of antibody in the initial stages in the establishment of infection of the mucosa. Eighty-two per cent of males with uncomplicated gonococcal urethritis have detectable amounts of secretory IgA in their urethral secretions by the time they present themselves to the clinic for treatment (58). The incidence and titre for patients with a history of previous infection is similar to those with their first infection. Whether this indicates a specific secretory response developed by the time the patient reports for treatment, or a cross reacting antibody elicited by another

organism is not yet known.

Similarly, IgA antibodies to N. gonorrhoeae can be demonstrated in the vaginal secretions of female patients with gonococcal cervicitis. The peak response of secretory IgA antedates serum IgA and the secretory IgA response tends to be of a much shorter duration (90).

IgA is capable of inhibiting bacterial adherence to epithelial surfaces (137) suggesting that it may serve as a mechanism to limit tissue invasion resulting in mucosal or asymptomatic infection; these are the common clinical manifestations of gonorrhoea.

#### Cell mediated immune response

Acquired immunity to some infectious agents differs from the classic mechanisms in which specific antibody interferes with infectivity or pathogenicity by combining with the invading organism or its toxic products. There is no evidence that virus, bacteria or protozoa are influenced in any way by antibody when they are contained within the host cell. A considerable amount of evidence is now available to indicate that changes in the host cellular environment, which lead to a resistance to infection with some intracellular parasites or resistance to infection with some parasites which are unaffected by antibody, are attributed to cell mediated immunity.

A number of bacteria have been shown to be capable of eliciting a cellular immune response. Evidence of such

a response has been demonstrated for streptococci (39), pneumococci (87), tetanus toxoid (35), typhoid-paratyphoid vaccine (35), diphtheria toxoid (35) and brucella (86,22). Host resistance to infection with certain bacteria, which includes M. tuberculosis and Listeria monocytogenes, has been shown to be dependent upon the development of a specific cellular immune response (30,73,74).

There is growing evidence to suggest that N. gonorrhoeae is resistant to antibody mediated bacteriolysis. It was first demonstrated by Ward et al (129) that gonococci in urethral exudates which were resistant to the bactericidal effects of serum lost their resistance on subculture. Certain DGI-associated strains of gonococci have been reported to be resistant to the bactericidal effects of sera (9,109). Ward et al (129) attributed the resistance to some cell surface factor. Brooks et al (9) and Schoolnik et al (109) suggested that it was a strain related phenomenon. An excellent investigation by McCutchan (83) has shown that one can in fact select forthese resistant cells. The results of Ward, Brooks and Schoolnik may reflect a residual spurious effect produced in an artificial environment, and may not necessarily reflect the in vivo course of events. A more likely explanation is that the host environment selects for the bactericidal resistant cells in the same way it selects for T1 cells. Both characteristics are rapidly lost in the in vitro non-selective passage.



Clearly, it becomes important to understand the role of the CMI response in an infection where the humoral response is deemed to be ineffective.

It has been suggested that certain of the clinical manifestations of complicated gonococcal disease may be a result of cell mediated immunity (65). Kraus cites as examples the protracted nature of pelvic inflammatory disease, fibrosis and abscess cavity formation. This aspect of gonococcal immunity has not been studied extensively.

Delayed type hypersensitivity skin reactions to gonococcal antigens were reported as early as 1912 (52), however, it was only recently that in vitro tests have been developed for the assay of the cellular immune response. The first in vitro evidence for a cellular immune response following gonococcal infection was reported by Kraus (65) who used whole sonicates of N. gonorrhoeae strain F62 (61) to measure the blastogenic response of lymphocytes. The response in patients with an initial infection was not significantly greater than in patients with two or more previous infections.

Grimble (46) with a small study group and using a variety of gonococcal antigens was able to demonstrate lymphocyte transformation among patients with gonococcal infection. Although the author has stated that only four of the ten controls had a significant blastogenic response, in fact, eight of the ten controls had transformation ratios greater than two with one or more of the gonococcal antigens.

The non-specificity of the antigenic preparations makes meaningful interpretation of the data difficult. The non-specific blastogenic response that can be elicited with LPS must also be considered in any antigen preparation.

An alkaline extract of F62 type 1 cells cross reacted with fewer of the control patients (3/24) and gave lymphocyte transformation ratios 2 with (19/24) the majority of patients who had multiple episodes of gonorrhoea. No immune response was detected in half of the patients with an initial gonococcal infection (65).

A sonicate of F62 type 1 cells, the soluble and insoluble fractions and the soluble material fractionated by ion exchange chromatography, have been compared for their specificity in eliciting a blastogenic response following gonococcal infection (38). This material was still relatively impure and all of the fractions which elicited a good response from the patients lymphocytes, also elicited a good response from many of the normal controls. Further purification of the reactive fractions will undoubtedly be required in order to elicit a highly specific response to N. gonorrhoeae. The cross reactivity of antigens is demonstrated by the rise and fall of the blastogenic response to a sonicate of N. catarrhalis following infection and successful treatment of a patient with acute gonococcal arthritis (38). Very little is known about the temporal relationship of infection

to the cellular response. In one patient with gonococcal arthritis the lymphocyte transformation response to gonococcal sonicate dropped from highly significant levels to normal ( 2) five days after successful treatment (38).

The demonstration of a measurable cell mediated immune response in patients to gonococcal antigens leads us naturally to ask whether this response plays any significant role in the protection of the host or in the pathogenesis of gonococcal disease. Much of the effort of our research has in fact been directed to this problem.

#### ANIMAL MODELS IN THE STUDY OF GONOCOCCAL DISEASE

##### Development of animal models

Because N. gonorrhoeae is highly host-specific it has presented a problem in the experimental study of host-organism interactions. The first successful experimental animal infection was the anterior chamber of the rabbit eye (82) where persistent infection resulted in the development of complement-fixing antibody.

Lucas (71) was able to demonstrate gonococcal urethritis in the chimpanzee when urethral exudate from males with gonococcal urethritis was transferred to the urethras of the other male chimpanzees. Natural transmission of gonorrhoea from male to female chimpanzees has also been demonstrated (11).

The need for a smaller research animal model led to

the development of subcutaneous implanted chambers (1,2,3). The earliest model used hollow, polyethylene golf balls, sterilized and surgically implanted into the subcutaneous tissue of the dorso-lumbar region of the rabbit. Following treatment of the animal with dexamethasone, a synthetic steroid, the chambers were inoculated with media containing N. gonorrhoeae. Stainless steel coils were used in a similar manner to infect guinea pigs, hamsters, rats and mice. Dexamethazone was not required to establish infection in these animals. Flynn (40) developed a similar model in the mouse using transparent vinyl tubing. Organ cultures (14) and human fallopian tubes (130) have been developed as laboratory models in the study of host parasite interaction.

#### The immune response and evidence for protection

The serological response of the chimpanzee following infection is similar to the human response (10). Sera tested in parallel using a semi-automated complement-fixation test (91), indirect fluorescent antibody test (134), microflocculation test (101), sediment flocculation (69) and a semi-automated microhemagglutination test, became reactive earliest in the complement-fixation, indirect fluorescent antibody and sediment flocculation tests. In one animal a positive serological test was obtained earlier than the positive urethral culture. The time sequence is important. As this study illustrates, the culture may be the only positive result, the culture and serological tests may be

positive, or the culture may be negative with one or more of the serological tests positive at any given point in the course of the infection (10).

The next step in the development of the experimental model has been the study of the protection of the host. Chimpanzees immunized with formalized gonococcal cells developed increased resistance to challenge with virulent gonococcal cells (3). This resistance is strain and dose dependent and can be overwhelmed by challenge with a strain different from the immunizing strain, or by increasing the number of cells in the urethral challenge.

A second important observation is that, if infected, the urethra of the immunized animal is colonized with fewer gonococci than the unimmunized control. There is some evidence to suggest that these animals, because of the small numbers of gonococci in the urethra, do not transmit the disease venereally.

Acquired immunity in chimpanzees also develops during the course of gonococcal urethritis and gonococcal pharyngitis (64), which is dose and strain dependent.

Limited protection of short duration that appears to be strain dependent is consistent with the apparent failure of humans to acquire immunity to gonococcal infection. Although it may appear that there is no significant protection when we see a history of repeated gonococcal episodes in certain individuals, it has been demonstrated epidemiologically

that only one in five males who experience a single sexual contact with an infected female develop gonorrhoea (51). Also, treatment terminating the infection may not allow sufficient time for the host to develop immunity capable of subsequent protection. The time required for spontaneous termination may exceed several months (80).

Whenever acquired immunity can be elicited, the feasibility of protection with a vaccine exists. However, many questions are still unanswered; namely, which of the parameters of the immune system are operative in protection and which bacterial antigens are involved in the stimulation of the significant response?

Using the subcutaneous guinea pig chamber, Scales and Kraus (106) demonstrated the development of immunity to reinfection in the course of an experimental gonococcal infection. This protection could be passively transferred with the 7S peak of gel filtered serum.

In this experiment the gonococci were preincubated with the immune serum before they were injected into the chamber. Gonococci, after culture on artificial media, are susceptible to bacteriolysis by immune serum. The serum used to transfer "immunity" was shown in fact to be bactericidal. It would appear that what was being measured in this experiment was the ability of the animal to adequately inactivate those gonococci which escape in vitro bacteriolysis.

In order to study the nature of the CMI response in human gonococcal infection, a suitable antigen for use in an in vitro assay was required. The results of the work of Kwapinski et al (66) and Cheng et al (19) suggested that the B<sup>-t</sup> antigen was a potentially important component of the gonococcal cell. It was reported to be a protein, present only in T1 cells and non-toxicogenic for the chick embryo. In addition, it was shown to be protective in the rabbit eye model. On the basis of this evidence, it was felt that studies should be carried out to determine whether a CMI response could be measured, using cytoplasmic components of the gonococcus.

To determine whether or not the cellular response was a significant factor in protection, it was necessary to have some means of suppressing the cellular response in the animal model. The anti-schistosomal compound, Niridazole, 1-(5-nitro-2-thiazolyl)-2 imidazolidinone has been shown to suppress the CMI response (29) while not affecting the production of antibody. Other studies have confirmed this (78,79,132). The low toxicity and easy administration indicated that Niridazole would be an appropriate agent to suppress CMI in the animal model for gonococcal infection.

CHAPTER III  
MATERIALS AND METHODS



## SOURCE AND PROPAGATION OF NEISSERIA GONORRHOEAE

N. gonorrhoeae F62 type 1 (61), supplied by Dr. Kellogg (CDC, Atlanta), was used for the antigen preparation, animal challenge experiments, and in the preparation of the cytoplasm for immunization. The F62 strain was passaged on GC Medium Base (Difco) with 2% of a defined supplement (68) and 0.005% ferric nitrate. The defined supplement was composed of cocarboxylase, 0.001 g; glutamine 0.5 g; and dextrose, 20.0 g in 100 ml of distilled water sterilized by filtration.

## ANTIGEN PREPARATION

Preparation of cytoplasm

The preparation of cytoplasm was carried out as previously reported by Cheng (19). Type 1 colonies from F62 were used to inoculate supplemented GC medium. After 18 hours incubation at 35°C in 5% CO<sub>2</sub> the cells were harvested by washing into 0.85% phosphate buffered saline (PBS) pH 7.1. Prior to harvesting each plate was checked for purity of culture and colony type. Any plate containing < 99% T1 colonies was rejected.

After centrifugation, the cell pellet was washed three times in PBS. Following the final washing, the cells were resuspended in five times their volume in PBS.

The cells were disrupted in a Biosonik BP-111 Ultrasonicator (Bromwill Scientific, Rochester, N.Y.) adjusted to 80% maximum output (2.2 Amp) for 15 minutes. The disrupted cells were then centrifuged at 20,000 xg for 30 minutes at 4°C. The final supernatant was removed and the centrifugation was repeated three times. The final supernatant was removed and filtered using a millipore filter system and a 0.22 membrane filter (Millipore, Gelman Co.). A portion of the filtered cytoplasm was removed for protein determination and the remainder was frozen at -70°C.

#### Preparation of the B-t protein component

The procedure reported by Kwapinski and Cheng (67) for the isolation of a cytoplasmic immunogen was followed.

Isoelectric focusing technique. Isoelectric focusing of the cytoplasm was carried out using the procedure of Vesterberg et al (122) in a LKB 8102 (440 ml) apparatus (LKB-Produkter AB, Stockholm). Ampholytes with a pH range of 3 - 10 and 3 - 6 were used (Ampholine, LKB Aminkemi).

The power supply was adjusted to 500V at constant voltage with the anode at the top of the column. The voltage was increased gradually to 700V. After focusing was completed the column was eluted and the fractions were collected in a Fractomat (Buchler Instrument Co.). The procedure was carried out at 4°C, and the pH of each fraction was determined immediately after the column was eluted. Following dialysis

against 0.1 M PBS pH 7.1 at 4°C, the absorbance given by each fraction was measured at 280 nm in a Pye Unicam spectrophotometer. The two major zones of protein concentration at pI values of 3.2 and 4.4, which correspond to the B and

fractions reported by Cheng et al (19), were collected. The isoelectric focusing was repeated using the material collected at pI 3.2 and using the material collected at pI 4.4 ( B and  $\alpha$  respectively) with ampholyte having a pH range of 3 - 6.

The resulting B and  $\alpha$  material was dialysed against 0.1 M PBS and then lyophilized to concentrate.

#### Preparative polyacrylamide gel electrophoresis

The B material recovered from isoelectric focusing was subjected to gel electrophoresis. The procedure designed by Raymond (99) was followed using the Mark 11 SAE-2782 model (Shandon Southern Instruments Ltd. Eng.). The column was filled with 7.5% acrylamide gel in 6M urea, pH 9.5. Eighty micrograms of the gonococcal protein (B) fraction in not more than 2 ml volume was applied to the polyacrylamide gel column. The power supply was adjusted to 30 mA at constant current. After one hour the electrophoresis current was increased to 80 mA.

The eluate was collected in 5 ml portions in a fraction collector and the absorbance given by each fraction was measured at 280 nm.

A single fraction showing peak absorption at 280 nm was observed. This fraction ( designated B<sup>-t</sup> (67) ) was dialysed against distilled water at 4°C. Following dialysis the amount of protein present was determined and the fraction was concentrated by lyophilization and stored at -70°C.

#### Gel-Electrophoresis

Block electrophoresis were performed as described in detail by Kaltschmidt and Wittman (57) using 5% and 10% polyacrylamide. The staining solution consisted of 1% bromphenol blue in HgCl<sub>2</sub> saturated 95% ethanol. The decolorizing solution was methanol-acetic acid-distilled water, 45:10:45.

#### KDO determination

2-keto-3-deoxyoctonic acid (KDO) was determined by the thiobarbituric acid reaction (127).

#### Protein determination

Total protein was determined using the method of Lowry et al (70). Bovine serum albumin (BSA Sigma Chemical Co.) was used as standard for the protein estimation.

#### Amino acid analysis

Amino acid analysis of the B and B<sup>-t</sup> material was performed by Dr. Frits Stevens (Biochemistry, U of M.) using the Beckman Automatic Analyzer Model 120C.

## LYMPHOCYTE TRANSFORMATION

Twenty ml of venous blood were collected from each subject in sterile tubes containing heparin without preservative. Using a mixture of sodium metrizoate (Nyegaard & Co., Oslo) and Ficoll (M.W. 400,000, Pharmacia, Uppsala, Sweden) a one-step centrifugal technique was used for the isolation of lymphocytes (7).

### Preparation of separation fluid

Ficoll solution (8.0 per cent w/v) was added to 29.4 ml sodium metrizoate solution (32.8 per cent) to a total volume of 100 ml. The solution was sterilized by autoclaving and stored at 4°C.

### Isolation of lymphocytes

Two ml of heparinized blood was mixed with an equal part of physiological saline and carefully layered with a pasteur pipette onto 3 ml of the separation fluid in a 10 ml tube. The tube was then centrifuged at 400 xg. The resulting cell layer was transferred to a 10 ml centrifuge tube and washed three times in Hanks balanced salt solution.

The final cell pellet was resuspended in 1 ml of medium 199 containing 20% autologous sera. Total lymphocyte count and per cent viability was determined by adding 0.1 ml of the cell suspension to 0.8 ml of Medium 199

containing 0.1 ml trypan blue and counting on a haemocytometer. The remainder of the cells were diluted with Medium 199 (Difco) containing 20% autologous serum to a final lymphocyte concentration of  $1 \times 10^6$  cells/ml.

#### Lymphocyte culture

The cell suspension was dispensed in 1 ml amounts into 12 x 75 mm plastic culture tubes (Falcon Plastics). 0.1 ml of antigen was added to the test and phytohemagglutinin (PHA-P, Difco) diluted one in ten with Medium 199, was used in 0.1 ml amounts in positive controls. No antigen was added to the cell control. The test and the positive and negative control tubes were incubated at 35°C in 5% CO<sub>2</sub> for five days. The cell control was incubated at 40°C.

