LYMPHOCYTE TRANSFORMATION BY

GROUP A STREPTOCOCCAL ANTIGENS

by

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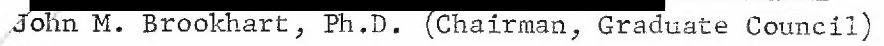

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INTRODUCTION

Statement of the Problem

This thesis is concerned with the transformation of sensitized human peripheral blood lymphocytes in the presence of specific bacterial antigens from Group A streptococci. This work is based on the observation that a small population of peripheral blood lymphocytes can be induced to undergo morphologic transformation and to divide in the presence of antigens to which the lymphocyte donor was sensitized *in vivo*. The specific bacterial stimuli used in this work are killed, intact Group A, Type 6 streptococci and pepsinized streptococci of the same group, which are devoid of their protein surface components.

The problems that were experimentally investigated are the following:

1. Will sensitized human peripheral blood lymphocytes transform in the presence of killed, Group A streptococci?

2. Will a higher degree of stimulation occur, using streptococci containing M protein and other protein surface antigens or using streptococci that have been pepsinized to remove these proteinaceous moieties?

3. How does the observed degree of transformation relate to the humoral antibody responses of the same individuals whose lymphocytes were used in transformation experiments and the same streptococci?

4. How do the lymphocyte transformation patterns vary in a simulated *in vivo* situation in which antibody is also present?

In order to answer these questions, an optimal tissue culture system was established for human peripheral blood lymphocytes, using phytohemagglutinin, and then streptococci or their extracellular products, as mitogens. Next, a series of experiments was run in which intact and pepsinized streptococci in varying dilutions were used as stimuli to lymphocytes from three individuals with histories of recent streptococcal infection. The observation was made that pepsinized streptococci were more stimulatory than Type 6, M protein-containing bacteria. Humoral antibody studies indicated low levels of humoral antibody in the same three human sera to Type 6 M protein. Lastly, a preliminary transformation experiment was performed with pepsinized streptococci and antibody directed against them. Patterns of lymphocyte transformation occurred that furnish a basis for speculation about *in vivo* human host response in streptococcal disease.

Survey of Literature

Group A Streptococci

Most human streptococcal infections are caused by one immunologic group, designated Group A by Lancefield (1). This group of beta-hemolytic cocci is responsible for or is implicated in acute suppurative diseases like streptococcal pharyngitis, scarlet fever, cellulitis of the skin and in the nonsuppurative diseases rheumatic fever and acute

glomerulonephritis (2).

Structural characteristics of these organisms, and immunity produced in response to them, will be reviewed. Figure 1 diagrammatically represents a Group A *Streptococcus pyogenes*.

Capsule

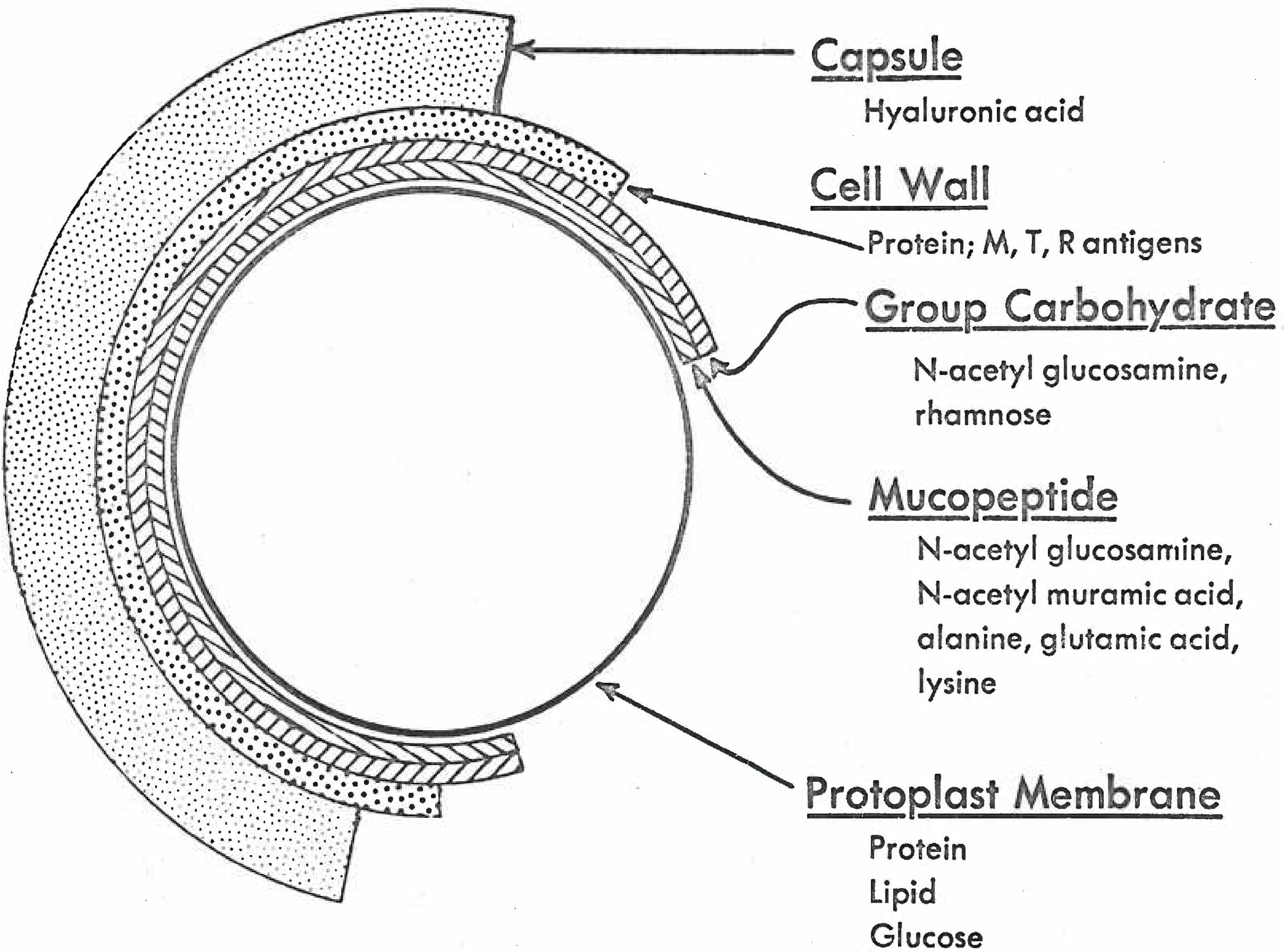
Freshly isolated, virulent strains of Group A streptococci have a hyaluronic acid capsule surrounding the cells. This component is nonantigenic. *In vitro*, it is found only in young, 2 to 4 hr cultures. One of the many extracellular enzymes produced by Group A streptococci is hyaluronidase. This product may serve to remove the capsule from older bacteria. What happens *in vivo* may be that the capsule remains intact long enough to exert an antiphagocytic effect, a property it shares with M protein (2).

Protein Coat

Group A streptococci are subclassified into immunological types, based upon differences in M protein antigens contained in their outer cell wall layer. Over 50 distinct types have been identified by the precipitin test, using a mild acid hydrolysis extract (3). Most Group A streptococci contain just one M type, but occasional strains contain more than one (4). Other protein surface antigens are the T and R antigens, detectable by agglutination tests and unrelated to virulence or immunity (5,6).

The protein fringe-like cover, attached to the cell wall of the Group A streptococcus, is accessible at the surface even when a capsule

Fig. 1 A schematic diagram of a Group A streptococcus (18)


Capsule
Hyaluronic acid
Cell Wall
Protein; M, T, R antigens
Group Carbohydrate
N-acetyl glucosamine,
rhamnose
Mucopeptide
N-acetyl glucosamine,
N-acetyl muramic acid,
alanine, glutamic acid,
lysine
Protoplast Membrane
Protein
Lipid
Glucose

is present. The fact that type-specific agglutination reactions proceed unhampered by a capsule attests to this. Electron micrographs show linear fimbriae extending from the cell walls (7). When proteolytic enzymes are added to the medium in which the organisms are held, these projections disappear; smooth-walled cocci remain. If an 0.1% trypsin solution is used as the enzyme, the bacteria remain viable, without their M protein. Upon removal of the trypsin, the streptococci regenerate their M protein.

The M antigen is generally accepted as one of the most important virulence factors in Group A streptococci; it is antiphagocytic, and antibody to it is protective (3,8). The presence of type-specific anti-M antibody in the blood of man prevents infection with streptococci that have the same M antigen, but not with nonrelated M types. There may be some cross-protection between antibodies to various M proteins. Bactericidal tests, which test for opsonic antibody,are recognized as the most reliable tests for type-specific antibody to Group A streptococci (8). Bactericidal tests furnish evidence of cross-protection between various M types (9,10). In general, however, immunity is type-specific.

In culture, streptococci produce variants which have no demonstrable M protein. Unlike the typical "matt" colonies, characteristically containing M protein, these variant colonies are generally glossy and smooth (3). Streptococci lacking M protein are readily phagocytized in normal human blood. Several passages through mice often serve to restore the M protein content of deficient streptococci.

As many as 50 passages may be required, however, to accomplish this (8).

Virulent, invasive strains of Group A streptococci contain ample M protein to which potential human hosts do not have circulating antibody. If antibody is present *in vitro*, the bacteria are engulfed by phagocytes and are destroyed. *In vivo*, selective pressure may operate to favor variants with new, antigenically different M proteins to which the general population is not already immune. Or, avirulent streptococci without demonstrable M protein may persist, apparently without harming their hosts (11). However, since mouse passage will restore the M protein and virulence of most strains, so, presumably, would human passage from carrier to another susceptible human. Up to 75% of the streptococcal strains recently isolated from the throats of carriers who were followed at the International Children's Centre in Paris, had no measurable M antigen (12). How the avirulent streptococci escape the blood phagocytes is an unanswered question.

Fox and others (13,14) are interested in the development of a highly purified M protein vaccine for protection against streptococcal infection. This preventive approach takes into account the fact that a small number of specific M types are responsible for most human infections (14,15). By vaccinating with these types, Fox and others hope to reduce the incidence of rheumatic fever and other complications in humans.

Major problems have prevented the realization of this goal. The purification of the M proteins has been difficult to achieve, and intense

hypersensitivity reactions have occurred in adult volunteers, either to the M protein itself or to contaminating substances. Recently, Fox has vaccinated a more tolerant age group, infants and children, with minute doses of alum-precipitated, highly purified M protein and has obtained positive bactericidal antibody tests in over half those vaccinated (13). There have been no toxic side effects. Whether this immunization, in minute dosage, is sufficient to produce lasting immunity is yet to be determined. Another approach being tried is treatment of adults with intranasal vaccine sprays. This treatment has induced secretory antibody, and, to a lesser extent, serum antibody. Time will also be needed to tell whether this route will provide immunity to subsequent streptococcal invasion.

The next step is to test polyvalent M vaccines in a large number of young children. Volunteers are difficult to obtain, however, because of Kaplan's finding, in a highly purified preparation of M protein obtained from Fox, of a cross-reactive antigen with human heart tissue (16). Fox and Grossman found no such cross-reactivity (17).

Carbohydrate and Mucopeptide Layers

Under the protein layer are the mucopeptide and group-specific polysaccharide layers. There is some question as to which is uppermost. Krause (18) believes the carbohydrate layer to be on top, because it protects the mucopeptide from the action of lysozyme and other muralytic enzymes. Osterland et al. (19), after their streptococcal vaccination of rabbits and subsequent analysis of antibodies obtained, concluded

that the carbohydrate moiety was the major antigenic surface determinant, following pepsinization. Fluorescent antibody studies (20), using antisera to group polysaccharide in the growth medium, show a fluorescent coating of newly synthesized portions of the outside walls of viable Group A streptococci. Other studies, however, seem to indicate that the mucopeptide layer is outermost. Barkulis (21) pictures this, because of enzymatic degradation of the walls and analysis of remaining pieces. Swanson _et al_. (7) recently published electron micrographs which give pictorial evidence that mild extraction of the major carbohydrate component of the cell wall eradicates a layer that is deep in the wall.

It is, of course, quite possible that the two layers are intermeshed, with a minor carbohydrate component interlaced with the mucopeptide, and the major polysaccharide moiety beneath.

The carbohydrate layer confers the specificity to the various groups of streptococci. The Group A polysaccharide is composed of rhamnose and N-acetyl glucosamine in a ratio of 5:2 (21). The specific antigenic determinant for Group A is the N-acetyl glucosamine, which is found at 60-70% of the terminal ends of the rhamnose side chains. In other streptococcal groups, the terminal sugar is different. The carbohydrate is extractable in a serologically-active form from the cell walls by the formamide method of Fuller (22), by mild acid hydrolysis, or by enzymatic means.

Group-specific carbohydrate, and various cell and membrane components, have been found by Kaplan and others to have a cross-relationship with mammalian tissue antigens, especially in the heart and

kidney (23). Furthermore, sera from patients with rheumatic heart disease and glomerulonephritis, as well as sera from patients recovering from uncomplicated streptococcal disease, have been shown to have antibodies that crossreact with both host tissue and streptococcal cell wall. The nature of this relationship is complicated and controversial, and it will not be gone into in this paper.

The mucopeptide of these bacteria is the familiar β 1, 4 linked N-acetyl glucosamine and N-acetyl muramic acid. Peptide tails, which are attached to the muramic acid residues, are cross-linked by alanine bridges, but not as extensively as in most gram positive bacteria. The mucopeptide is linked to the carbohydrate by phosphate-containing bridges, composed of glycerol or glyceryl-rhamnoside (21).

This mucopeptide layer gives rigidity to the streptococcus and is protective to the cell membrane, which is beneath the wall and acts as an osmotic barrier. At the same time, the mucopeptide skeleton is sufficiently porous to allow the release of many macromolecular components into the external environment. Among these are hyaluronidase, streptolysin S and O, erythrogenic toxin, DPNase, streptokinase, DNAses, and proteinases. As many as 20 of the extracellular products are antigenic to man; many are toxic (2,24). Streptolysin S (SLS) is an oxygen stable cell lysin and is non-antigenic. Streptolysin O (SLO), on the other hand, is an oxygen labile lysin and is antigenic. Because SLO is a strong antigen, and immunity to it can be accurately determined, the anti-SLO titer is a useful index of recent streptococcal infection in humans (11).

Circulating antibodies to the carbohydrate moiety are found in the serum of normal human adults (25) as are antibodies to the mucopeptide of Group A (26). Higher levels are found to both in various streptococcal disease sequelae although there is some overlap with the normal range.

Role of Hypersensitivity to Streptococcal Antigens

Partially purified M protein generally induces an Arthus-type hypersensitivity with circulating antibodies in man. However, at the beginning of immunization or persistently when immunization is done with low doses, hypersensitivity is of the delayed type (12). Lawrence (27) showed in one study that 80-90% of normal humans tested had a positive delyaed cutaneous hypersensitivity (DCH) to heat-killed Group A streptococci. (Intact cells, partially purified M protein, and streptokinase were injected intradermally.) This delayed hypersensitivity was transferable by peripheral blood lymphocytes from positive donor to negative recipient. Fox _et al_. found that delayed cutaneous hypersensitivity was elicited to purified M protein from three types of Group A streptococci in 80% of adults and 8% of infants tested (28).