On the third day of incubation, 0.1 ml of tritiated thymidine ( (6-<sup>3</sup>H) thymidine, 0.5 uCi/tube, specific activity 5Ci/mmol Amersham/Searle Corp.) was added to each tube. On the fifth day all tubes were centrifuged, the supernatant was decanted and the cells were resuspended in normal saline. One ml of 10% trichloroacetic acid was added, and the cell suspension was filtered through 2 cm diameter glass filter pads (GF/C, Whatman). The tubes were washed twice with 5% trichloroacetic acid, and the washings were poured through the filters. The filters were washed twice with 10 ml of methanol ( Fisher Scientific ), placed in scintillation vials and dried at 60°C. Six ml of toluene

scintillation fluid (Econofluor, New England Nuclear) was added to each scintillation vial and counted in a Beckman liquid scintillation system. For each experimental subject the degree of DNA synthesis induced by the gonococcal antigen was expressed as a function of DNA synthesis in cultures without antigen (37°C control). The degree of lymphocyte transformation with the gonococcal antigen was therefore expressed by the following equation:

$$\text{Transformation ratio} = \frac{(\text{counts per minute with antigen cultures}) - (\text{counts per minute with 20°C control cultures})}{(\text{counts per minute with 37°C control cultures}) - (\text{counts per minute with 20°C control cultures})}$$

#### PERIPHERAL LEUKOCYTE MIGRATION INHIBITION TEST

##### Preparation of culture medium

Buffered medium was prepared from 100 ml Medium 199 (TC-199 10x, Difco), 6.2g Hepes, 500,000 iu penicillin, 500 mg streptomycin, and distilled water to a final volume of 1 litre. The pH of the medium was adjusted to 7.2 with 5N NaOH and sterilized by filtration.

##### Preparation of agarose medium

The agarose medium was prepared as described by Clausen (23). The final medium which was prepared immediately prior to use contained 1% agarose (Sigma), 10% horse serum, 60 iu penicillin, and 50 ug streptomycin (Difco) in medium 199. Sodium bicarbonate was added to maintain a pH between 7.2

and 7.4.

Five ml of the agarose medium was pipetted into disposable plastic Petri plates (50mm, Falcon). Immediately before use 8 wells were punched in the agarose with a 2.3 mm steel punch.

#### Preparation of leukocyte suspension

Ten ml of peripheral blood was collected by venipuncture in 10 ml tubes containing 250 iu heparin. The red cells were sedimented by the addition of 6% dextran 250 (M.W. 250,000, Pharmacia, Uppsala) in saline. Blood and dextran were mixed in the proportion 4:1. After sedimentation for one hour the supernatant plasma-leukocyte layer was removed and centrifuged at 220 xg for five minutes. The cell pellet was washed three times in Hanks balanced salt solution (Difco). The washed leukocytes were resuspended in culture medium containing 10% horse serum to give a final concentration of  $2 \times 10^8$  leukocytes per ml.

#### Performance of the leukocyte migration inhibition test

Antigen ( $B^{-t}$ ) in Medium 199 was added to half of the cell suspension and the same volume of Medium 199 without antigen was added to the remaining half of the cells. The antigen concentration in the leukocyte suspension was 100 ug/ml.

Following thirty minutes incubation of the cell



suspension at 35°C in 5% CO<sub>2</sub> in air, 7 ul (1.4 x 10<sup>6</sup> leukocytes) were placed in each well in the agarose medium. Each plate constituted a single test with four wells containing cells with antigen and four control wells containing cells without antigen.

The plates were incubated at 35°C in 5% CO<sub>2</sub> in a humidified incubator for eighteen hours.

#### Reading of the test

The diameter of cell migration was measured by means of a tissue microscope equipped with a stage micrometer. The amount of migration inhibition was expressed as a migration index (MI) that is a ratio of the average area of antigen containing cultures and control cultures.

$$MI = \frac{\text{mean of four areas of migration with antigen}}{\text{mean of four areas of migration without antigen}}$$

### ANTIMICROBIAL SUSCEPTIBILITY TESTING

#### Organisms

The strains of N. gonorrhoeae used for susceptibility testing were isolated from 100 patients with acute urethritis

and 12 patients with disseminated gonococcal infection. Three reference gonococcal strains obtained from Dr. Thornsberry (CDC, Atlanta) were included as controls for the determination of penicillin minimum inhibitory concentration (MIC)

#### Media

Penicillin and Niridazole susceptibility testing were performed on Mueller-Hinton agar (Difco) supplemented with 1% Isovitalex (BBL).

#### Antimicrobial agents

To prepare agar dilution plates containing Niridazole 500 mg of Niridazole was dissolved in 2 ml N,N-Dimethyl Formamide and 3 ml distilled water. Serial 2-fold dilutions were made in foetal calf serum. 1.5 ml of Niridazole-sera dilutions were added to 13.4 ml of agar to yield a final concentration of 10 mg/ml to 0.039 mg/ml Niridazole. Penicillin was tested in 2-fold dilutions ranging from 0.004 iu/ml to 8 iu/ml.

#### Agar dilution technique

Mueller-Hinton broth was inoculated with organisms from an overnight culture grown on Chocolate agar in 5% CO<sub>2</sub>. to a density equivalent to that of a 0.5 McFarland standard ( $10^8$  cells/ml). Using a Steers-Foltz replicator,

the cultures were applied to the surface of the series of Mueller-Minton agar plates containing Penicillin and Niridazole.

Control plates without antimicrobial agent were included with each series.

The plates were incubated in 5% CO<sub>2</sub> at 35°C for 24 hours. The minimum inhibitory concentration was the lowest concentration of antimicrobial agent which produced an abrupt change from uninhibited growth to slight or no growth.

#### ANTI-PILI ANTIBODY DETERMINATION

The quantitative determination of antibody to gonococcal pili was performed by Dr. T. Buchanan (Seattle, Washington). The assay, utilizing purified pili labeled with 125<sub>I</sub> in an antigen binding technique, was carried out according to procedures described elsewhere (12).

#### NEISSERIA GONORRHOEAE AUXOTYPE

Auxotyping of 100 strains of N. gonorrhoeae was performed by Dr. Frank Young (Rochester, New York) as part of an ongoing study of the epidemiology of the gonococcus (personal communication).

## STUDY POPULATION

### Lymphocyte transformation experiments

The study group included 22 patients with uncomplicated gonorrhoea who attended the Primary Health Care Clinic of the Health Sciences Centre. The diagnosis of gonorrhoea was confirmed by the demonstration of gram negative intracellular diplococci in smears of urogenital exudate and the growth of oxidase positive, gram negative diplococci which were identified as N. gonorrhoeae by specific antibody staining (91).

The controls consisted of 14 volunteers among the laboratory and medical staff without history of gonococcal infection.

### Peripheral leukocyte migration experiments

The study included 91 patients with either uncomplicated or disseminated infection.

### Uncomplicated infection

Seventy-seven patients with uncomplicated gonorrhoea participated in the study. The number of previous gonococcal infections and the number of days the patient was symptomatic prior to treatment were determined by history. They were asked to return to the clinic at weekly intervals following antibiotic treatment. Nasopharyngeal and urethral or cervical specimens were obtained for culture for

N. gonorrhoeae on the 28 patients who returned.

#### Disseminated gonococcal infection

Fourteen patients had disseminated infection based on the typical clinical syndrome (arthritis and/or dermatitis) in conjunction with the isolation of N. gonorrhoeae from one or more of the following sites; cervical, urethra, pharynx, rectum, blood or synovial fluid. Informed consent was obtained from all patients.

In addition, 58 laboratory and other hospital employees without history of gonococcal infection, three patients with meningococcal disease, two N. lactamicus carriers, and ten asymptomatic carriers of N. meningitidis were studied.

### EXPERIMENTAL ANIMAL STUDY

#### Animals

White, female, Hartley strain guinea pigs (Bio-Lab, Minn.) were used in all experimental work.

#### Immunization

An emulsion of gonococcal cytoplasm and complete Freund's adjuvant (CFA, Difco) was used for immunization. Cytoplasm and CFA in a ratio of 1:1.4 was emulsified in a syringe emulsifier (BBL). The cytoplasm was diluted so that the emulsion would contain 100 ug gonococcal protein

/0.2 ml.

Each foot pad was injected with 0.05 ml resulting in an immunizing dose of 100 ug gonococcal protein per animal.

#### Delayed hypersensitivity skin testing

The cytoplasmic gonococcal material used for immunization was also used for skin testing. The antigen was diluted to 100 ug protein/ml. Prior to skin testing the hair was removed from the abdomen by shaving followed by application of a commercial depilatory (Neet). The skin was carefully washed, cleaned with 70 per cent alcohol and allowed to dry. Using a sterile 1 ml tuberculin syringe the tip of the needle was inserted, bevel up, just under the surface of the skin and 0.1 ml of the antigen solution was injected (intracutaneous). This resulted in a bleb about 5 mm in diameter.

The site of the infection was marked with a marking pencil and the results were read at 4, 24, and 48 hours. The diameter of induration and erythema was measured in two directions and recorded in mm.

#### Chamber implant

A technique similar to that described by Arko (2,3) was used to insert subcutaneous chambers. The chambers were Tygon flexible plastic tubing (R-3603) that measured

11 mm in diameter with a 2 mm wall. The tubing was cut in 25 mm lengths and autoclaved. Six chambers were surgically implanted in the subcutaneous tissue of the dorso lumbar region of each animal. The wounds were closed with wound clips (Autoclips, Clay Adams).

#### Sterility check

Three weeks after the chambers were implanted, fluid was aspirated from each chamber and cultured on blood agar for sterility.

#### Counterimmunoelectrophoresis

The aspirated fluid from the chamber implants was subjected to counterimmunoelectrophoresis for the detection of antigenococcal antibody. The antigen used in the procedure was F62T1 gonococcal cytoplasmic material. Immunelectrophoresis was carried out in 1% agarose (Sigma) in Veronal buffer pH 8.6.

Clear glass microscope slides 75 mm x 25 mm were evenly coated with agarose. Sample wells 2.5 mm in diameter were punched in the agarose. Each slide contained two wells 10 mm apart. After application of the chamber aspirate and antigenic material (approximately 7 ul) the slides were placed in the electrophoretic chamber. Filter paper wicks were immersed in the buffer chambers and the power supply was connected.



FIGURE 1

PLASTIC TUBING USED FOR SUBCUTANEOUS CHAMBER IMPLANTS





After completion of the electrophoresis the slides were viewed under diffuse transmitted light.

#### Niridazole treatment

Fifty animals were given 100 mgm Niridazole (Ambilhar, Ciba) by oral feeding.

#### Gonococcal challenge

Three days after Niridazole treatment, the animals were challenged with F62T1 gonococci. The organisms were grown on supplemented GC agar and, after the colony type was confirmed, were suspended in Trypticase Soy Broth (TSB) with 10% horse serum (Grand Island Biologicals). The culture was suspended to a density to correspond with the McFarland 0.5 standard, or  $10^8$  cells/ml. The optical density was determined at 550 nm, adjusted to an optical density of 0.3 and the appropriate dilutions were made in TSB with 10% horse serum. The number of colony-forming units (CFU) in the suspensions was determined by plating 0.1 ml of the higher dilutions on GC medium. The sample with an optical density of 0.3 contained approximately  $2 \times 10^8$  CFU per ml. Each chamber was inoculated with 0.2 ml of TSB inoculum containing  $1 \times 10^3$  CFU of N. Gonorrhoeae. Counts of the dilutions were made before and after the challenge to confirm the viability of the organisms throughout the experiment.

Chamber aspiration

All chambers were aspirated three days after the challenge with gonococci. The aspirates were withdrawn with a 1ml tuberculin syringe and cultured on supplemented GC agar and Thayer Medium (BBL) incubated in 5% CO<sub>2</sub> at 35° C.

## CHAPTER IV

### RESULTS

ANALYSIS OF THE GONOCOCCAL PROTEIN FRACTION B<sup>-t</sup>

Following isoelectric focusing of the gonococcal cytoplasm the B material (pI 3.2), as described by Cheng et al (19) was subjected to electrophoresis. Using preparative gel-electrophoresis a single peak, shown in Figure 2, determined by absorbance at 280 nm, was obtained. A small second peak reported by Cheng et al (19) to be present in some of the assays was not detected.

Results of the amino acid analysis of this fraction (B<sup>-t</sup>) are summarized in Table 1. The amino acid composition was consistent with a previous reported analysis of the B<sup>-t</sup> fraction (66). There are some differences, however, in the amino acid composition of this assay and the amino acid composition of B<sup>-t</sup> reported in an earlier publication (67). While there is a qualitative similarity, several significant differences are noted. In the present analysis, and in a recent publication by Kwapinski et al (66), glycine and lysine were the two major constituents. They were present in a molar ratio of 0.86. Threonine was found to be present in only trace amounts. Kwapinski and Cheng (67) reported that glycine and serine were the major components of the B<sup>-t</sup> protein and the glycine/lysine ratio was 0.18. Threonine was present in approximately the same molar ratio

as lysine. Some alteration in the technique used in the earlier work may account for the quantitative differences noted.

The  $\alpha$ , B and B<sup>-t</sup> material was subjected to gel-electrophoresis after treatment with 2-mercaptoethanol. The B<sup>-t</sup> fraction was composed of at least two protein components. The  $\alpha$  and B material was resolved into 25 and 10 proteinaceous bands respectively.

The thiobarbituric acid reaction indicated that KDO was present in the  $\alpha$  and B material. However, KDO was not detected in the B<sup>-t</sup> fraction with this method.

FIGURE 2

THE PROTEIN FRACTION ( $B^{-t}$ ) OBTAINED FROM PREPARATIVE  
POLYACRILAMIDE-GEL ELECTROPHORESIS OF THE B MATERIAL

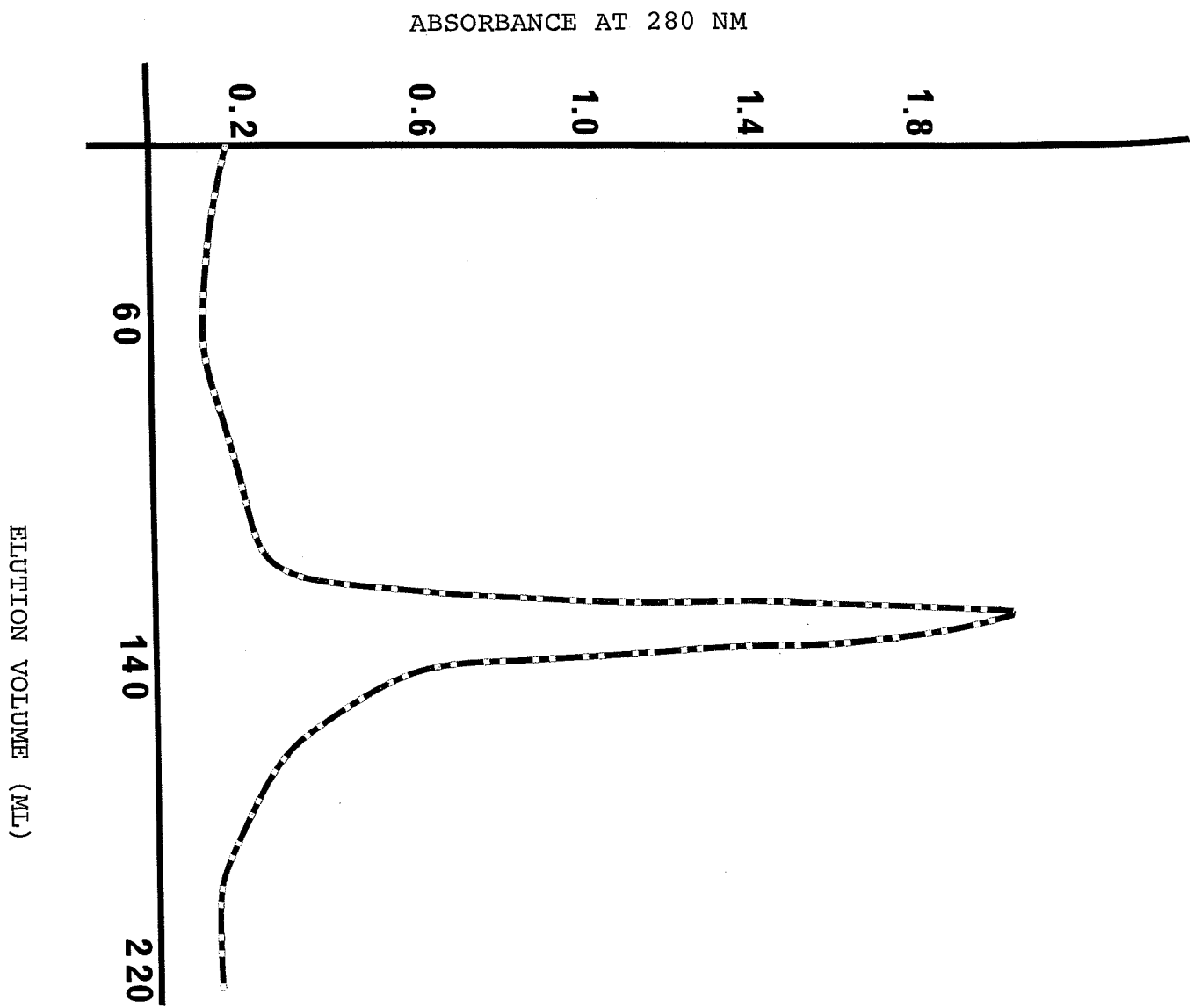




TABLE 1

## AMINO ACID ANALYSIS OF THE GONOCOCCAL PROTEIN FRACTION B-t

<u>AMINO ACID</u>	<u>CONCENTRATION (NANOMOLES)</u>
Lysine	19.46
Aspartic Acid	3.69
Threonine	trace
Serine	5.62
Glutamic Acid	6.52
Glycine	22.72
Alanine	3.73
Leucine	1.96
Histadine	0
Argenine	0
Proline	0
Valine	0
Methionine	0
Isoleucine	0
Tyrosine	0
Phenylalanine	0

## LYMPHOCYTE TRANSFORMATION RESULTS

Lymphocytes from Control subjects without a history of gonococcal infection were examined for blastogenic response to gonococcal antigens. The transformation ratios for subjects in the non-gonorrhoea and gonorrhoea groups are presented in Tables 2 and 3. These tables outline the blastogenic response to cytoplasm and the  $\alpha$  and B fractions of that cytoplasm.