Purified Group A carbohydrate is not antigenic (29) and it has not been found to evoke delayed hypersensitivity (12). This moiety does have biologic activity, however. As already noted, it protects the mucopeptide layer of the streptococcus from the action of lysozyme and other degradative enzymes. When the carbohydrate, still linked to the mucopeptide layer, is injected intradermally into rabbits, chronic,

relapsing lesions of the dermal tissue result. When mucopeptide is injected alone, the lesions are acute and of short term. Group-specific polysaccharide alone produces no lesions (30).

Immunosuppressiveness of Group A Streptococcal Components

Group A streptococci have also been discovered to have components that are immunosuppressive. Hanna and Watson (31) found that streptococcal pyrogenic exotoxin, when injected intravenously after sheep red blood cells, suppressed the 19S response in rabbits as measured by the Jerne system. These workers had previously found that the exotoxin suppressed phagocytic function in the blood of rabbits (32).

The second immunosuppressant was found by Malakian and Schwab (33). A fraction from washed and disrupted Group A streptococci, when injected into mice before an injection of sheep red blood cells, was found to suppress both primary and secondary responses in a Jerne assay.

Hirata and Terasaki (34) found that purified M protein of Type 1, extracted by Fox's method, crossreacted strongly with histocompatibility antigens on human peripheral blood lymphocytes. That is, the antiserum to lymphocytes was inhibited from cytotoxic action by the presence of the purified M 1 protein. No inhibition was observed from other M types tested, including type 12, a common nephritogenic type, nor type 6, the type used in the experiments to be described in this paper. No inhibition was observed from Group A and other bacterial polysaccharides.

It is presumed from these experiments that Type 1 M protein is structurally similar to histocompatibility antigens. Further work has shown that this specific immunosuppressant is not anticomplementary and does not act by coating the lymphocyte's surfaces.

Another substance that may turn out to be immunosuppressive *in vivo* is a mitogen associated with SLS. *In vitro*, this mitogen is nonspecific in its action, much like phytohemagglutinin. In the presence of complement, it might be cytotoxic to lymphocytes, as is antilymphocyte serum, now used therapeutically as an immunosuppressant (35).

Lymphocyte Transformation

Because circulating lymphocytes are thought of as representative of a heterogeneous population of cells that circulates through the blood and lymphatic channels of the body and acts directly or indirectly as agents of immunological competency and memory, a study of them is of interest. Since it has been found that certain streptococcal antigens induce transformation of peripheral blood lymphocytes in humans, it is of special concern to those interested in human streptococcal disease to determine the nature of this response and to determine which bacterial components are involved.

Nature and Scope of Lymphocyte Transformation

Several survey articles are available which give a history of lymphocyte transformation (36,37,38). Basically, it has been found

that certain substances called *mitogens* induce striking morphological changes in human peripheral blood leucocytes. Large blast-like cells appear in cultures that were predominately small lymphocytes; these transformed cells go on to division. *Nonspecific* mitogens induce transformation in previously nonsensitized individuals. Some examples of nonspecific mitogens are phytohemagglutinin (PHA), an extract of red kidney bean (*Phaseolus vulgaris*); staphylococcal filtrate; SLS associated mitogen (36); antiglobulin serum (39); and antigen-antibody complexes (40). These nonspecific stimuli induce most human lymphoid cells in culture to incorporate tritiated thymidine into newly-synthesized DNA and to divide. Other mitogens are termed *specific* and require previous *in vivo* contact and are stimulatory to a much smaller population of lymphocytes. Lower levels of tritiated thymidine incorporation are recorded than with nonspecific stimulation.

SLO is an example of a specific mitogen. It induces circulating antibody production; it also produces low level transformation in most human adult peripheral blood lymphocytes, but not in cord blood lymphocytes from newborns. SLS-associated mitogen, on the other hand, stimulates both cord and adult blood (41).

In vitro sensitivity to specific stimuli have been demonstrated with bacterial toxoids of tetanus and diphtheria; with penicillin and other drugs; with viruses, such as vaccinia (36); and with allergens such as alternaria, ragweed pollen (42), and timothy (43). These agents have been associated with both cellular and humoral antibody-mediated hypersensitivity.

Antibody to heterologous lymphocytes, called antilymphocyte serum (ALS) has also been shown to be mitogenic, in the absence of complement (36). When complement is present, lysis of lymphocytes occurs. The finding that ALS is mitogenic suggested to Coulson (44) that special recognition sites are located on the surface of lymphocytes and that ALS recognizes heterologous sites, attaches, and triggers the chain of events that is called transformation.

Bain *et al.* (45) have shown that when lymphocytes from two unrelated individuals are cultured together, transformation occurs. Studies with monozygotic twins and nonrelated individuals indicated that the degree of transformation observed is related to the genetic differences between lymphocytes of the donors. Mixed lymphocyte culture is now used as a histocompatibility test. It has been found that the degree of stimulation in a mixed lymphocyte culture is inversely related to the survival time of a graft between the same individuals (46).

That the small lymphocyte is the cell that responds to mitogenic stimulation has been demonstrated (47). The number of cells other than this type in culture at 24 hr is too small, mathematically, to account for the proliferation seen at the peak of activity, after PHA stimulation. Autoradiographic data of Cooper *et al.* supports this conclusion (48).

Circulating lymphocytes from many animals have been found to respond to PHA and other mitogens. However, as a practical matter, survival of lymphocytes of most species *in vitro* is poor. Rabbits and pigs are good sources of usable lymphocytes (36). Monkey lymphocytes

have been used successfully for week-long cultures (38).

Cells other than circulating lymphocytes have been used in transformation experiments, and these cells show varying degrees of responsiveness. Lymph node, spleen, thymus, appendix, and tonsil lymphocytes have been successfully cultured and used (36).

Technique of Lymphocyte Culture

Techniques of lymphocyte culture and harvest for liquid scintillation spectrometry have been reviewed (36,49,50). The culture methods do not differ in substance from culture of other tissue cells. Lymphocytes do not form monolayers, however, and can be either grown in suspension or can be allowed to settle to the bottom of the tube. The leucocyte cultures used contain not only lymphocytes but other cell types as well. It has been found that less response is obtained, under some conditions, with pure lymphocyte cultures. Most of the red blood cell mass and many of the polymorphonucleur leucocytes (PMNs) are removed for optimal conditions. The serum additive is very important to the realization of maximal activity in transformation experiments. The serum concentrations most used in culture of human lymphocytes are 15-20%. Autologous serum is thought by many to be best, because no foreign antigens are added (36). However, since short supply usually prevents use of autologous serum, other human or animal serum is often used.

No matter what tissue culture system is used, conditions must be worked out that give optimal stimulation of the cells to be cultured with the mitogen to be used as stimulus over the necessary length of

incubation time needed to get peak response. A preliminary series of experiments is required to establish such things as optimal cell density, antigen dosage, medium and serum content and concentration, whether cultures are to be rotated or are to remain stationery, and the proper harvest conditions that will give the most reproducible results. These conditions have been outlined for rhesus lymphocyte culture (38).

Streptococcal Mitogens

The findings that indicate that there is a high degree of immunity to Group A streptococcal structural components or products in the general population have already been discussed.

With the development of lymphocyte transformation techniques, reports of mitogenic and suppressive Group A-associated antigens began to be published. As already noted, Hirschhorn *et al.* (41) found that SLO is a specific antigen. SLS, on the other hand, was found to act like a nonspecific stimulator in normal infants and adults.

Fox and Pachman (51) found that purified M proteins of types 1, 3, 5, and 12 were mitogenic to normal adult lymphocytes. They found that DCH, hemagglutination titers, and degree of transformation were not related.

Francis *et al.* (52) found that intact, killed types 12 and 24 streptococci containing M protein stimulated normal human lymphocytes to a greater degree than type 14 that had lost its M protein by passage in artificial medium. Still, the type 14 without its M protein gave considerable transformation, suggesting that some M was still present,

although not detectable by precipitin test, or that other cell wall antigens were also capable of inducing blastogenesis. Francis et al. (52) claimed this experiment gives evidence that M protein increases the mitogenic power of Group A streptococci. It was unfortunate that type 14 with its M protein coating was not used in the experiment, and that bactericidal tests were not run on the serum of the donors of the lymphocytes to see if they had positive tests for the three M types of streptococci involved. If present, opsonic antibodies would have indicated previous contact with one or more of the types, and then a better evaluation of the results of the transformation experiments could have been made.

In another experiment, Francis's group used a mild hydrolysate from type 12 Group A streptococci that contained the M protein and the group-specific carbohydrate antigens (52). It was found that the hydrolysate stimulated normal human lymphocytes to a greater degree than the intact, whole organisms or purified type 12 M protein did. This result might be due to a more mitogenic molecular size in the hydrolysate than in either the whole cells or the purified protein material. Or, it might be that a dose dependency exists in this system, and the optimum doses were not tried for the purified M or whole cells.

Zabriskie et al. found very little stimulation of peripheral blood lymphocytes, from patients with acute glomerulonephritis and patients with unrelated renal disease, to soluble cell wall material, containing M and T proteins and group-specific carbohydrate (53). These

workers *did* find significant *in vitro* blastogenesis in the lymphocytes of the glomerulonephritis patients in the presence of Group A protoplast membrane and cell wall preparations with 30% membrane contamination. Much less stimulation was found with the cells of the non-nephritic group, although the degree of stimulation over control tubes with no antigen was significantly increased. Both nephritics and others exhibited normal responses to PHA and PPD. A crossreaction has been demonstrated between glomerular basement membranes and Group A membranes, however, so the results are difficult to evaluate.

Other experiments have been reported in which depression of blastogenesis was obtained with streptococcal antigens. Hirschhorn *et al*. (41) found that SLS produced less stimulation in lymphocytes of persons with acute rheumatic fever than those of normal persons, while the response to PHA was the same in both cases. When the rheumatic fever patients were treated with penicillin, their cells became responsive to SLS. Since rheumatic fever is a disease with high familial incidence, it was theorized that a genetic defect causes loss of ability to neutralize SLS, which is then able to produce the tissue damage associated with the disease. Oettgen *et al*. (54) were able to increasingly absorb out the stimulus of SLS mitogen by pre-incubation of SLS with increasing numbers of normal, tonsillar lymphocytes. Tonsil lymphocytes from rheumatic fever patients were not tried for their adsorptive capacity.

Francis and Oppenheim (55) used intact, heat-killed Group A streptococci of types 12, 14, and 24 and nonpathogenic streptococci as mitogens for cells from normal persons and those with several disease

states, including rheumatic heart disease. It was found that rheumatic heart patients showed no greater stimulation by pathogenic Group A whole cells or extracts than by the non-pathogens, a significant difference from the normal persons used who showed much greater stimulation by the pathogens. The rheumatics showed normal responses to PHA and smallpox vaccine. Thus, there appeared to be a depressed level of response in rheumatic heart disease patients who had histories of severe, prolonged streptococcal involvement toward the virulent group of organisms responsible.

This was reminiscent of the work of Oettgen *et al*. with tonsillar lymphocytes (54). It was found that lymphocytes from tonsils of children 4 to 12 years of age were stimulated in a manner similar to peripheral blood lymphocytes with PHA, SLS, and specific antigens to which the children had been immunized. However, the lymphocytes were not significantly stimulated by SLO. It was theorized that since streptococci are a common bacterial type found in tonsils, the depressed response to one of their extracellular products, SLO, could represent immunological unresponsiveness to an antigen always present. Another possibility to be considered is that the tonsils, upon surgical removal, are rarely sterile, and streptococci may be present when the *in vitro* culture is begun. The lack of difference between control and tests might represent maximum stimulation by SLO in control tubes.

In summary, specific and nonspecific mitogenic stimuli have been found that are associated with Group A streptococcal extracellular products and cell walls. Immunosuppressants to other immune assay

systems, the Jerne plaque assay and lymphocyte cytotoxicity assays, have been found associated with cell wall components and in the mitogen associated with SLS. Lymphocytes from humans with certain diseases states, sequelae to streptococcal contact, showed less than normal transformation to streptococcal walls and products.

MATERIALS AND METHODS

Streptococcal Strains

Streptococcus pyogenes strains which were used in these experiments were all identified as belonging to Lancefield's Group A (1). The strains were tested by Mrs. Mary Willis of the Pediatric Microbiology Laboratory of the University of Oregon Medical School (UOMS) Hospital, using Difco Fluorescent Antiserum for Group A streptococci.

1. Streptococcus A 1

This culture was taken from the lyophilized stock of Dr. Lyle Veazie of the Microbiology Department, UOMS, and it was used to make the rabbit antiserum. The culture was obtained from Miss Janet Gunnison of the University of California Medical School at San Francisco. It was sent, originally, by Dr. Rebecca Lancefield of the Rockefeller University in New York.

2. Group A, Type 6 (ATCC 12348)

Most of the experiments described in this thesis used Group A, M and T Types 6. This strain was received in a lyophilized state from the American Type Culture Collection in Rockville, Maryland. It was cultured and maintained on blood agar slants. Mouse passage was used in 1970, to maintain the matt phase, characteristic of streptococci containing M protein.

3. Student Strains

Streptococci showing beta hemolysis on blood agar plates were recovered from the throats of each of three medical students during their laboratory course in Medical Microbiology at the UOMS. The students are William Hosack, Robert Nelson, and Paul Wardlaw. Their streptococcal strains were identified as Group A by cultural and morphological characteristics, bacitracin sensitivity, fluorescent typing, and by fibrinolysin activity. There were also characterized as Group A by precipitin tests in which strongly positive reactions were obtained when formamide extracts of the streptococci were reacted with A 1 antiserum. Two of the strains, those of Nelson and Hosack, were lyophilized in May of 1968, after having been in culture approximately seven months. The third strain, that of Wardlaw, isolated in October of 1968, was not lyophilized during the course of this investigation.

The three student strains were serially passed through at least three mice, after they were removed from culture or from the lyophilized state. From cultures of each final mouse's peritoneum, single colonies were isolated of each student streptococcus. Blood agar slants, containing pure cultures of each strain, were sent to the Communicable Disease Center (CDC) of Atlanta, Georgia for typing. Dr. Richard R. Facklam of the Streptococcus Unit at CDC reported that the three strains were untypable for M protein. The two 1967 strains, Nelson and Hosack, were both positive for T-Agglutination Type 12, while the 1968 strain of Wardlaw was positive for T-Agglutination

Type 3/13. No specific antisera was available at CDC to check the R-protein antigen types.