Marked blastogenesis was induced by the  $\alpha$  fraction for each of the six control subjects tested. This type of a response is consistent with that produced by non specific mitogens rather than a response elicited by binding of the antigen to antigen specific sites on sensitized lymphocytes.

A blastogenic response ( $T \geq 2$ ) to the B fraction was present in seven of the nine control subjects. Cytoplasm induced a blastogenic response in two of the six control subjects. Similarly, as can be seen in Table 3, a number of gonorrhoea subjects have transformation ratios greater than two. Three of the eight responded to the B fraction and two of the six gave blastogenic response to the cytoplasm.

Results of lymphocyte transformation tests for control and gonococcal patients using the purified B-t protein are summarized in Figure 3. Fewer responses than

were obtained with  $\alpha$ , B and cytoplasm are noted, with only one of the controls having a transformation ratio greater than 2 with the 100 ug of antigen, and none of the control subjects tested responded to 50 ug of antigen. Of the twenty-three patients with acute gonococcal infection, six had transformation ratios greater than two in response to 100 ug of antigen and two of the nineteen patients tested responded to 50 ug of antigen.

Table 4 summarizes the lymphocyte transformation ratios for four patients obtained at the time of treatment and one week following therapy. In two patients a blastogenic response to the  $B^{-t}$  was detected on the follow-up that was not present at the time of treatment.

There is no statistically significant difference between the controls and the patients in their response to the  $B^{-t}$  antigen. Such a difference, however, might appear if larger study groups were used. The  $B^{-t}$  antigen does stimulate lymphocyte transformation, and there is a suggestion that the patients have greater transformation ratios. Because of the suggestive nature of these results, we have used the  $B^{-t}$  antigen to determine whether it would also stimulate the production of lymphocytes. We have used the antigen in an in vitro test to study its effect in stimulating the production of leukocyte migration inhibition factor.

TABLE 2

RESULTS OF TRANSFORMATION IN CONTROL SUBJECTS WITH SELECTED  
GONOCOCCAL ANTIGENS

Subject	Antigen*			
	$\alpha$	B	Cytoplasm	PHA
1.	51.16**	2.33	2.25	54.72
2.	32.54	3.00	2.25	21.57
3.	9.15	1.00	0.71	18.02
4.	127.1	2.81	1.53	54.36
5.	119.2	2.25	0.81	40.72
6.	65.64	3.02	0.62	11.60
7.		2.19		18.71
8.		1.13		34.72
9.		2.23		23.57

\* the culture medium contained 100 ug protein/ml of the indicated antigen

\*\* a transformation ratio  $> 2$  is regarded as significant

TABLE 3

RESULTS OF TRANSFORMATION TESTS IN GONORRHOEA SUBJECTS WITH  
SELECTED GONOCOCCAL ANTIGENS

Subject	Antigen*		PHA
	B	Cytoplasm	
1.	0.7**		5.2
2.	3.39	0.91	20.44
3.	1.69	0.70	6.12
4.	3.39	2.24	5.72
5.	0.74	0.54	11.73
6.	0.93	1.07	128.4
7.	0.81		63.9
8.	2.45	3.77	16.15

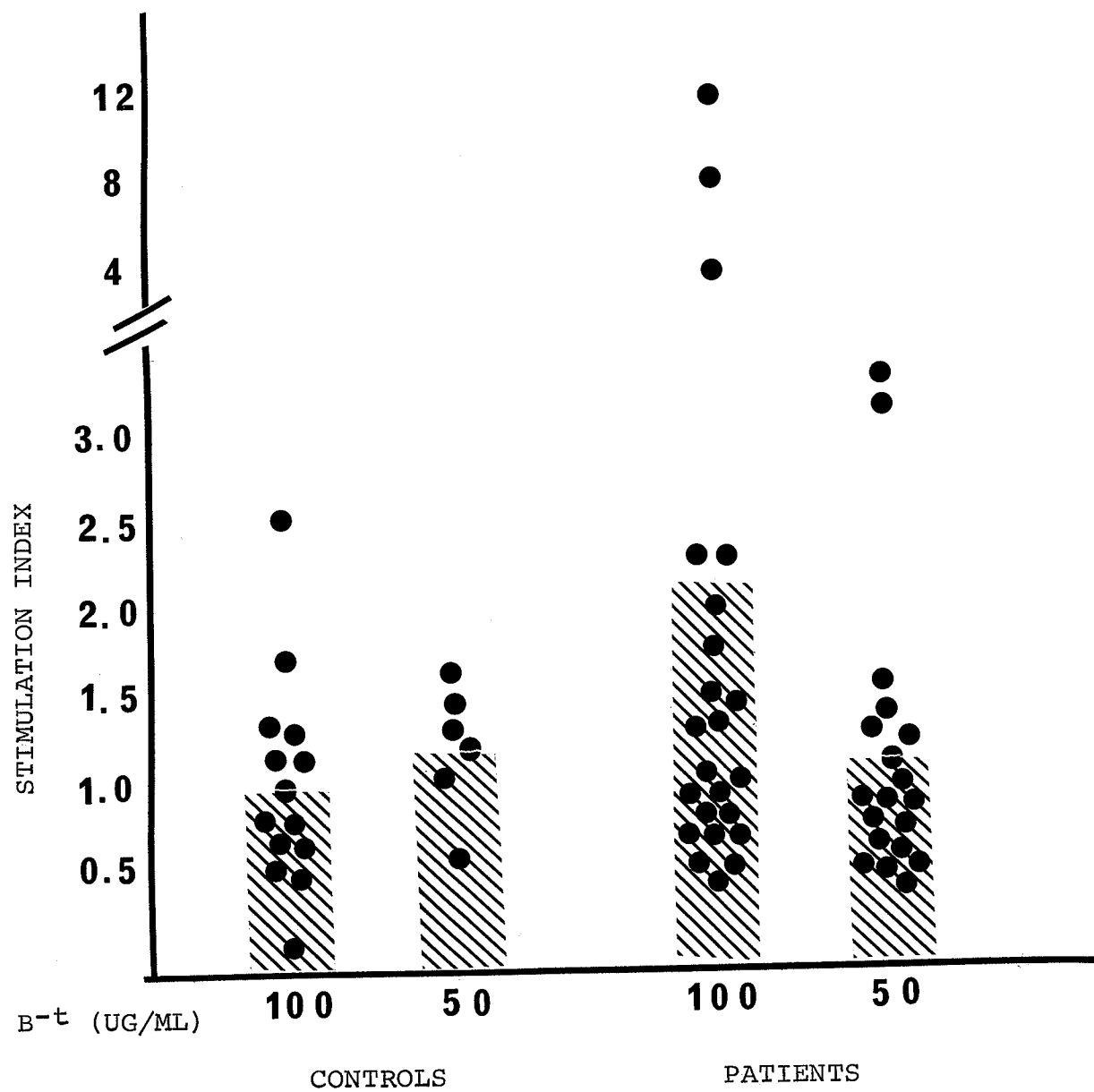
\* the culture medium contained 100 ug protein/ml of  
the indicated antigen

\*\* a transformation ratio  $> 2$  is regarded as significant

FIGURE 3

LYMPHOCYTE TRANSFORMATION RATIOS OBTAINED FOR PATIENTS AND  
CONTROL SUBJECTS WITH 100 UG AND 50 UG OF B<sup>-t</sup> ANTIGEN.

The shaded area represents the mean.



## PERIPHERAL LEUKOCYTE MIGRATION INHIBITION

Granulocyte - MIF procedure

The leukocytes migrate from the wells between the agar and plastic surfaces and are seen as a halo around the well (Figure 4). Following fixation with alcohol, the agar is readily removed leaving the cells intact on the plate surface. They were then stained with Wright's stain in order to identify the migrating cells.

The migrating cell population appears to consist almost entirely of granulocytes (Figures 5, 6, and 7).

When red cells are placed in the wells either alone or in combination with leukocytes, the red cells do not appear in the area surrounding the well but remain within, indicating that the granulocytes actually migrate.

Inhibition is demonstrated in Figure 8. A small area of migration is seen around the four wells containing leukocytes pre-incubated with B<sup>-t</sup> gonococcal antigen. A larger area of migration is apparent around the wells containing cells pre-incubated without antigen.

It was necessary to determine to what extent variation in the diameter of migration from the four individual control wells, or the four individual test wells, affected the calculated migration index.



TABLE 4  
CELLULAR RESPONSE TO GONOCOCCAL ANTIGENS  
IN PATIENTS AT THE TIME OF TREATMENT AND ONE WEEK FOLLOWING  
TREATMENT

Patient		At time of treatment	1 week post- treatment
A	Cytoplasm *	ND**	ND
	B	0.70**	ND
	B <sup>-t</sup>	0.94	1.16
B	Cytoplasm	1.07	1.94
	B	0.93	2.96
	B <sup>-t</sup>	1.54	4.11
C	Cytoplasm	2.45	4.61
	B	3.77	5.00
	B <sup>-t</sup> (100ug)	2.17	2.47
	B <sup>-t</sup> (50 ug)	1.26	3.35
D	Cytoplasm	ND	ND
	B	1.00	0.74
	B <sup>-t</sup>	0.64	0.78

\* inducing antigen. All antigens were used in a concentration of 100 ug protein/ml of culture medium unless otherwise indicated

\*\* test not performed

\*\*\* transformation ratio

FIGURE 4

LEUKOCYTE MIGRATION TEST AFTER 18 HOURS INCUBATION.

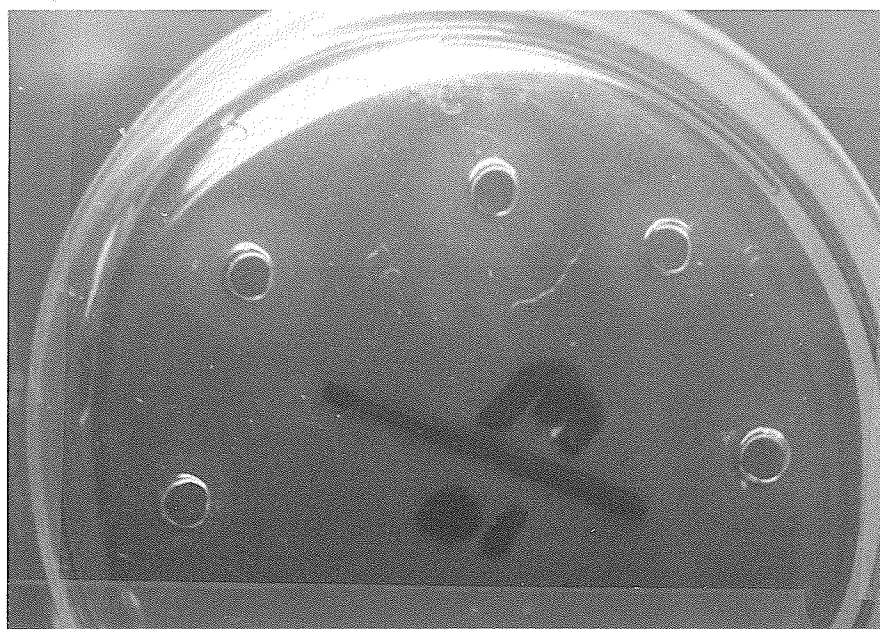


FIGURE 5

MIGRATING CELL POPULATION X125. The edge of the well is seen in the lower right hand corner. In preparation for photography, the cells were fixed by flooding the plate with 95% alcohol. The agarose was then removed and the cells were stained with Wright's stain.