Streptococcal Vaccine Preparation

A vaccine was made from Streptococcus A 1, according to the method of Osterland et al. (19). Streptococci were grown 18-24 hr in 1000 ml Todd Hewitt Broth (Difco), collected by centrifugation and washed three times in sterile pH 7, 0.85% NaCl (saline). The bacteria were resuspended in 10 ml sterile pH 2 saline, containing 1 mg/ml pepsin (obtained from Dr. Richard Jones) and were incubated for 2 hr at 37°C. Pepsinized bacteria were collected and were washed two times in sterile pH 7 saline. After the last wash, the streptococci were resuspended in 50 ml formalinized saline (0.3 ml formalin in 100 ml pH 7 saline). The vaccine was stored in the refrigerator. The vaccine contained gram positive cocci, predominately in chains, characteristic of streptococci.

Rabbits (two New Zealand White and a Belgian Giant) were obtained from the Animal Care Department of the UOMS. These animals had not previously been immunized and weighed between 3.9-4.6 kg. Rabbit antisera were prepared using the schedule and route (i.v.) of immunization previously described (19). Rabbits were bled by cardiac puncture 5-7 days after the last immunization injection. The serum was separated by centrifugation, and was kept frozen at 0°F until used.

Preparation of "M" and "P" Streptococci for Transformation Studies

Group A, Type 6 was used in all transformation studies. Bacteria were grown and treated as above, except that part of the

streptococci were not pepsinized, and none of the bacteria was formalinized.

The protocol was as follows: After three washes in sterile pH 7 saline, half the bacterial cells were resuspended in pH 7 saline and were incubated for 2 hr at room temperature. The other half were treated as previously described for the vaccine preparation: they were incubated in pH 2 saline containing 1 mg/ml pepsin for 2 hr at 37°C. Afterward, both batches were washed two times in pH 7 saline. Both sets were made up to the same volume with pH 7 saline and were incubated for 30 min at 60°C to kill bacteria that were still viable.

Samples of each preparation, "M" and "P", were dispensed into sterile test tubes, capped, and stored at -70°C until used.

Culture purity was checked by blood agar growth characteristics and by gram stain before and after the above procedures. Both final preparations were non-viable.

Antisera

1. Streptococcus A 1 antiserum preparation has already been described. This antiserum was used in the last transformation experiment, either untreated or pre-incubated with either L-rhamnose (Calbiochem) or N-acetyl D-glucosamine (A grade, Calbiochem).

2. Type 6 antiserum was obtained in two batches from Dr. Morris Suggs, of the CDC Biological Reagents Section. A description, accompanying both lyophilized batches, stated that the rabbit antisera were specific for Type 6 M protein with no crossreactions

between M protein Types 1-51, less 9, 27, 28, 44, 48, 49, and 50.

3. Student Sera: When blood was drawn from Hosack, Nelson, or Wardlaw for transformation experiments, an additional sample was often taken for indirect bactericidal tests and anti-streptolysin O (anti-SLO) titers.

4. Normal Sera:

A. Normal human serum was used in indirect bactericidal and anti-SLO tests. Blood from 85 medical students was drawn in October of 1969. The serum was stored at 0°F and was later thawed, pooled, sterilized by Millipore filtration, and refrozen in amounts convenient for one experiment.

B. Normal rabbit serum, fron nonimmunized rabbits, was obtained in two batches. Both were sterilized by Millipore filtration and were stored until used at 0°F. The second batch was used in the last transformation experiment and was found to be somewhat cytotoxic for lymphocytes by the trypan blue dye exclusion test (56).

Biochemical Tests

1. Rhamnose assays were determined by the method of Dische and Shettles (57). They were read on a Beckman DU Spectrophotometer, Model 2400.

2. Protein assays were carried out by a modified Folin-Ciocalteau method (58).

Immunological Reactions

M protein extraction was done by the acid-heat method of Lancefield

as described in a CDC streptococcal bulletin (59).

Formamide extraction of Group A carbohydrate was run on whole streptococci by the method of Fuller (22) at 180°C for 20 min.

1. Precipitin Test: The capillary technique, also described in a CDC bulletin (59), was used for the precipitin tests to check for the presence of Type 6 M protein, using CDC antiserum and hot acid extract. The precipitin test was also used to test for the presence of Group A carbohydrate in formamide extract, using *Streptococcus A 1* antiserum.

2. Bactericidal Test: The method used is basically that of Lancefield (6) for the detection of type-specific M protein opsonic antibodies.

Sera: For indirect bactericidal tests, sera were decomplemented for 30 min at 56°C.

Blood: Human blood was aseptically drawn and mixed with phenol-free heparin (S/P Ammonium Salt Heparin) at 10 units or less per milliliter of blood. The tube containing the blood was iced and was used within two hours.

Streptococci: In order to try to select streptococcal matt variants that have been found suitable for bactericidal tests, serial mouse passages were used (6). After three or more mouse passages, the streptococci to be used in bactericidal tests were grown in Todd Hewitt Broth, were concentrated by centrifugation, and were frozen at -70°C until use. For the tests, dilutions were made of two-hour cultures transferred from overnight broths, which had been inoculated

from the frozen stock. These Todd Hewitt dilution tubes were iced until used.

Direct bactericidal tests were made by adding 0.1 ml of each streptococcal dilution and 0.3 ml heparinized human blood to 15 x 125 mm tubes. Test tubes were sealed and were incubated on a rotating culture drum, set at 1 revolution per minute (rpm). Incubation was at 37°C for 3 hr. For small volumes, Kahn pipettes were used.

The indirect bactericidal tests were performed by adding 0.05 ml of the serum to be tested to 0.1 ml streptococcal broth dilution and 0.3 ml heparinized human blood. The same incubation and plating procedures were followed for both tests.

Plate counts were made at the beginning of the incubation period of 0.1 ml of streptococcal broth dilutions that were used in setting up the tests. Post-incubation counts were taken in 0.025 ml amounts from bactericidal culture tubes, shaken lightly. All platings were done in triplicate, onto the surfaces of blood agar plates. After overnight incubation, plates were read, using a dissecting microscope to count colonies.

Indices were calculated from the countable dilution(s) in each series (60).

3. Antistreptolysin O Titers were performed by the staff of the Serology Department, Clinical Pathology, UOMS.

Lymphocyte Transformation Experiments

1. Special Techniques: All tissue culture glassware was

washed in 0.5% Microsolv (Microbiological Associates), and was then rinsed ten times in tap water and ten times in deionized distilled water. All tissue culturing was done under a hood, pre-exposed to ultraviolet light.

2. Lymphocyte Cultures: For transformation experiments 35 to 50 ml human blood were drawn; a small portion was allowed to clot. The serum was separated and was frozen until used to evaluate circulating antibody. The remaining 30 to 45 ml was mixed in a sterile culture bottle with phenol-free heparin in a concentration of 20 units/ml blood. The heparinized blood was incubated at 37°C for 3 1/2 hr, with occasional mixing, to permit rouleau formation of the red blood cells and to facilitate separation of leucocytes.

Lymphocyte separation was accomplished essentially by the method of Coulson and Chalmers (61). Difco Gelatin was mixed with saline in a concentration of 3% w/v, and was heated to 50°C until the gelatin dissolved. The gelatin solution was sterilized by a Millipore Sterifil Aseptic Filtration System with an 0.45 μ filter (HAEG, 047, AO). The resultant sterile gelatin solution was kept at 37°C to prevent gellation; later when the heparinized blood was added, and during the separation of lymphocytes, a temperature of 37°C was maintained.

The heparinized blood was mixed with the gel-saline in a 100 ml Nalgene tube in a ratio of three parts blood to one part

of the gelatin solution and was allowed to stand for 30 to 45 min.

After the settling period, most of the red cells and many of the polymorphonuclear leucocytes had settled, and a cloudy plasma supernatant could be aspirated from the top. This lymphocyte-rich layer was pipetted into Nalgene 50 ml centrifuge tubes, and three volumes of warm Eagle's Minimum Essential Medium (MEM) with Hank's salts (GIBCO, Grand Island Biological Company, New York) were added. Tube contents were mixed and centrifuged at 300 g for 15 to 20 min at 37°C.

The cell pellets were mixed in warm MEM, and an aliquot was counted on a hemocytometer. Polymorphonuclear contamination averaged about 35% of the cells present. Small lymphocytes made up the bulk of the cell population used in the cultures and were 98% viable by the trypan blue dye exclusion test (56).

Mononuclear leucocytes were diluted in enriched MEM to the desired concentration of 1×10^6/ml. Each 100 ml of enriched MEM contained 15 ml Fetal Calf Serum (BBL lyophilized Fetal Calf Serum, Batch number 70-000), 2mM L-glutamine (GIBCO), 10,000 units Penicillin and 10,000 units Streptomycin (GIBCO).

Two milliliter samples of the tissue culture were transferred to Wallis-Melnick screwcap culture tubes (15 x 125 mm). Mitogens were added to appropriate tubes in 0.1 ml amounts. The tubes were tightly capped and were incubated at 37°C in a roller culture rack set at 1 rpm, at an 11° angle. The initial pH of the medium was 7.2, on

a phenol red indicator tube, and no adjustment of the pH was done throughout the incubation period.

3. Mitogens

Phytohemagglutinin (PHA): A highly purified sample of PHA was kindly provided by Virginia Tisdale and Dr. Demetrios Rigas, UOMS, Department of Experimental Medicine. This PHA was used, initially, to set up the optimum conditions for these lymphocyte cultures, and was used subsequently in each transformation experiment as a positive control. PHA has an optimum harvest time of 72 hours; consequently, PHA controls and two unstimulated controls were harvested as near to 72 hours as was possible, in each experiment.

"M" and "P" Streptococci: The major thrust of these experiments was toward finding which of the two streptococcal preparations, "M" (protein-containing) or "P" (pepsinized) bacteria would cause the maximum transformation of human lymphocytes, when equivalent amounts of each antigen were used.

The preparation of these cultures for transformation experiments has already been described. Rhamnose content was used as a measure of streptococci added. Several concentrations of "M" and "P" were used in most experiments.

4. Quantitation of Lymphocyte Response with Tritiated Thymidine:

Twelve hours before harvest, a pulse of tritiated thymidine (New England Nuclear Corporation, 6.7 c/mM, sp. act.) in a final concentration of 0.5 μc/ml was added to appropriate

tubes. At harvest, further incorporation of the labelled thymidine was stopped by the addition of 7 volumes of iced 0.9% NaCl (0.9% saline). The cells were centrifuged at 300 g for 15-20 min. After removal of the supernatant, recentrifugation was done as before with iced 0.9% saline. The cellular debris, in about 0.5 ml saline, was then frozen at $0^{o}F$ until a later time. The elapsed interval was never more than a week and was usually much less.

An equal volume of 20% trichloracetic acid (TCA) was added to the cells in 0.9% saline in the culture tubes. Tubes were allowed to thaw, were vortexed vigorously to break up cells, and were placed in an ice bath for 1 hr to precipitate. The precipitate was layered on Millipore filters (HAWG 02500, 0.45 μ porosity) in a filtration apparatus with light negative pressure. Filters were washed in iced 5% TCA and iced 95% isopropyl alcohol; they were placed in scintillation vials and were dried in a $40^{o}C$ oven. The material collected upon the filters was treated with several drops of 30% H_2O_2 to decolorize the hemaglobin present. Five ml of scintillation fluid (Spectrafluor, Nuclear Chicago, final concentration of 4 g PPO, 0.05 g POPOP/liter toluene) was added.

Radioactivity in the vials was counted on a Beckman Liquid Scintillation Counter and a Packard Automatic Liquid Scintillation Counter. In the latter instrument, the degree of quenching was determined by automatic external standardization. No corrections to 100% efficiency was done, and Counts Per Minute (CPM) were

compared within an experiment, counted on one day, and not between several experiments. Counting was always done for at least 10 minutes, and usually 20 minutes, in two cycles.

RESULTS

Lymphocyte Transformation Studies

Before transformation assays could be run on Group A streptococcal-stimulated lymphocytes from human donors, a reliable culture method was needed that would maintain human blood lymphocytes for a week or more.

Initial experiments were designed to establish the optimal conditions for culture of human peripheral blood lymphocytes, using the conditions previously established for PHA in the rhesus monkey by Mackler _et al_. (63). Table I shows an experiment in which three concentrations of Fetal Calf Serum (FCS) were compared. The effect of continuous rotation on cultures in 15% FCS was also tested; harvest was at 72 hr. On the basis of the results obtained, 15% FCS was used in all subsequent experiments, and a roller apparatus was approved. Because the streptococcal-stimulated cultures were to be incubated 4 days or more after the termination of the PHA cultures, the enriched content of 15% FCS was selected rather than 10%.

Another experiment was done in which three densities of mononuclear leucocytes were cultured, using the 15% FCS, the roller tube method, and PHA stimulation. The purpose of the experiment was to determine the cell density that would give optimum levels of thymidine incorporation. Cell densities used were 0.5, 1.0, and 2.0 x 10^6

Table I. The effect of three concentrations of Fetal Calf Serum (FCS) and roller or stationary culturing on PHA stimulation of human peripheral blood lymphocytes. The cultures were harvested at 72 hr after a 12 hr pulse of tritiated thymidine.

Serum concentration		Roller tubes CPM	Stationary CPM
10% FCS	+ PHA	11,467	
		14,054	
		10,156	
		24,288	
	- PHA	247	
		330	
15% FCS	+ PHA	11,260	5,005
		14,200	6,875
		12,890	
		10,216	
	- PHA	257	51
		212	49
20% FCS	+ PHA	7,506	
		7,187	
		8,481	
		14,760	
	- PHA	75	
		191	

mononuclear leucocytes/ml of culture. It was found that either 1 or 2 x 10^6/ml cell concentrations gave acceptable results. The latter were slightly higher, but the harvesting of such a dense cell mass could not be accomplished with the filtration system available in a reasonable time. The 1 x 10^6/ml density was the highest that would allow a sufficient number of tubes to be set up, using 35-50 ml human blood. This density was used, thereafter.

Next, a pilot experiment was run with four different Group A streptococcal antigens as stimuli in the now-established culture system. Blood came from a medical student with a recent history of untreated streptococcal infection. The stimuli used were 0.1 ml of the following: heat-killed, washed, whole Group A streptococci isolated from the student's own throat 5 months beforehand; supernatant from the broth culture in which his bacteria were grown; autoclaved sediment of the same washed streptococci; and supernatant from the autoclaved culture, containing solubilized intracellular and wall components. Figure 2 shows that all four stimuli were capable of transforming peripheral blood lymphocytes from this individual, given the optimum incubation period and dosage.

Heat-killed, whole Group A streptococci were used as stimuli for the remainder of the transformation experiments, with and without M protein. Group A, Type 6 was selected, because it is a common M type. "M" bacteria gave positive precipitin tests with Type 6 antiserum from CDC, "P" streptococci gave negative results, under the

Figure 2 - A pilot experiment using streptococcal stimuli with human peripheral blood lymphocytes from an individual with a history of recent streptococcal pharyngitis. Dilutions represent final concentrations per milliliter of culture medium.