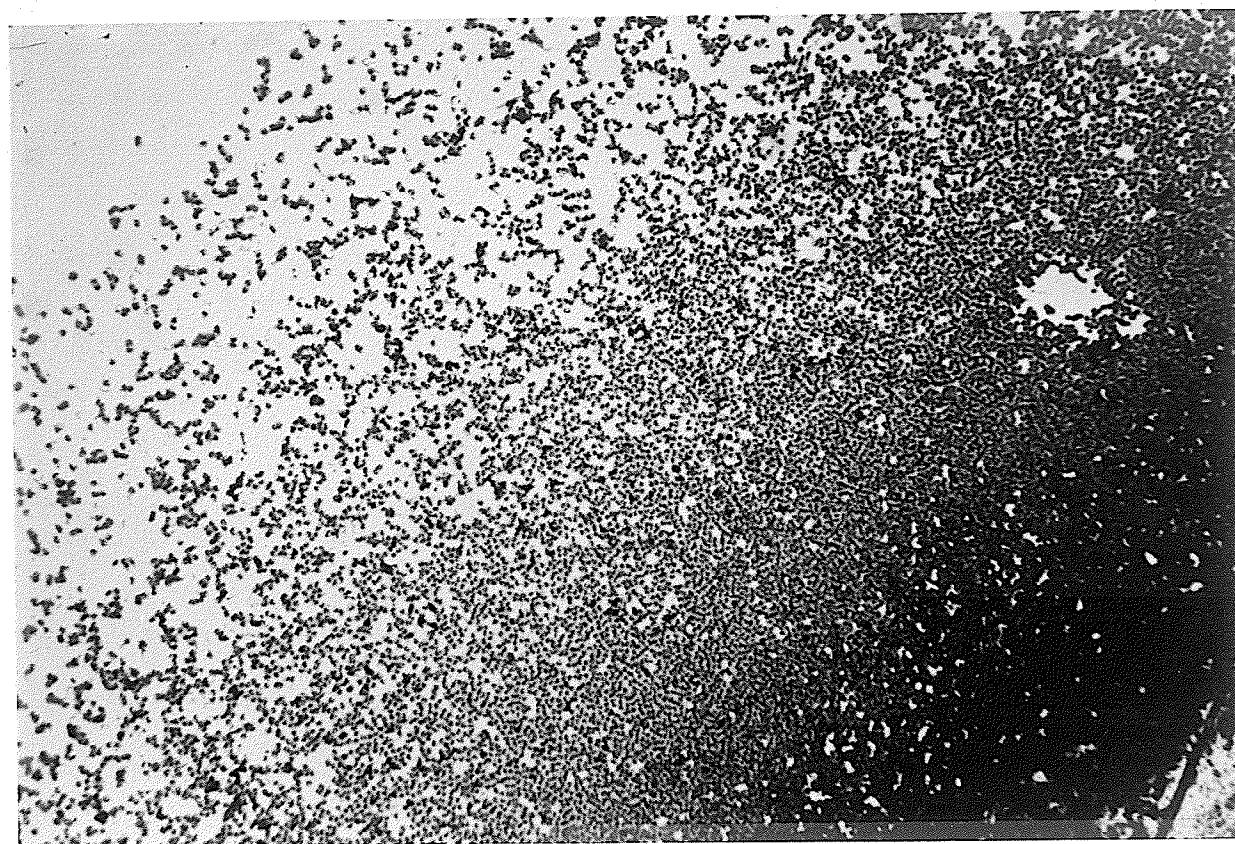


FIGURE 6

MIGRATING CELL POPULATION X500

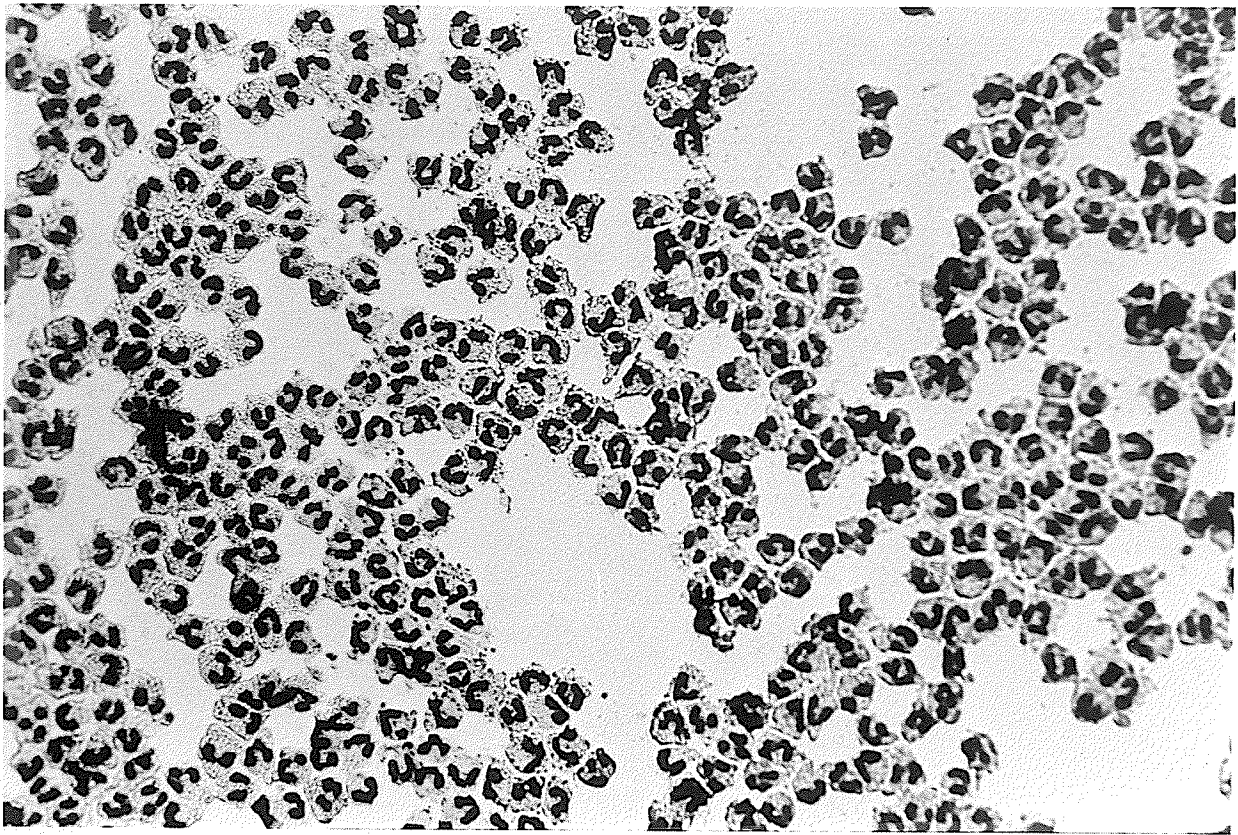
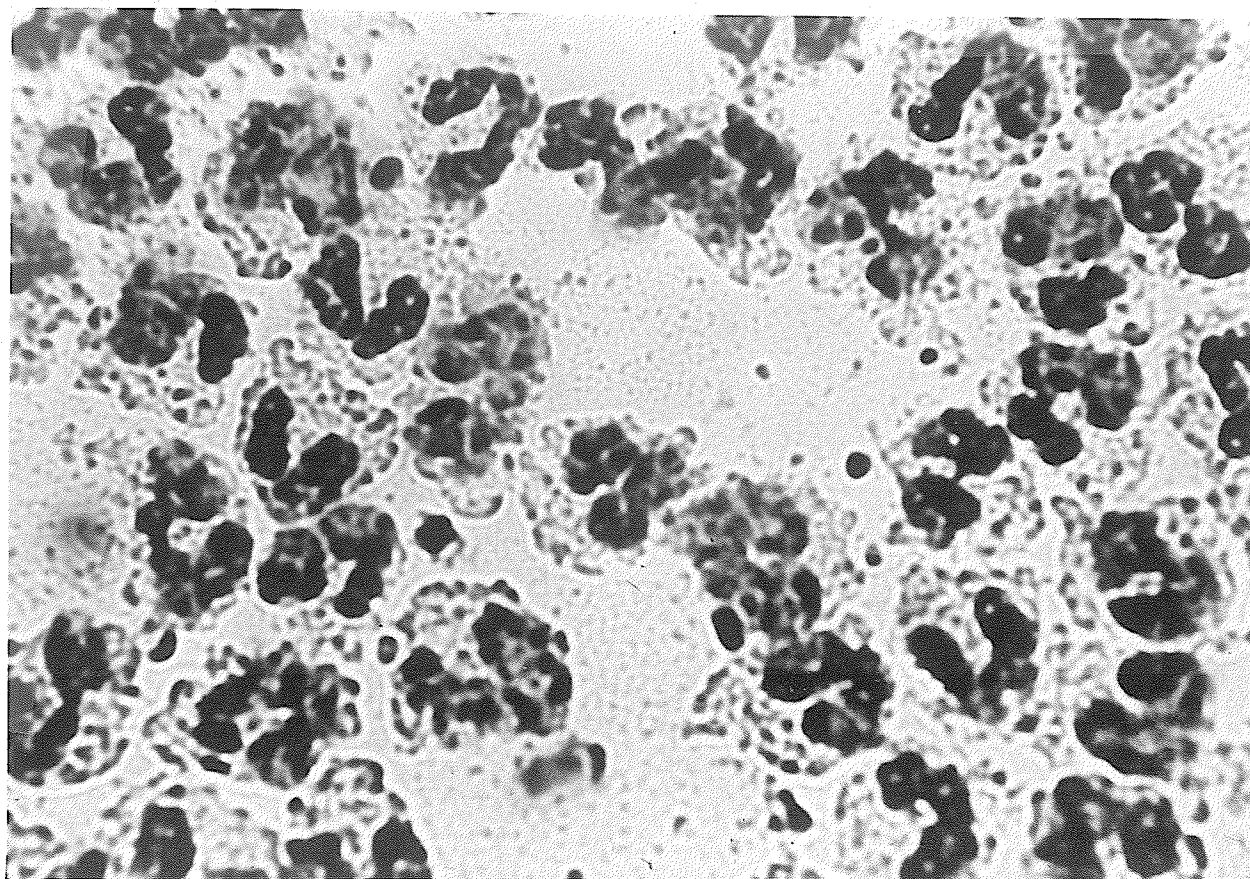




FIGURE 7

MIGRATING CELL POPULATION X1250





Since no significant correlation was found between the individual diameter variation and the calculated migration index ( $r = 0.310$   $p = 0.01$ ) it was possible to estimate the reliability of the calculated migration index (MI). A pooled estimate of the standard deviation (i.e. reliability) of the estimate of the MI for each person was  $\pm 0.05$ .

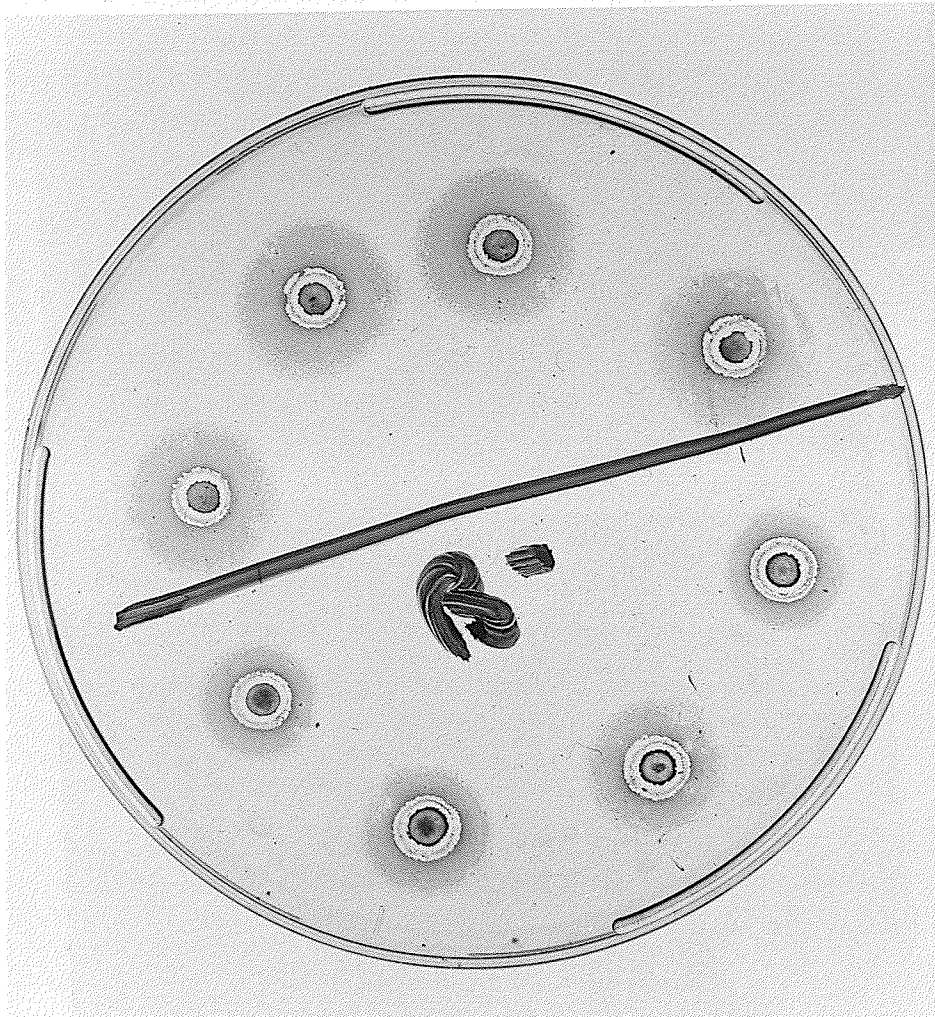
The cellular immune response indicated by the granulocyte-MIF procedure

The agarose leukocyte inhibition test was used to determine the specificity of the immune response to gonococcal protein antigen ( $B^{-t}$ ). The migration indices obtained for the 72 control subjects and the 89 patients with gonococcal infections are summarized in Figure 9.

Group I, which consists of 72 controls, had a mean migration index of 0.91. Group II includes the MI obtained from 76 patients with acute, uncomplicated gonorrhoea, at the time of their initial visit to the clinic. Forty-two of these patients had a history of at least one gonococcal infection, and thirteen had a history of two or more previous infections. The duration of symptoms before the blood was drawn varied from one to seven days with a mean of 2.6 days' duration. The mean MI for this group was 0.87.

FIGURE 8

LEUKOCYTE MIGRATION INHIBITION TEST SHOWING MIGRATION INHIBITION BY GONOCOCCAL PROTEIN ( $B^{-t}$ ). The cells on the half of the plate labeled  $B^{-}$  have been pre-incubated with gonococcal antigen while the control cells on the other half of the plate were pre-incubated in medium 199 without antigen.



Statistical analysis of the migration indices (Chi square analysis) revealed that a significantly greater number of patients demonstrated migration inhibition less than 0.79 than control subjects ( $\chi^2 = 6.71$   $p < 0.01$ ). For the purposes of discussion of these results,  $MI \leq .78$  was considered to indicate inhibition.

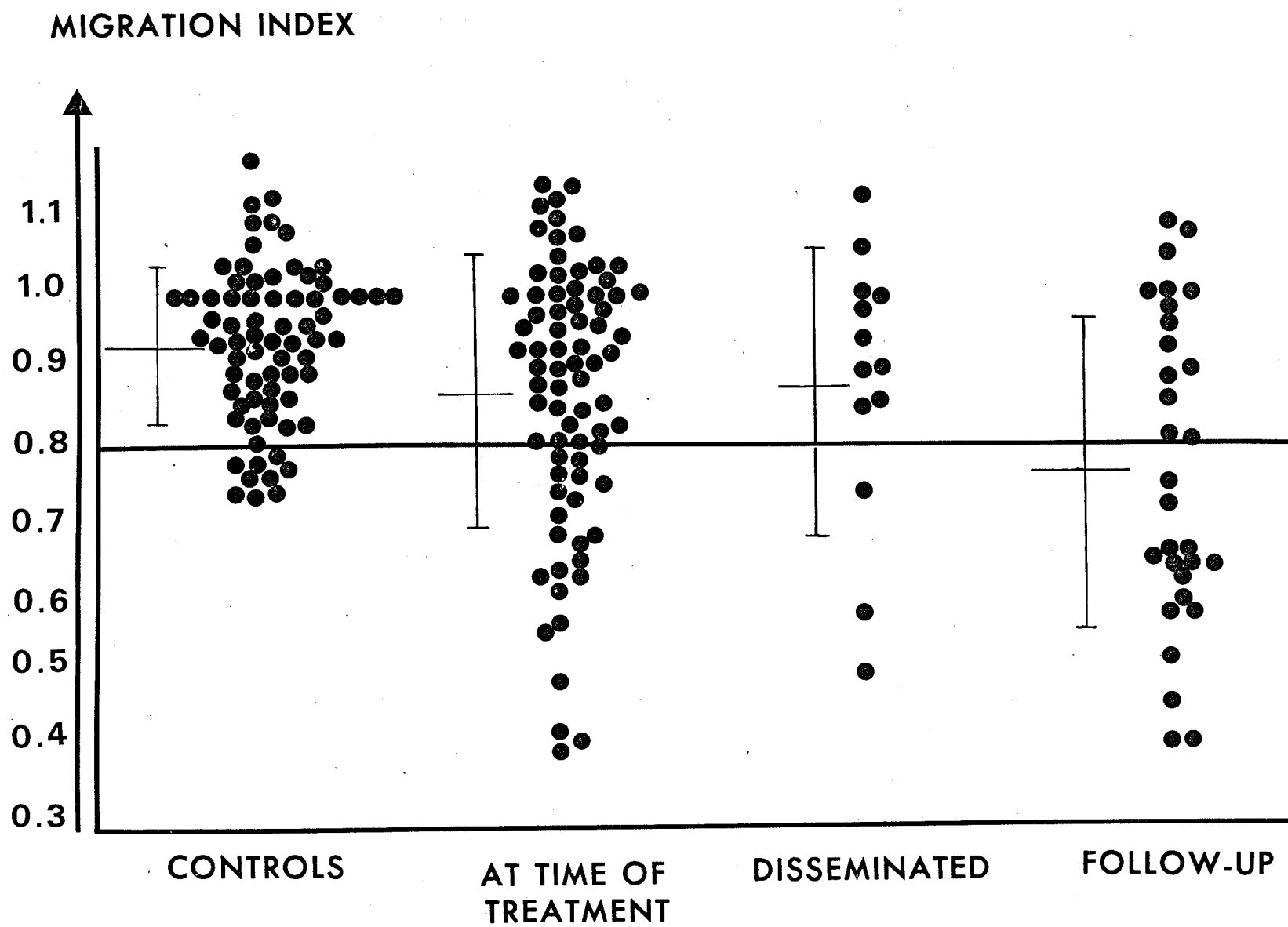
Seven of the controls had MI less than .78 but all of the indices were  $\gg .74$ . Twenty-one patients demonstrated inhibition greater than 0.78 and sixteen of those patients had migration indices which were lower than any of the control subjects.

The leukocyte migration inhibition results for fourteen patients with disseminated gonococcal infection is presented in Group III (Figure 9). Neisseria gonorrhoeae was isolated from the blood of the two patients, and the synovial fluid of an additional two patients. The remaining ten patients all had positive cervical or urethral and/or rectal cultures with joint symptoms. Three patients had typical skin lesions and all responded favourably to penicillin. The mean migration index for this group was 0.87, and three patients had migration indices of less than 0.78

While marked migration inhibition occurred in some instances, the majority of the patients were unresponsive

FIGURE 9

LEUKOCYTE MIGRATION INDICES. Group 1 consists of 72 control subjects, group 2 consists of 76 patients with acute gonococcal infection, group 3 includes 14 patients with disseminated gonococcal infection and the fourth group are 30 patients with acute gonococcal infection one week after antibiotic therapy.



to the gonococcal antigen. There was not a significantly greater degree of inhibition in the total gonorrhoea group than in the non-gonorrhoea controls (Student's t-test,  $p > 0.01$ ). Failure to detect leukocyte inhibition factor may have been due to the short infection period, which, in some patients, was not sufficient time for sensitized leukocytes to accumulate to a level that could be detected. In order to test this hypothesis, thirty-two patients with uncomplicated gonorrhoea had blood drawn one week after treatment (seven to ten days following the onset of symptoms).

The mean migration index for this group was 0.88, and sixteen of the thirty patients had MI less than 0.78. Statistical analysis (Student's t-test) of the MI revealed a significantly greater degree of inhibition in the total gonorrhoea group than in the non-gonorrhoea controls ( $p < 0.01$ ).

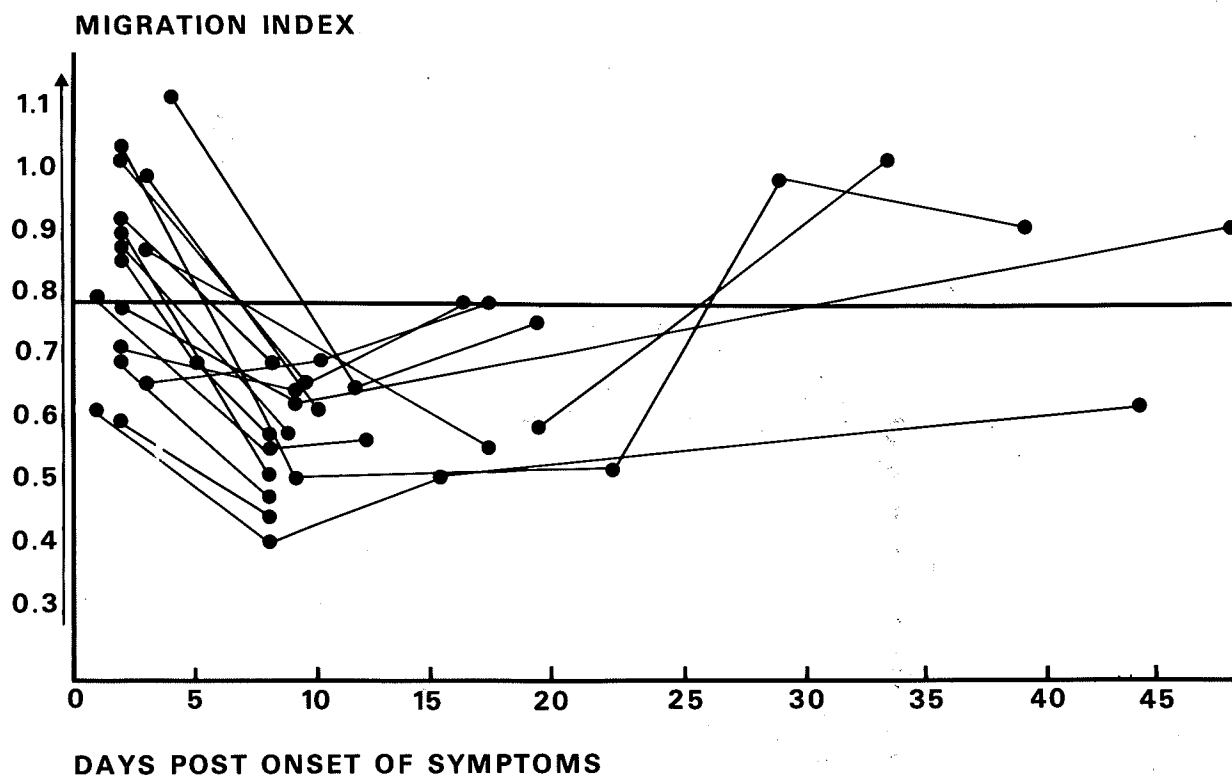
#### Time course of the development of the CMI response

Patients in whom an immune response was detected were examined for the time relatedness of that response (Figure 10). Maximum inhibition was observed seven to ten days after the onset of symptoms. Two patients who demonstrated inhibition at three weeks were unresponsive to the gonococcal antigen four weeks after the onset of symptoms. One patient who demonstrated inhibition forty-eight hours after the onset of symptoms, continued to show migration inhibition for the six



FIGURE 10

TIME COURSE OF THE DEVELOPMENT OF CMI RESPONSE



weeks that he participated in the study. This patient had a history of eight previous infections.