1

Whole streptococci

Rhamnose = $\sim$ 30 μg/ml in undiluted sample

2

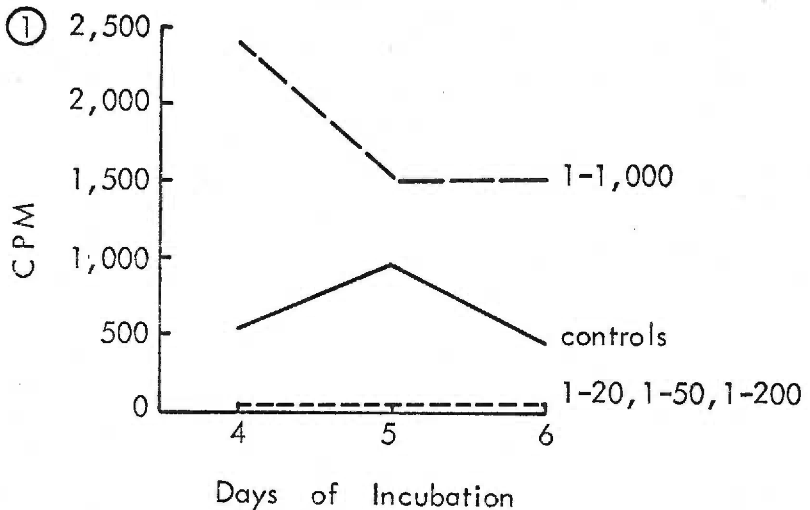
Supernatant from 35 ml streptococcal broth which was concentrated by centrifugation, diluted to 0.5 ml with saline, and autoclaved

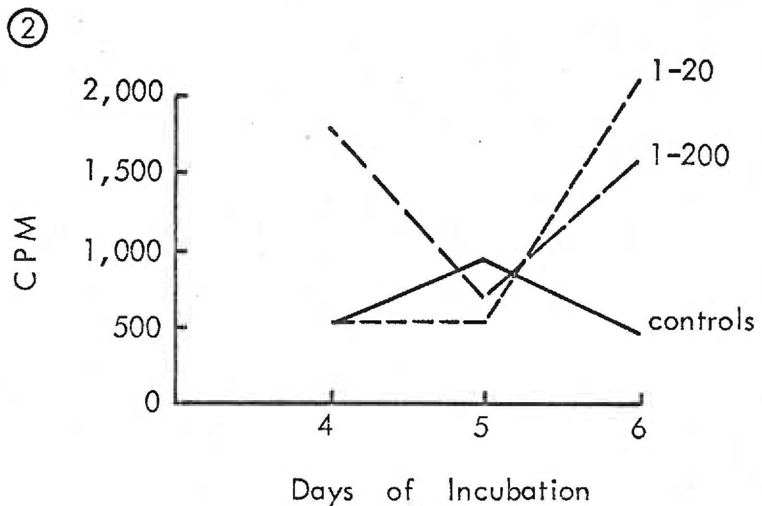
3

Filtrate of broth in which whole streptococci were grown

4

Autoclaved sediment from broth concentrate, described above


1
2,500
2,000
1,500
1,000
500
0
CPM
1-1,000
controls
1-20, 1-50, 1-200
4
5
6
Days of Incubation


2
2,000
1,500
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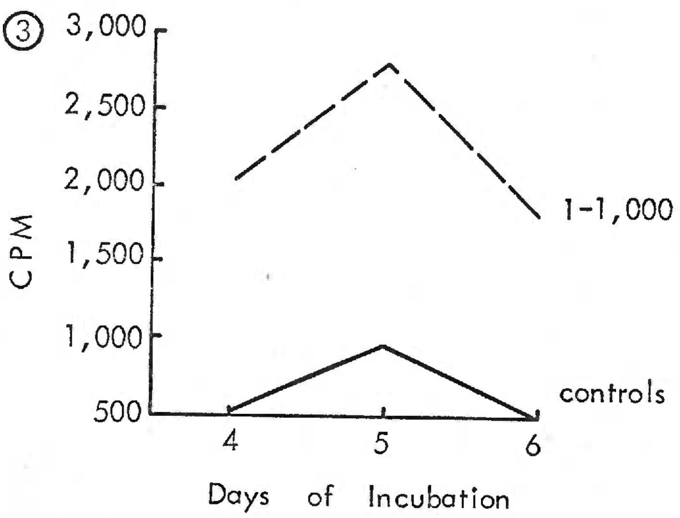
CPM
1-20
1-200
controls
4
5
6
Days of Incubation


3
3,000
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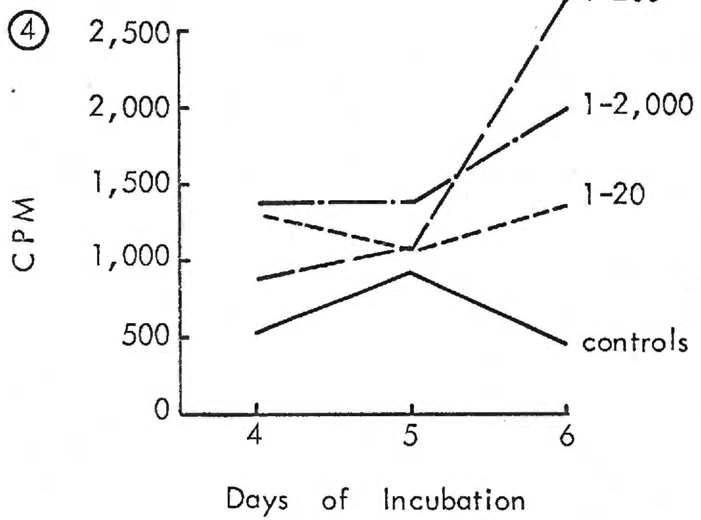
CPM
1-1,000
controls
4
5
6
Days of Incubation

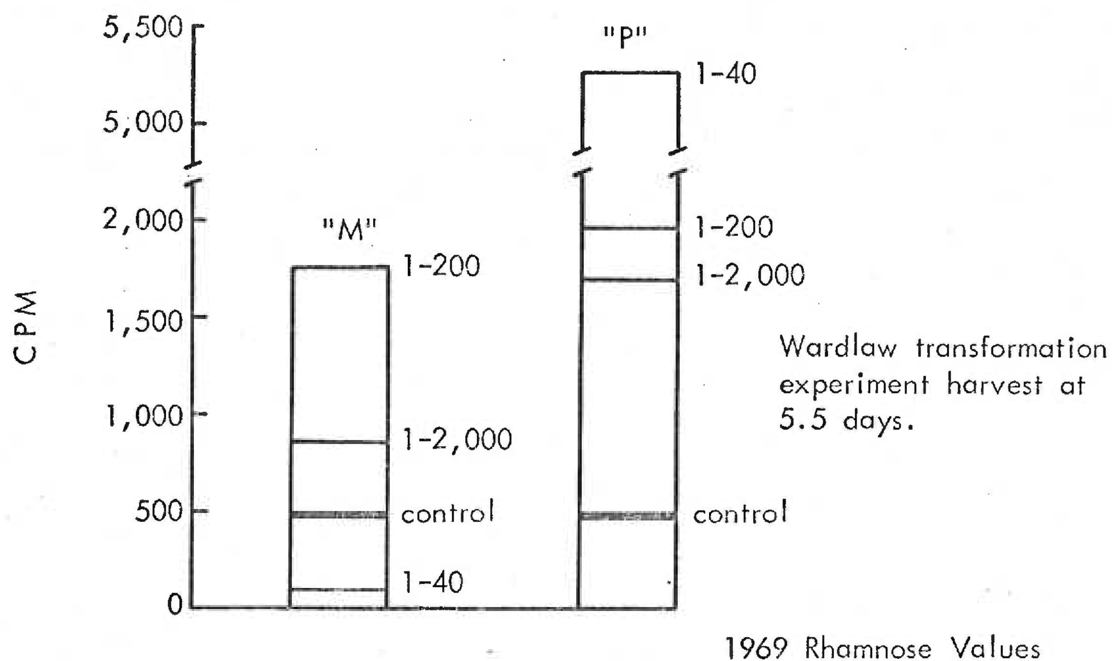

4
2,500
2,000
1,500
1,000
500
0
CPM
1-200
1-2,000
1-20
controls
4
5
6
Days of Incubation

same conditions. "M" and "P" antigens were cultured with lymphocytes from the three medical students who had histories of Group A infections, as has already been indicated. The preparation of the "M" and "P" antigens is described in the Materials and Methods section.