Significant factors in the development of the CMI response. Several factors, which included a history of previous gonococcal infection, a history of two or more previous gonococcal infections, and males versus females, were examined for significance of correlation with the development of a cell mediated immune response following infection with N. gonorrhoeae. These results are summarized in Table 5. A history of two or more episodes of gonococcal infection was found to correlate with the development of a measurable leukocyte inhibition factor ( $\chi^2 = 7.39$   $p < 0.01$ ).

"Early" migration inhibition. The area of cell migration in control wells, that is cells not preincubated with antigen, was calculated after four hours, eighteen hours, and twenty-four hours incubation for eighteen patients and thirty-two non-gonorrhoea control subjects. Almost 80% of the migration occurs in the first four hours of incubation and is complete at eighteen hours (Figure 11).

Migration indices for the thirty-three control subjects and eighteen patients with gonococcal infection were calculated after four and eighteen hours of incubation. "Early" migration inhibition was defined as a migration index of  $< .78$  at 4 hours and  $> .78$  at 18 hours with a difference  $> .05$  between the 4 and 18 hour index.

TABLE 5  
FACTORS EXAMINED FOR ASSOCIATION WITH MIGRATION INHIBITION

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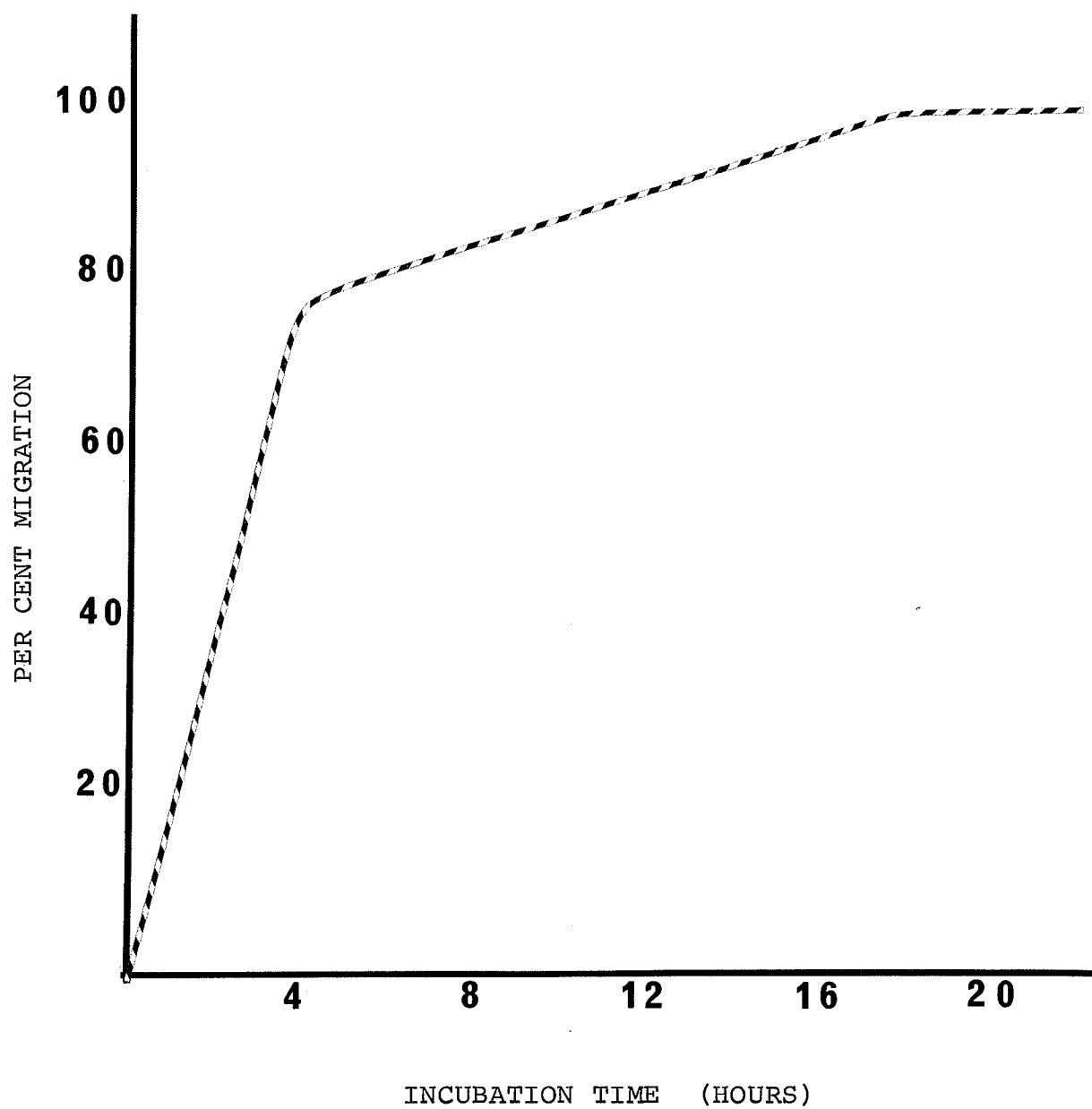
1.	Males	30/89*		
	Females	9/19	$p > 0.10$	
2.	History of previous gonococcal infection	24/56		
	No history of previous gonococcal infection	10/37	$p > 0.10$	
3.	History of 2 previous infections	13/20		
	History of > 2 previous infections	21/73	$p < 0.01$	

---

\* number with migration inhibition/total number examined

FIGURE 11

TIME COURSE OF LEUKOCYTE MIGRATION



Nine patients demonstrated migration inhibition after four and eighteen hours. "Early" migration inhibition was demonstrated in five patients, while none of the patients had migration inhibition after eighteen hours that was not also apparent at four hours (Table 6).

Of the thirty-three controls tested, none had migration inhibition at four or eighteen hours (Table 7).

The development of anti-pili antibody and the CMI response. Seventy-four sera specimens were collected from forty-five patients who had a granulocyte-MIF assay performed the same day.

Specific antibody to gonococcal pili was determined by a radio-immune method, and those results were compared with the migration indices. Specific antibody level of 1 ug/ml and migration index of  $\leq 0.78$  were considered significant for anti-pili antibody and LIF factor respectively.

There was no correlation of the development of significant levels of anti-pili antibody with the development of measurable LIF (Figure 12;  $r = 0.068$   $p > 0.10$ ).

TABLE 6

## MIGRATION INDICES AFTER FOUR AND EIGHTEEN HOURS INCUBATION

Patient	<u>At time of treatment</u>		<u>1 week post-treatment</u>	
	4 hr.	18 hr.	4 hr.	18 hr.
1.	.71*	.96	.80	.96
2.	.91	1.03		
3.	.82	.80		
4.	.71**	.64		
5.	.73**	.68		
6.	1.00	1.08		
7.	.74	.79	.75**	.64
8.	.67**	.67	.76**	.71
9.	1.00	1.14	.72**	.66
10.	1.00	1.13	.72**	.66
11.	.74*	.91		
12.	.59*	1.09	.95	.81
13.	.56**	.63		
14.	1.00	1.13		
15.	.70*	.81		
16.	.63**	.50		
17.	.90	1.03		
18.	1.00	1.00	.71*	.78

\* "early" migration inhibition

\*\* migration inhibition



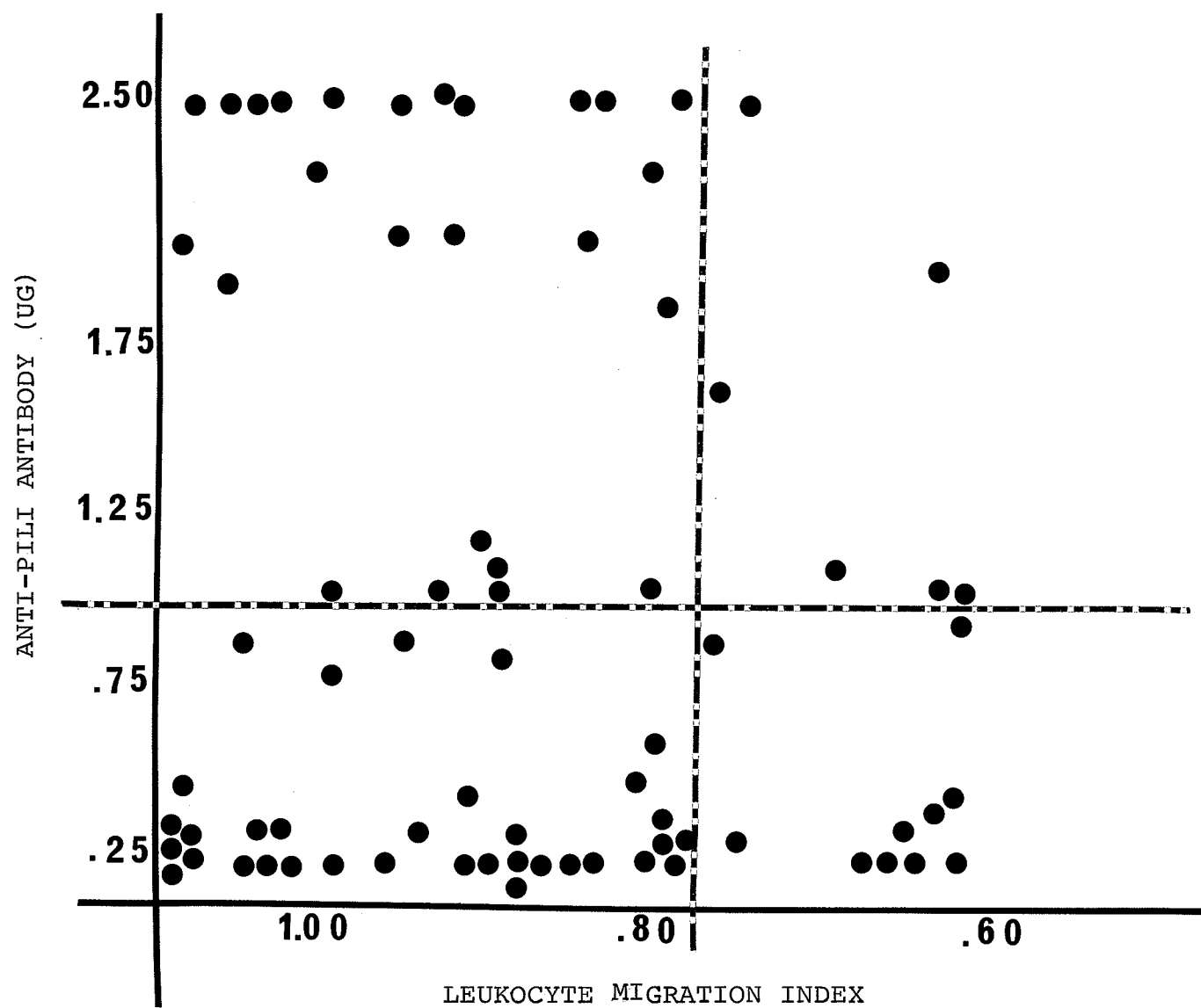
TABLE 7

## MIGRATION INDICES AFTER FOUR AND EIGHTEEN HOURS INCUBATION

Control	4 hr.	18 hr.	Control	4 hr.	18 hr.
1.	.87	.92	2.	.90	.95
3.	.96	.92	4.	1.04	1.04
5.	.94	.96	6.	1.00	1.09
7.	1.00	1.00	8.	1.13	1.18
9.	1.00	1.02	10.	1.00	1.00
11.	1.02	1.00	12.	.88	.96
13.	1.04	1.10	14.	.90	.83
15.	1.00	1.00	16.	1.09	1.03
17.	1.10	1.00	18.	1.00	1.00
19.	1.00	.98	20.	1.04	.83
21.	1.00	.88	22.	1.20	1.13
23.	1.00	.92	24.	1.00	1.04
25.	.85	.87	26.	.95	.95
27.	1.05	.96	28.	1.09	1.00
29.	.91	1.02	30.	1.00	1.00
31.	.86	.84	32.	1.00	.95
33.	1.11	.91			

FIGURE 12

THE RELATIONSHIP OF ANTI-PILI ANTIBODY LEVELS AND MEASURABLE  
LEUKOCYTE INHIBITION FACTOR.



## PENICILLIN SUSCEPTIBILITY OF NEISSERIA GONORRHOEAE ISOLATES




Strains of N. gonorrhoeae causing disseminated infection have been reported to be more sensitive to penicillin G than strains isolated from patients with uncomplicated infections (136,62). This suggests that DGI may be a result of infection with a unique population of gonococci. If this is true for the population under study, it may be reflected in their ability to induce a CMI response or in our ability to detect that response.

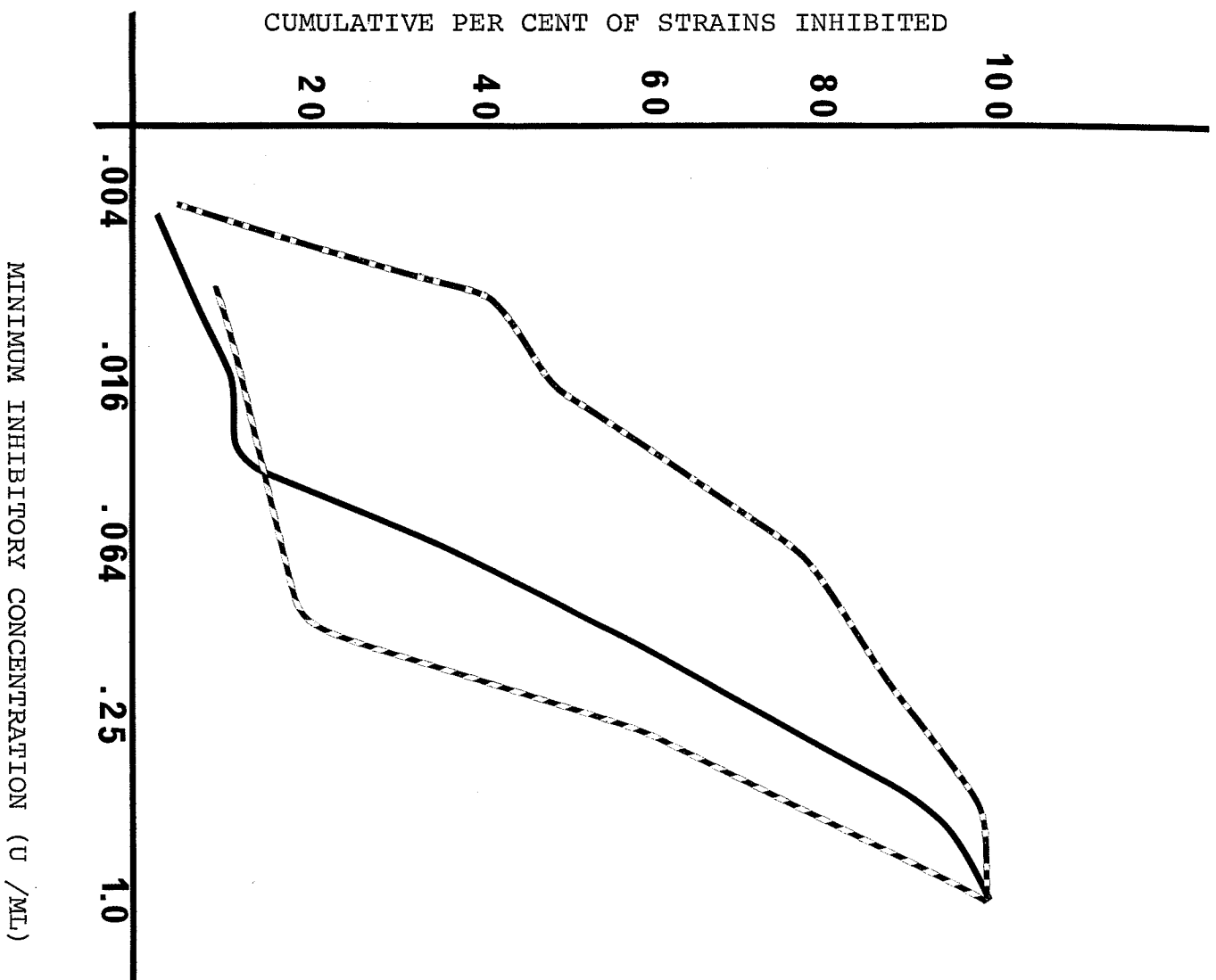
The MIC of penicillin was determined for 100 consecutive isolates from patients with acute uncomplicated gonorrhoeae. Approximately 50 per cent of the isolates were inhibited by 0.125u/ml of penicillin but six per cent of the isolates had an MIC of 1.0 u/ml (Figure 13).

The MIC of penicillin for the ten strains isolated from patients with DGI were determined. In contrast with recent reports in the literature, (62), they appear to have the same distribution of MIC's as organisms isolated from patients with acute uncomplicated infection. However, the fourteen isolates with the auxotype arg- hyx- ura- isol- were found to be more sensitive to penicillin than other strains. Eleven had MIC's  $\leq$  0.063 and all were inhibited by  $\leq$  0.5 u/ml penicillin. Of the fourteen strains

FIGURE 13

PENICILLIN SUSCEPTIBILITY PATTERN.

-  100 strains isolated from patients with acute uncomplicated gonococcal infection
-  10 strains isolated from patients with disseminated gonococcal infection
-  14 strains with auxotype  $\text{arg}^-$ ,  $\text{hyx}^-$ ,  $\text{ura}^-$ ,  $\text{iso}^-$ .



tested two were isolated from patients with DGI.

#### NUTRITIONAL CHARACTERIZATION OF *N. GONORRHOEAE*



It has been proposed that gonococci with unique nutritional requirements possess increased capacity to produce DGI (62). It was important to the total view of the immunological response to determine whether the organisms isolated from patients in the present investigation with DGI represent a unique group of organisms.

Auxotyping was kindly performed by Dr. Frank Young. The results of the nutritional requirements of eighty-nine strains isolated from patients with uncomplicated gonococcal infection and ten strains isolated from nine patients with DGI are presented in Figure 14.

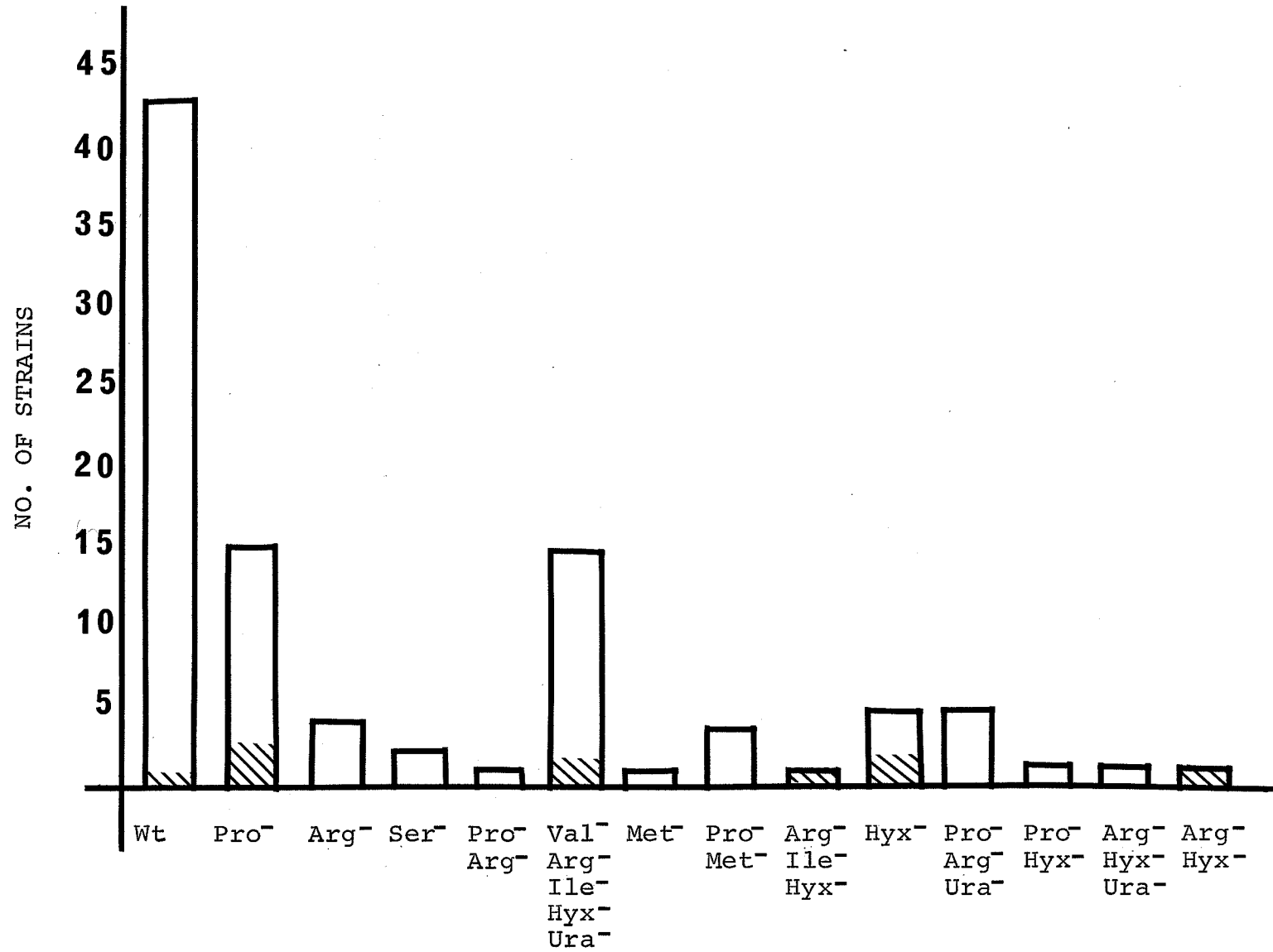
The organisms were tested for their requirement for proline, arginine, serine, valine, isoleucine, hypoxanthine, uracil and methionine. Strains which were prototrophic for all of the above amino acids (wt) represent almost half of the isolates. The strains isolated from DGI cases were nutritionally more dependent. The single isolate of a "wt" strain from a patient with DGI was from an anal culture. This patient, in addition, had a proline-requiring strain isolated from the same culture. Whether or not the "wt" strain (or the pro-strain) was actually the disseminated

FIGURE 14

AUXOTYPE OF 99 STRAINS OF NEISSERIA GONORRHOEAE.

-  strains isolated from patients with disseminated gonococcal infection (10 strains from 9 patients).
-  strains isolated from patients with acute uncomplicated gonococcal infection
- Wt are strains which grow on the basal medium without the addition of proline, arginine, serine, valine, isoleucine, hypoxanthine, uracil or methianine.





strain has not been demonstrated.

The data would support the conclusion that DGI associated strains are nutritionally more dependent than are those associated with uncomplicated infections.

(Fisher's Exact Test for 2x2 tables  $p=0.02$ )

The majority of DGI associated strains isolated in Seattle belonged to the auxotype designated as 11 and 14 by Carifo and Catlin (13,15). The strains in Winnipeg, while nutritionally dependent, represent a larger number of different auxotypes. The nine strains are included in auxotypes 2, 14, 13 and a Hypoxanthine-requiring strain.

Granulocyte-MIF results were available for only three of the patients whose organisms were auxotyped. None of these patients demonstrated migration inhibition. Migration inhibition was demonstrated in three patients at the time of treatment and in a fourth patient two weeks after successful therapy. The organisms from these patients were not available for auxotyping.

#### ANIMAL MODEL EXPERIMENTS

##### Development of an immune response as measured by dermal sensitivity

A group of thirty-eight guinea pigs were observed for the development of dermal hypersensitivity following immunization with cytoplasm-CFA or chamber infection with

N. gonorrhoeae.

The guinea pigs were immunized with F62 T1 cytoplasm and all infecting N. gonorrhoeae were F62 T1. The control group consisted of six animals without immunization or prior infection. No dermal response (erythema or edema) was noted in any of these animals.

Eight guinea pigs were immunized with CFA-cytoplasm and skin tested fifty-five days post immunization. No induration or erythema was apparent at four hours, however a strong dermal reaction was elicited in all the immunized animals at twenty-four hours which persisted for seventy-two hours (Table 8).

The immunized animals were subsequently infected with N. gonorrhoeae. The chambers were cultured at weekly intervals. The last date on which the culture of any chamber fluid was positive was used to calculate the total number of days the chamber was infected. Chambers were infected for fourteen to fifty days. Some erythema and edema was noted at four hours in three of the seven animals (Table 9) following intradermal injection of gonococcal antigen.

All of the animals demonstrated significant dermal response at twenty-four hours which was somewhat increased at forty-eight hours (Table 10).

The six animals with chamber infections without

TABLE 8  
DERMAL SENSITIVITY FOLLOWING IMMUNIZATION WITH GONOCOCCAL  
CYTOPLASM

Animal *	Diameter of induration		
	24 hrs.	48 hrs.	72 hrs.
5	15 **	15	15
5E	25	25	15
6	15	15	15
6E	20	20	20
7	15	15	15
7E	15	15	15
8	15	15	15
8E	15	15	15

\* the cytoplasmic gonococcal material used for immunization was also used for skin testing. Each of the eight guinea pigs was injected subcutaneously with 10 ug protein/ml

\*\* the diameter of induration was measured in two directions and recorded in mm.

TABLE 9

DERMAL SENSITIVITY FOLLOWING IMMUNIZATION AND INFECTION  
WITH N. GONORRHOEAE

Animal *	Days Infected	4 hr. reaction at 10 and 20 ug site
8	50	no erythema; edema approx. 5 mm at the 20 ug site
8E	21	Negative
7	21	5 mm erythema and edema at both sites
7E	50	no erythema; edema 5 mm at 20 ug site
6	21	Negative
6E	14	Negative
5	50	Negative

\* the cytoplasmic gonococcal material used for immunization was also used for skin testing. Each of the seven guinea pigs was injected subcutaneously with 10 ug protein/ml.

TABLE 10

DERMAL SENSITIVITY FOLLOWING IMMUNIZATION AND INFECTION WITH N. GONORRHOEAE

Animal *	24 hrs. 10 ugm	20 ugm	48 hrs. 10 ugm	20 ugm
8	6**	10	7	7
8E	8	11	10	10
7	10	13	10	10
7E	9	12	10	12
6	7	12	13	13
6E	7	12	10	12
5	15	15	13	13

\* the cytoplasmic gonococcal material used for immunization was also used for skin testing. Each of the seven guinea pigs was injected subcutaneously with 10 ug protein/ml.

\*\* the diameter of induration was measured in two directions and recorded in mm.

prior immunization had minimal or negative skin response to the gonococcal cytoplasm (Table 11). It is evident that immunization with cytoplasm-CFA induces a CMI response in the guinea pig as indicated by the dermal sensitivity. Chamber infection with N. gonorrhoeae, on the other hand, produced little or no CMI response.

#### Detection of a humoral response in the subcutaneous chamber fluid

Three weeks after subcutaneous chambers were implanted, there was complete healing of the suture. The embedded chambers became filled with a serous fluid which could be readily aspirated with a needle and syringe (Figure 15). Before an animal was used in the study, a portion of the aspirated fluid was cultured on blood agar to check for sterility. In addition, the fluid was examined for anti-gonococcal antibody using counterimmunoelectrophoresis. A drop of fluid from a number of chambers was placed on a microscope slide, dried, and subsequently stained with Wright's stain to examine for cellular constituents. The aspirate was a clear, straw-colored fluid, acellular in the absence of bacterial contamination. Antibody to gonococcal cytoplasm was not detected in any of the animals prior to infection and/or immunization with N. gonorrhoeae. Three weeks following immunization with cytoplasm-CFA, counterimmunoelectrophoresis of the chamber fluid

TABLE 11

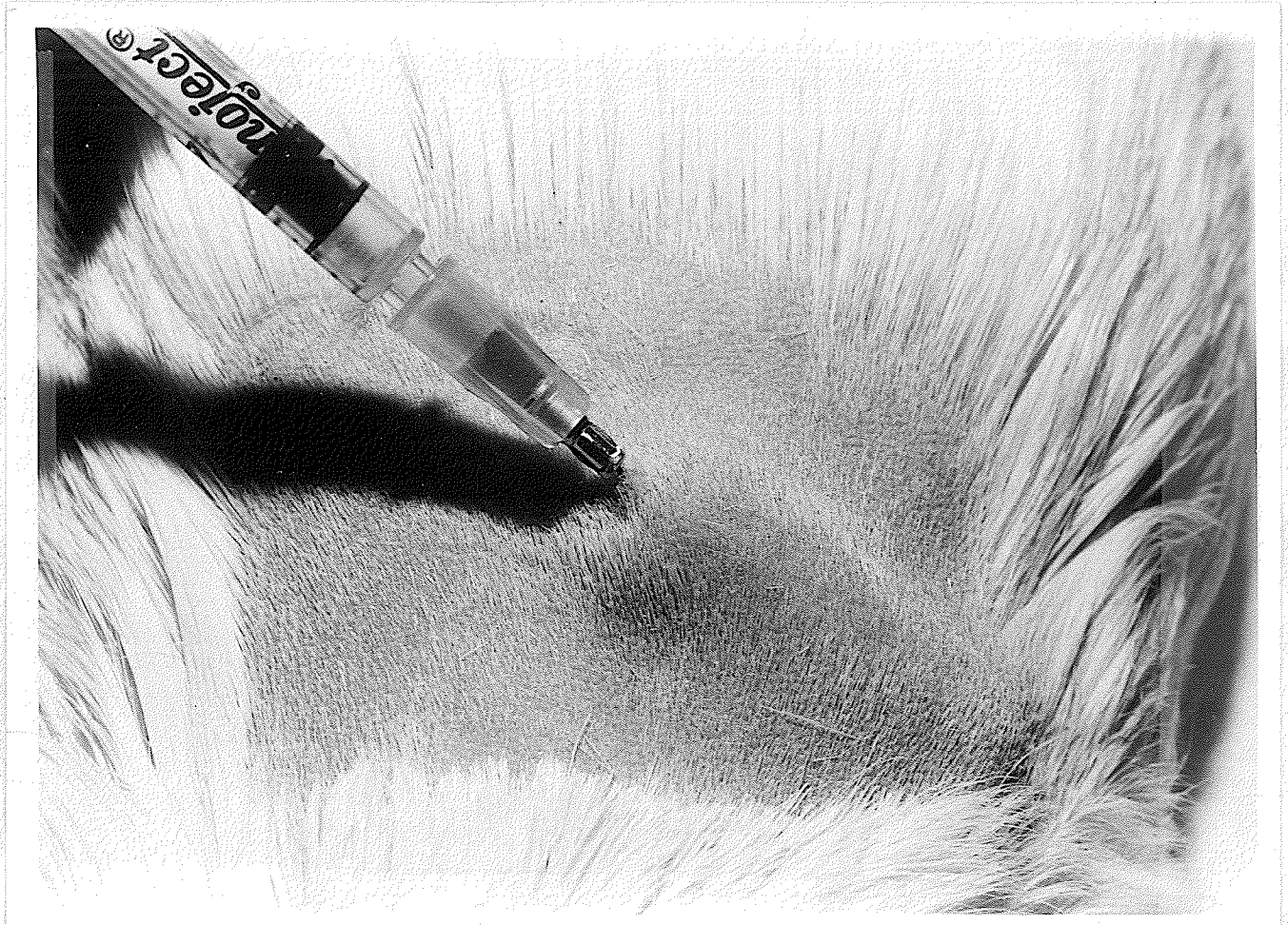
DERMAL SENSITIVITY FOLLOWING INFECTION WITH N. GONORRHOEAE  
WITHOUT PRIOR IMMUNIZATION

Animal	Days Infected	4 hr. reaction at 10 and 20 ugm site	
4	50	no erythema or edema noted at any of the injection sites	
4E	50		
3	14		
2	50		
1	14		
1E	14		
Animal	24 hrs.	10 ugm	20 ugm
4E	negative	less than 5mm/	no erythema
3	negative	less than 5mm/	no erythema
2	negative	less than 5mm/	no erythema
1	negative	less than 5mm/	no erythema
1E	negative	less than 5mm/	no erythema
4	negative	5mm/	no erythema
Animal	48 hrs.	10 ugm	20 ugm
4E	negative	negative	negative
3	less than 5mm/ no erythema	less than 5mm/ no erythema	less than 5mm/ no erythema
2	less than 5mm/ no erythema	less than 5mm/ no erythema	less than 5mm/ no erythema
1	5mm/ no erythema	5mm/ no erythema	5mm/ no erythema
1E	less than 5mm/ no erythema	5mm/ no erythema	5mm/ no erythema
4	negative	negative	negative



FIGURE 15

ASPIRATION OF FLUID FROM THE SUBCUTANEOUS CHAMBER.



against the cytoplasmic material was performed. Anti-gonococcal antibody was detected in all of the immunized animals at this time.

#### Effect of niridazole on the dermal response

Three weeks after immunization, thirty-five guinea pigs were skin tested with gonococcal antigen. Niridazole (100 mgm/ animal), was administered orally to eighteen of the animals and three days later skin testing was repeated.

Niridazole significantly decreased the forty-eight hour dermal response (Table 12).

Counterimmunoelectrophoresis of chamber fluid aspirated at this time indicated the presence of anti-gonococcal antibody.

#### Effect of niridazole on infection with *N. gonorrhoeae*

In order to determine the effect of niridazole on chamber infection with *N. gonorrhoeae*, sixteen guinea pigs were challenged with gonococci. Eight of the animals were given 100 mgm niridazole immediately before the gonococcal challenge. Each chamber was inoculated with  $1 \times 10^3$  organisms. Chamber fluid was aspirated seventy-two hours after the challenge and inoculated onto Thayer-Martin medium and supplemented GC agar.

The results of that challenge are summarized in Table 13. Of the animals treated with niridazole, 50 per

TABLE 12

## EFFECT OF NIRIDAZOLE ON THE DERMAL RESPONSE TO GONOCOCCAL ANTIGEN

Animal	1st intradermal test diameter of induration	Treated**	2nd intradermal test diameter of induration
5E	15 mm	yes	5 mm
5LF	15 mm	yes	5 mm
6E	20 mm		15 mm
6LF	20 mm		20 mm
7E	20 mm	yes	5 mm
7LF	15 mm	yes	5 mm
8E	25 mm		25 mm
8LF	15 mm		20 mm
9E	25 mm		20 mm
9LF	20 mm		20 mm
10E	20 mm		20 mm
10LF	15 mm		20 mm
11E	15 mm		15 mm
11LF	15 mm		20 mm
12E	15 mm	yes	5 mm
12LF	15 mm	yes	5 mm
15E	15 mm		20 mm
15LF	25 mm		20 mm
23E	20 mm	yes	5 mm
23LF	15 mm	yes	ND*
24E	25 mm	yes	ND
24LF	20 mm	yes	10 mm
25E	20 mm		25 mm
25LF	15 mm		15 mm
26E	20 mm		15 mm
26LF	15 mm		15 mm

29E	20 mm	yes	10 mm
29LF	25 mm	yes	died
30E	25 mm		15 mm
30LF	15 mm		20 mm
35E	10 mm		15 mm
35LF	20 mm		15 mm
36E	15 mm		15 mm
36LF	15 mm		15 mm
37E	15 mm		15 mm
37LF	15 mm		20 mm
38E	15 mm	yes	5 mm
38LF	20 mm	yes	5 mm
39E	15 mm	yes	10 mm
39LF	15 mm	yes	5 mm
40E	20 mm	yes	5 mm
40LF	15 mm	yes	5 mm
42E	15 mm		15 mm
42LF	25 mm		15 mm
15LH	25 mm		25 mm

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\*ND Skin test not done

\*\* 100 mgm Niridazole by oral feeding

TABLE 13

## EFFECT OF NIRIDAZOLE ON CHAMBER INFECTION

<u>Treated Group</u>		<u>Untreated Group</u>	
<u>Animal</u>	<u>Infected Chambers</u>	<u>Animal</u>	<u>Infected Chambers</u>
8E	1/2*	7E	2/2
8	2/2	7	1/2
6E	1/2	5E	2/2
6	1/2	5	2/2
4E	1/2	3E	2/2
4	1/2	3	2/2
2E	0/2	1E	2/2
2	2/2	1	2/2
9	0/2	9E	2/2

\* infected chambers / number of chambers inoculated

cent were infected, while 94 per cent of the untreated animals were infected. The number of chambers infected in the untreated animals is significantly greater than the number infected in the treated group ( $\chi^2 = 6.63$   $p < 0.01$ ).

The effect of chamber fluid on the growth of N. gonorrhoeae after oral feeding with niridazole was observed for a seventy-two hour period.

Two animals were given 100 mgm of niridazole. The chamber fluid was aspirated at the time of treatment and every four hours for the first twelve hours and every twelve hours thereafter.

The aspirates were then assayed for their inhibitory effect on the growth of N. gonorrhoeae. The results of this assay are presented in Table 14. No inhibition of growth was apparent at the time of treatment. Inhibition was noted, two, three and seventeen hours after treatment, but no inhibition occurred with the fluid aspirated twenty-four, forty-eight, and seventy-two hours after the niridazole was given.

#### Minimum inhibitory concentration of niridazole for N. gonorrhoeae

The minimum inhibitory concentration (MIC) of niridazole was determined for 100 isolates of N. gonorrhoeae. The MIC was 0.008 to 8 ug/ml (mean 0.259) (Figure 16).

TABLE 14

INHIBITORY EFFECT OF CHAMBER FLUID AFTER NIRIDAZOLE  
TREATMENT

Time of fluid aspiration	Zone of inhibition
At the time of treatment	no inhibition
Post treatment (hours)	
1	no inhibition
2	10 mm
3	25 mm
17	8 mm
24	no inhibition
48	no inhibition
72	no inhibition

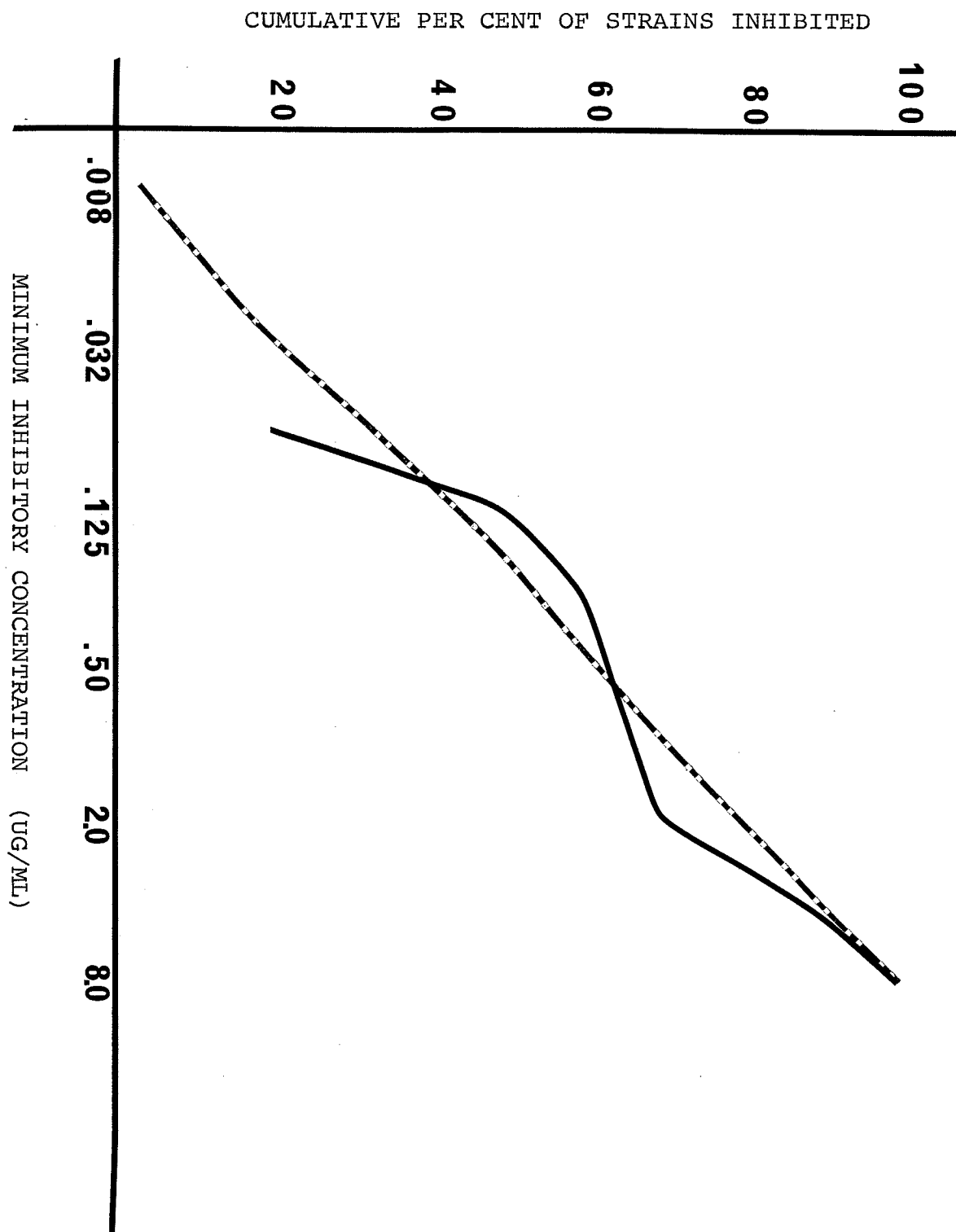


FIGURE 16

SUSCEPTIBILITY PATTERN OF NEISSERIA GONORRHOEAE TO NIRIDAZOLE

----- 90 strains isolated from patients with acute uncomplicated gonococcal infection.

———— 10 strains isolated from patients with disseminated gonococcal infection.



There was no difference observed in the MIC's of niridazole for DGI associated strains.

There was no correlation of the niridazole MIC with the penicillin MIC (Figure 17) ( $r = 0.153$   $p > 0.05$ ).

Effect of niridazole treatment on chamber infection seventy-two hours post therapy

Depression of the CMI response by niridazole was evident seventy-two hours post therapy, as determined by dermal hypersensitivity response. However, inhibition of growth of N. gonorrhoeae by chamber fluid was not evident at this time. It was felt that these two events were separable by time, when challenge experiments were carried out seventy-two hours after treatment with Niridazole.

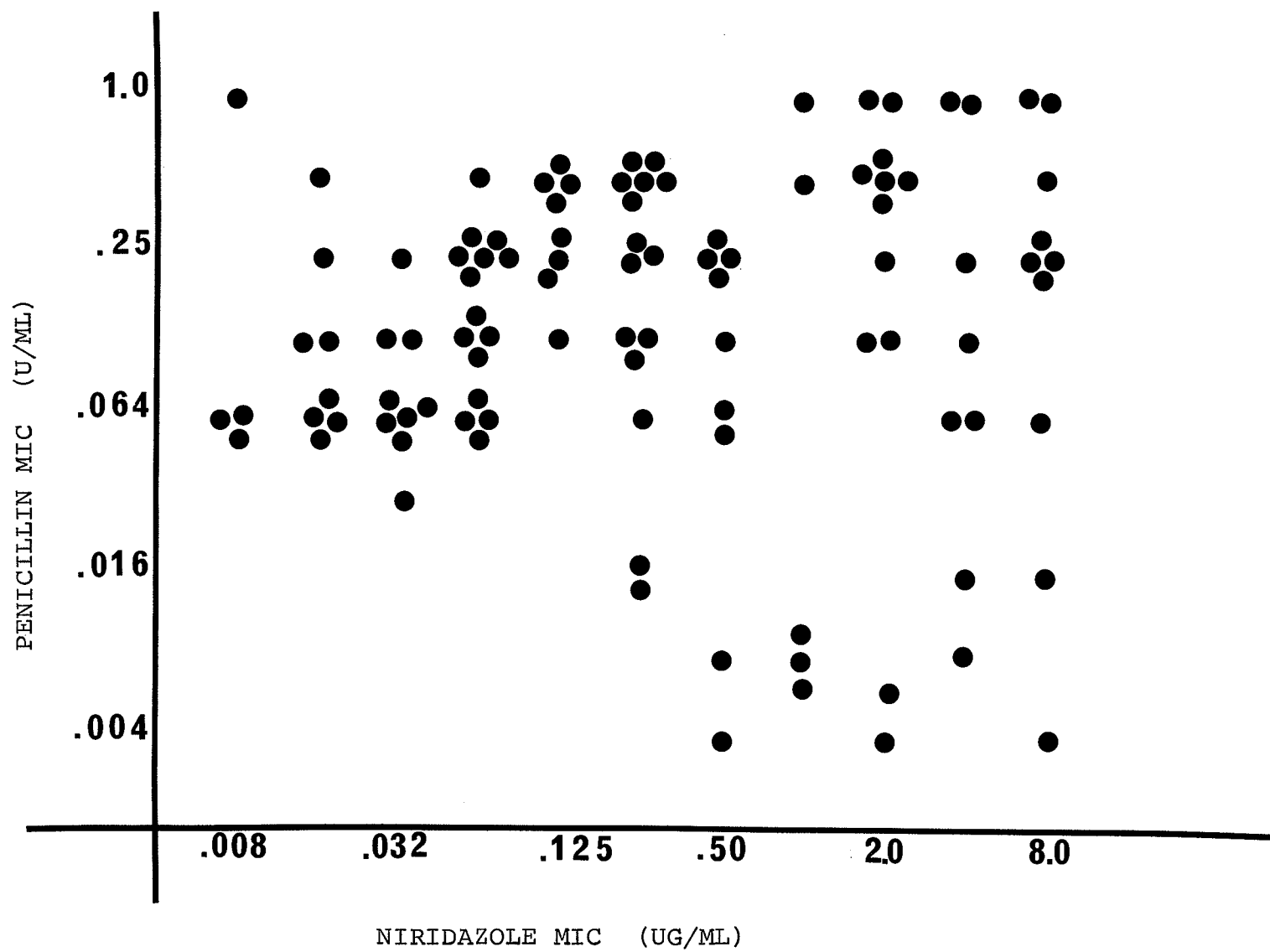
The last phase of the animal infection model experiments was to determine the effect of niridazole induced CMI depression on the infectivity rate.

Half of the study group which consisted of 100 guinea pigs were immunized with CFA-cytoplasm as previously described. Chambers were implanted, after complete healing of the suture and sterility of the chamber had been determined, twenty-five of the immunized and twenty-five of the non-immunized animals were given Niridazole. Each of the treated animals received 100 mgm of Niridazole.

Dilutions of F62 T1 gonococci were prepared so that the final inoculum would contain  $1 \times 10^3$  cells / 0.2 ml.

FIGURE 17

SCATTER DIAGRAM OF THE MIC OF PENICILLIN AND NIRIDAZOLE FOR  
102 STRAINS OF NEISSERIA GONORRHOEAE.



Counts were made of the inoculum to determine the actual inoculum size. Chambers inoculated with  $5 \times 10^2$  -  $1 \times 10^3$  CFU/chamber were included in the final tabulation.

Chambers were inoculated seventy-two hours after the administration of Niridazole. The aspirate was cultured at seventy-two hours and at one week following the challenge. The isolation of N. gonorrhoeae from either sample was considered a positive result.

The culture results obtained from the four study groups (Table 15) were subjected to statistical analysis. The infection rate in the total immunized group was compared to the infection rate in the non-immunized group ( $\chi^2 = 4.43$   $0.025 < p < 0.05$ ). When the infection rate of the immune group was compared to the infection rate of the non-immune treated group the result was similar ( $\chi^2 = 4.51$   $0.025 < p < 0.05$ ). The infection rate for the four groups was analysed with a four by two Chi square test ( $\chi^2 = 6.10$   $p > 0.10$ ).

Our hypothesis was that CMI is a significant component in protection of the host from gonococcal infection. Therefore, depression of the CMI response by Niridazole would increase the chamber infection rate. Using this as our basic assumption, the groups of animals could be ranked from most resistant to challenge to the most susceptible to gonococcal challenge. Our rank order

TABLE 15

EFFECT OF NIRIDAZOLE ON CHAMBER INFECTION 72 HOURS AFTER  
TREATMENT

Group		Infected/ Total *	% Infected
I	Immune	43/75	57
II	Immune plus niridazole	38/59	64
III	Non-immune	67/95	71
IV	Non-immune plus niridazole	57/76	75

\* the number of chambers infected/the total number  
of chambers challenged with N. gonorrhoeae

was (Table 15) immune, immune plus Niridazole, non-immune, and non-immune plus Niridazole.

The Trend Test (81) was then used to test the hypothesis of the a priori ranking (Chi-square test with one degree of freedom). We can assume the hypothesis to be correct with 97.5 per cent confidence (Chi square 6.03  $0.01 > p > 0.025$ ). In addition, the difference between the four and two chi-square (Chi-square 6.10 3DF) and the Trend Test (Chi-square T-6.03 1DF) represents the amount of deviation from a straight line relationship. The per cent of chambers infected increases in an almost linear fashion from the lowest to the highest.



## CHAPTER V

## DISCUSSION

The major portion of this investigation has been concerned with the development of a system to measure the cellular immune response of the host to gonococcal infection. The antigen used in such a system is, of course, crucial to the sensitivity and specificity of the assay. This was most apparent in the comparison of the blastogenic response obtained from assays using gonococcal cytoplasm, and the  $\alpha$  and B fractions separated from that cytoplasm as the stimulating antigen. Clearly, the cytoplasm contains a "non-specific" mitogen which was concentrated in the  $\alpha$  fraction by isoelectric focusing.

"Non-specific" mitogens transform from 60 to 90% of lymphocytes from all normal individuals independent of immunization. Other stimulants, classified as antigens, stimulate lymphocytes only from sensitized donors. Antigens usually activate only a small clone of the previously sensitized lymphocyte population, and even after five to seven days' incubation and repeated cell division, only 5 to 35% of the lymphocytes will be transformed (44). The high stimulation indices, and the non-specificity of the reaction in response to the  $\alpha$  fraction are consistent with that of a non-specific mitogen.

Lipopolysaccharides are known to be mitogenic; presumably an interaction between the lipid A portion of the lipopolysaccharide and the lymphocyte cell membrane

induces blastogenesis (53,87,88). The presence of KDO in the  $\alpha$  fraction suggests this may be the mechanism for the response of the control subjects.

Using the B fraction as the stimulating antigen, the majority of the controls and 54% of the patients had a blastogenic response  $>2$ . This lack of specificity could be a result of low levels of the same non-specific mitogenic material present in the  $\alpha$  fraction or some other cross reacting antigens associated with the CMI. The heterogeneity of the protein components of this antigen, (twenty-five bands were resolved by gel-electrophoresis), increases the possibility of cross reacting antigens. This is supported by the observation of reactive-delayed skin tests with gonococcal antigens in patients with meningococcal meningitis (52). Cross antigenicity, determined by blastogenic response of N. catarrhalis, an organism normally present in the upper respiratory tract and N. gonorrhoeae, has also been demonstrated (37).

With cytoplasm as the inducing antigen, two of the patients and two of the controls have stimulation indices  $>2$ . Although only a small number of patients and controls were tested, considering the mitogenic activity of the  $\alpha$  fraction, and the lack of specificity of the B fraction, the cytoplasm would be unlikely to be appropriate antigenic material.

Kraus et al (65), using disrupted gonococcal cells as the stimulating antigen, was unable to distinguish patients with a single episode of gonorrhoea from the controls. Using F62 T1 sonicate, Kearns et al (58), found little difference between patient and control blastogenic responses. A third report of blastogenic response to gonococcal cytoplasm (46) suggested that while blastogenic response was apparent in the controls, the stimulation indices were higher in the gonorrhoea subjects. No attempt was made to determine whether, in fact, the difference was statistically significant. One or two additional points need clarification. Grimble and McIllmurray (46) reported that one of the patients with a stimulation index of 99.4 to a gonococcal preparation, had no response to PHA which presumably was being used as a control of the system. In addition, the history of one of the gonorrhoea subjects stated, "said by G.P. [General Practitioner] to have salpingitis; no clinical evidence; no bacterial evidence of gonorrhoea". One would need to question the inclusion of these results to support the conclusion that the gonorrhoea group had higher stimulation indices than the controls in response to gonococcal cytoplasm.

The lymphocyte transformation results obtained with the B<sup>-t</sup> fraction indicated that this may be a more appropriate antigen to use in the study of the development of

specific CMI response to gonococcal infection. In a small trial, six of twenty-three patients tested were shown to have a blastogenic response at the time of treatment (Figure 3). The development of a blastogenic response to  $B^{-t}$  one week following therapy, that was not present at the time of treatment, was also demonstrated in one additional patient (Table 4).

Dissociation of cutaneous delayed hypersensitivity, in vivo mediator production, and lymphocyte transformation, has been reported (33,101,102,103). Some antigen components may induce only lymphocyte transformation, whereas others induce only mediator production (110,5).

The next phase of the research was concerned with the ability of  $B^{-t}$  to induce mediator production as well as lymphocyte transformation. The agarose technique was used in a direct method to assay for granulocyte-MIF. The antigen sensitive cell in the leukocyte migration inhibition test is the lymphocyte, whereas the polymorph is merely an indicator cell which migrates (22,23,25,26).

Fixation and staining of the migrating cells indicated that they are predominantly neutrophils. This is consistent with the findings of the only other report in the literature (36). Erard reported that eosinophils could not be seen further than 30 per cent of the maximum migration distance. Lymphomonocytes could be seen as far

as 80 per cent of the maximum distance but their proportion was very low.

The movement of the cells has been shown to be a true migration and not simply mechanical diffusion between the agar and plastic surface. When erythrocyte suspensions were placed in the well there were no erythrocytes seen beneath the agarose gel after twenty-four hours incubation. This indicates that the spreading of the leukocytes beneath the agarose is caused by cell migration and not diffusion of the cells. Also, when leukocytes which migrate normally at 35°C were placed in agarose plates and incubated at 4°C, no migration occurred.

The results of this investigation, with a large number of patients with gonococcal infection, have indicated that the B<sup>-t</sup> fraction does induce the production of leukocyte-MIF. Migration inhibition was demonstrated at the time of treatment in twenty-two of the seventy-six patients with acute gonococcal infection (Figure 9). Sixteen of these patients demonstrated greater inhibition than any of the controls. In spite of the presence of significant inhibition in some of the patients, the majority of them remained unresponsive to the gonococcal antigen. One plausible explanation for this may be that the pool of circulating sensitized lymphocytes was small, particularly early in the course of infection. In order to test this hypothesis,

granulocyte-MIF assays were performed at weekly intervals following the initial diagnosis and treatment. We were able to demonstrate, as predicted, that more of the patients were producing measurable amounts of granulocyte-MIF at seven to ten days after the onset of symptoms than initially at the time of treatment. It is proposed that the curves presented in Figure 10 represent the time required, from onset of infection, to develop a sufficient pool of circulating sensitized lymphocytes to produce a measurable amount of leukocyte-MIF in response to the B<sup>-t</sup> antigen. Maximum inhibition is shown to occur seven to fourteen days after the appearance of symptoms. The duration of the response will again be related to the total number of sensitized lymphocytes that are circulating, and whether or not reinfection occurs.

The results would indicate that the mediator may be detectable up to four weeks after therapy. The long duration of measurable MIF for one patient may be attributed to the history of multiple episodes of gonococcal infection.

The only other study of the duration of CMI response following gonococcal infection reported a loss of blastogenic response to gonococcal antigen within five weeks of successful therapy (38). Esquinazi and Streitfeld (38) measured the blastogenic response for gonococcal patients to gonococcal sonicate, and the insoluble, soluble and

ion-exchange fractions of that sonicate. The results were based on the number of antigenic preparations which produced significant transformation. Lymphocytes from five of the six patients in the study group were stimulated by two or more of the antigenic preparations three weeks after therapy. This represented the maximum response obtained. By five weeks, three of the six patients responded to one of the preparations. The use of antigenic mixtures as inducers of lymphocyte transformation resulted in a number of non-specific reactions. Four of the seven controls responded to at least one antigen which makes interpretation of the data difficult.

Esquinazi and Streitfeld (38) also reported on one case of gonococcal arthritis. Significant transformation levels detected at the time of admission to the hospital dropped to normal ( $< 2$ ) five days after the initiation of antibiotic therapy. Such an abrupt drop in CMI response was not observed in the granulocyte-MIF assay in the present investigation. The results of the present investigation have, in addition, shown that a history of two or more gonococcal infections correlates with the measurable granulocyte-MIF (Table 8). A similar conclusion for the blastogenic response was reported by Kraus et al (65), who found that greater transformation occurred with two or more infections than in those with their initial



infection, and greater transformation occurred in those with three or more infections than in those with one or two infections. The reason for the lack of measurable cellular response after the initial infection cannot be explained by our own data although it may possibly reflect the amplification of the cellular response by repeated stimulation.

A second possibility for the lack of granulocyte-MIF production or lack of blastogenic response may be attributable to strain variability of the gonococci. All of the  $B^{-t}$  used in this study was prepared from F62. If the  $B^{-t}$  antigen is not present in all gonococci, we would not expect to detect sensitized cells in patients infected with ( $B^{-t}$ )- strains. Patients with multiple infections would have a greater probability of having been infected with a ( $B^{-t}$ )+ strain, thus a greater number would produce granulocyte-MIF in response to  $B^{-t}$ .

The molecular weight of  $B^{-t}$  was estimated to be 23,000 daltons, which is very close to the weight of gonococcal pili (based on personal correspondence from Dr. T. Buchanan, Chief Immunology Research Laboratory, University of Washington). It is possible that the process of sonication may remove the pili from the cell surface allowing pilar protein to be resolved in the soluble fraction. Strain variability of pili (85,122) would be an important factor in the ability to detect the CMI response

in all patients using a pili antigen. Immunological properties of the B<sup>-t</sup> suggest, however, that it is unlikely to be pili antigen. Anti-F62 T1 pili antibody did not react with the B<sup>-t</sup> antigen in gel-immunoelectrophoresis, nor was any reaction noted when anti B<sup>-t</sup> sera and F62 T1 pili were reacted in a similar system (based on personal correspondence from Dr. T. Buchanan, Chief Immunology Laboratory, University of Washington). In addition, no correlation could be demonstrated between the development of anti-pili antibody and the development of measurable granulocyte-MIF in response to B<sup>-t</sup>.

Although there is a difference in the amount of detectable antibody produced by males and females in response to gonococcal infection (63), the data from this study do not demonstrate a significant difference in the cell mediated immune response of males and females.

The results of the auxotyping of Winnipeg isolates of N. gonorrhoeae support the findings of Knapp and Holmes (62), who suggested that DGI associated strains represent a sub-group of the strains isolated from patients with uncomplicated gonococcal infection. This sub-group is nutritionally more dependent when compared with the non-DGI associated isolates. The DGI associated strains isolated from the Winnipeg study group, however, were not confined to the auxotype 11 and 14 as were the Seattle

strains (62). The important factor in dissemination of infection may simply be related to nutritional dependency and not restricted to the pro-arg-hyx-uracil and arg-hyx-uracil requiring strains as was suggested by Knapp and Holmes.

Penicillin susceptibility testing indicated that increased susceptibility is related to auxotype. Whether or not the disseminated strains have increased susceptibility to penicillin as reported by Knapp and Holmes will depend upon the particular auxotype causing DGI in the geographical area from which they were selected. The DGI strains in Winnipeg had penicillin MIC's which are similar to the non-DGI strains, although the auxotype 14 strains did demonstrate increased susceptibility to penicillin.

There is not sufficient data from the present study to suggest that the DGI associated strains differ immunologically from other strains. While none of the patients whose organisms were available for auxotyping demonstrated migration inhibition, inhibition was demonstrated in other patients with DGI. Further studies would be required to determine if there was any correlation of the auxotype with the CMI response.

The phenomenon of "Early" migration inhibition reported by Clausen (21) in Mantoux positive patients in response to PPD has also been demonstrated in the present

investigation. Granulocyte-MIF detected in some patients was found to be present in four hours, but after further incubation the leukocytes somehow overcome the inhibitor and migrate normally. It is not clear whether this is a labile, or short lived mediator, or if it represents some function of the leukocytes which allows them to escape inhibition. It appears to be associated only with patients with a history of gonococcal infection, since it was not observed in any of the thirty control subjects. It would be prudent to read all granulocyte-MIF tests at four hours in addition to eighteen hours when performing an assay for "inhibition". However, whether the four and eighteen hour results represent an assay for different cell functions, or different mediators, remains to be elucidated.

The route of immunization or the site of infection may be important in the final outcome of the immune response to the gonococcus. Recently, the concept of local cell mediated response has been proposed. Waldman (123), Henney (48), and Nash et al (84) have shown that antigens instilled into the upper respiratory tract will produce a local cell-mediated immune response. A lesser response, with a later time course for maximal effect was obtained from peripheral blood. Local CMI may be a matter of dose and distribution of antigen. Countey and Hand (16) have shown that in rabbits infected with Listeria monocytogenes or Strepto-

coccus pneumonia, local and systemic CMI developed after the introduction of the organisms into the lower respiratory tract. There was no evidence of CMI however after other routes of immunization unless the lung was involved in the infectious process. CMI associated with the gastrointestinal tract has also been demonstrated. Oral exposure of swine to TGE virus (41) produced local and systemic CMI response while subcutaneous injection of the virus produced negligible CMI response from lamina propria lymphocytes.

Further studies are required to determine whether local CMI response occurs in those gonococcal patients in whom peripheral leukocyte assay for granulocyte-MIF was negative.

The second portion of the study was concerned with the development of a model to study the role of the cellular immune response in the host defence mechanisms.

It was possible to demonstrate a good dermal delayed type hypersensitivity in guinea pigs immunized with gonococcal cytoplasmic material with complete freund's adjuvant. While immunization produced DTH, chamber infection alone was not sufficient to produce a dermal response in the guinea pigs. This suggests that there may be some basic difference in specific T-cell stimulation in the natural host as compared to the guinea pig chamber infection.

The anti-schistosomal compound, Niridazole, 1-(5-nitro-2-thiazolyl)-2-imidazolidinone, given in 100 mg amounts was shown to decrease the dermal response. Recently, Daniels (29) reported similar results using 100 mg dosage. While DTH is suppressed, Niridazole apparently does not affect the non-specific inflammatory response (29).

Other studies have shown that Niridazole suppresses hypersensitivity profoundly. Administration to mice inhibits granuloma formation around Schistosoma mansoni eggs (78,79) and retards allograft rejection (79). Therapeutic doses of Niridazole suppress cutaneous delayed hypersensitivity to a number of antigens and suppress antigen-induced lymphocyte transformation in man (132).

The mechanism by which the CMI response is suppressed by Niridazole has not been determined. Higashi et al (50) found a reduction in T-cell rosettes following Niridazole therapy while B-cell rosettes remained unchanged. Metabolites of Niridazole, but not the parent compound, have been shown to prevent MIF production by lymphocytes (29).

Since we were able to produce a DTH response in guinea pigs in response to the cytoplasmic gonococcal material as well as impair that response by treatment with Niridazole, this became our model to study the effects of that impaired CMI on the guinea pig chamber infection rate.

As reported in the literature, there is a wide spectrum of antiparasitic and antibacterial activity associated with nitroheterocyclic compounds (63). The present study has been able to show that Niridazole has a significant antibacterial effect on N. gonorrhoeae in addition to its effect on various members of the Enterobacteriaceae (63).

In order to study the effect of the depressed CMI response, it was necessary to dissociate the antibacterial effect of the Niridazole from the depression of the CMI response. It appears that these two events are separable, It has been demonstrated that DTH responses are depressed three days after treatment while no gonococcal inhibitory effect can be detected in the sera at that time. Daniels et al (29) reported that the CMI suppressive activity appeared early (one to two days after treatment), peaked by day three or four and was no longer detectable at seven to nine days after treatment.

Evidence has been presented for the protective role of the CMI response in gonococcal infection. Treatment of the animals with Niridazole and the resulting suppression of the CMI system produced an increase in the chamber infection rate. It was assumed that both humoral and cellular immune responses would contribute to the protection of the immunized animals. It was further assumed that both branches of the immune system would contribute,

albeit to a much lesser extent, to the protection of the unimmunized animal (i.e. natural immunity). It was then possible to rank the animal groups from most resistant to most susceptible to gonococcal infection after treatment with a suppressor of CMI response.

It was demonstrated that the resulting impairment of the CMI response correlated with a significant ( $0.01 < p < 0.025$ ) increase in chamber infection.

While the data presented support the hypothesis that CMI plays a partial role at least in protection, it must be understood that what has been demonstrated is an associative relationship. The in vivo system represents a very complex milieu. It can not be assumed from the data presented, or indeed from other published reports of CMI suppression that the only expressed effect that Niridazole has on the animal treated is a depression of the cellular immune response.



## CHAPTER VI

### SUMMARY

The objectives of the research had been to delineate the cellular immune response to gonococcal infection, and to evaluate the role of that response in host protection.

The conclusions of the research are, in summary:

1) Gonococcal cytoplasm, prepared by sonication and differential centrifugation, contains a non-specific cell mitogen.

2) The B<sup>-t</sup> fraction of the gonococcal cytoplasm acts as a specific inducer of lymphoblast transformation and lymphocyte mediator production.

3) Evidence has been presented to indicate that the B<sup>-t</sup> material is distinct from that of pili protein.

4) Specific granulocyte-MIF production in response to B<sup>-t</sup> can be detected in approximately fifty per cent of patients following gonococcal infection. This response is more frequently elicited in patients with a history of previous episodes of gonorrhoea. Maximum inhibition was obtained seven to ten days following the onset of symptoms, and the duration of the response varied from fourteen to more than forty days.

5) The phenomenon of "early" migration inhibition has been shown to occur following B<sup>-t</sup> stimulation of lymphocytes of patients with gonococcal infection. The mechanism which allows the granulocytes to escape inhibition has not been investigated.

6) Strains of N. gonorrhoeae causing disseminated gonococcal infection in Winnipeg represent a nutritionally distinct group of organisms. However, sufficient data were not available to indicate whether this affected the induction of, or the ability to detect, the cellular immune response.

7) Increased susceptibility to penicillin correlates with a specific, nutritionally dependent strain of gonococci but increased susceptibility to penicillin was not characteristic of the strains causing disseminated gonococcal infection in the Winnipeg area.

8) There was a development of DTH following immunization of guinea pigs with gonococcal cytoplasm-CFA. However, DTH could not be elicited by chamber infection with N. gonorrhoeae.

9) Niridazole effectively suppresses the DTH response in the guinea pig to gonococcal antigen. The antibacterial effect of Niridazole on N. gonorrhoeae has also been demonstrated.

10) Evidence was presented for the effect of the CMI response on the protection of the host from gonococcal infection. Suppression of the host DTH response with Niridazole increased the infection rate to gonococcal challenge in the guinea pig chamber model.

A local CMI response following antigenic stimulation

of the respiratory and gastrointestinal system has recently been reported in the literature. Whether or not a similar phenomena occurs in the genitourinary system of patients with gonococcal infection has not been documented. It is speculated that in the case of a recent or a single infection, a local response, not detectable in the peripheral blood lymphocytes could be possible.

The presence of DTH following immunization but not following chamber infection in the guinea pig may be the result of a similar phenomenon.

Further investigation is required to determine whether a local CMI response occurs following infection with N. gonorrhoeae. The presence and duration of such a response would conceivably be an important factor in host protection.

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## APPENDIX

## ABBREVIATIONS

CFA	- complete Freund's adjuvant
CFU	- colony forming units
CMI	- cell mediated immunity
DGI	- disseminated gonococcal infection
GC	- gonococcal
GCFT	- gonococcal complement fixation test
MI	- migration index
MIC	- minimum inhibitory concentration
MIF	- migration inhibition factor
PBS	- phosphate buffered saline
PHA	- phytohemagglutinin

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