The 1969 transformation studies indicated that the degraded bacteria, the pepsinized form, were more mitogenic than the "M" protein-containing streptococci, in dilutions that were thought to be equivalent. Figure 3 illustrates two experiments, harvested at 5 days. It was necessary to determine if the same number of bacteria was present in these two batches of streptococci. A microscopic examination revealed that comparative counts of the two batches would not be accurate. The "P" cells were much more evenly dispersed than the "M" bacteria. The latter were often in clumps, and the number of individual cocci in a clump would be difficult to ascertain. Optical density on a Spectrophotometer could not be used to determine equivalence, because the two batches were of different character; they would have different light-scattering properties. A rhamnose determination was decided upon for the quantitation of cell wall present. This sugar should not be degraded by pepsin, and the "M" and "P" cocci in equal number should contain equal amounts of rhamnose. Rhamnose assays were done on the remaining aliquots of "M" and "P" cells from the 1969 transformation experiments. Figure 3 gives the rhamnose values of antigens used in the 1969 experiments.

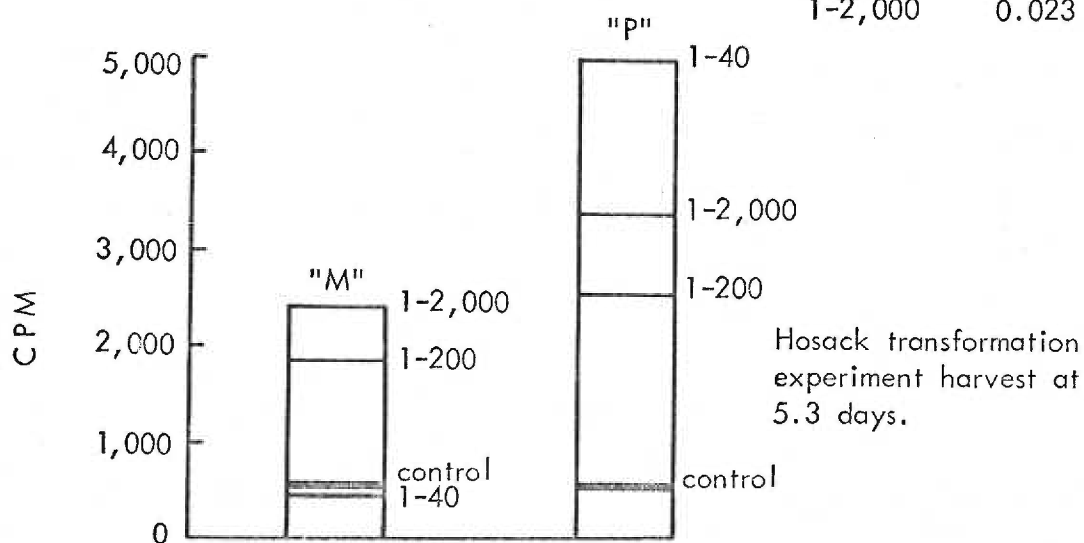
Pepsinized streptococcal preparations, undiluted, measured 46 μg rhamnose/ml. "M" bacteria in undiluted form were 19 μg/ml.

Figure 3 - Two transformation experiments of 1969, using peripheral blood lymphocytes from Wardlaw and Hosack. Mitogens were "M" and "P" whole streptococci of Group A, type 6.



1969 Rhamnose Values

Antigen	Dilution	Rhamnose µg/ml culture
"M"	1-40	0.48
	1-200	0.096
	1-2,000	0.0096
"P"	1-40	1.15
	1-200	0.23
	1-2,000	0.023



A number of possibilities existed as to why these results were obtained: 1) The most obvious conclusion was that "P" dilutions contained more bacteria than "M" dilutions. The 2.4 times difference in rhamnose content of the two similarly treated aliquots of the same original batch of streptococci was hard to accept. 2) Another possibility to be considered was that heat-inactivated pepsin adhered to the surface of the "P" streptococci. This substance might not only contribute to the rhamnose assays but might also be a mitogen. 3) A third possibility was that pepsinization of the bacteria altered them in such a way that more rhamnose was available to the reagents.

The second alternative, that pepsin or something associated with it contained something measurable as rhamnose, was tested. Heat-inactivated pepsin at 1 mg/ml in saline was found to give an unmeasurably low amount of rhamnose. Furthermore, in a single experiment without replicate cultures, 0.1 ml of heat-inactivated pepsin, except at 1 mg/ml, did not give significant stimulation to lymphocytes. Table II depicts this. This concentration was too high to have been present on the surface of pepsinized and twice-washed streptocci. Todd Hewitt Broth, the liquid medium in which streptococci are grown prior to use in these experiments, and another possible adherent, was also tested to its mitogenic properties. Table II shows that 0.05 ml is suppressive.

The possibility that "P" streptococci are altered in such

TABLE II. The effect of heat-inactivated pepsin, diluted in 0.85% NaCl solution (saline), and Todd Hewitt Broth on human peripheral blood lymphocytes in culture.

Duration of culture	Culture Stimulus	Quantity added	CPM
4.5 days	Pepsin 1 mg/ml	0.1 ml	3,273
	Pepsin 0.1 mg/ml	0.1 ml	2,064
	Pepsin 0.01 mg/ml	0.1 ml	1,647
	Controls		2,242
			2,659
			3,241
5.6 days	Todd Hewitt Broth undiluted	0.05 ml	60.8
			50.6
	Controls		6,069
			5,202
			4,841

a way that more rhamnose is accessible was tested as follows: A new (1970) batch of "M" and "P" streptococci were prepared; rhamnose content of each was determined. It was found that "P" contained about 1.55 times more rhamnose than "M" cells. A sample of these antigens was sonicated on a 10 kc Bronwill Biosonik sonic oscillator for 20 minutes. Another sample was autoclaved for 15 min at 15 lb pressure. Rhamnose determinations were performed on these samples. Table III illustrates that after sonication or autoclaving, the P/M ratio is still close to the original 1.55/1 value. Accessibility of rhamnose is apparently not the reason for the discrepancy between the two batches. Protein determinations of the two 1970 batches gave mean values of 44 μg/ml and 110 μg/ml protein for "M" and "P" antigens, respectively, in 1-50 dilutions.

That "P" dilutions contained more cells than "M" remained as the most plausible explanation of why the pepsinized bacteria were more mitogenic and contained more rhamnose and protein.

A carefully monitored pepsinization of a fresh batch of Type 6, Group A streptococci was carried out. The same preparation method as had been used in 1969 and 1970 was used, except that centrifugation speed was less. It was noted that the supernatants of saline washes of "M" cells contained more rhamnose than the "P" supernatants. In addition, "M" bacteria adhered to the sides of the test tube. Table IV follows the course of this experiment. With washing, more cell wall material was lost in the "M" preparations than in the "P". Pepsinization itself did not alter the

TABLE III. Effect of sonication and autoclaving on M protein-containing ("M") streptococci and pepsinized ("P") streptococci. All preparations were diluted 1-50.

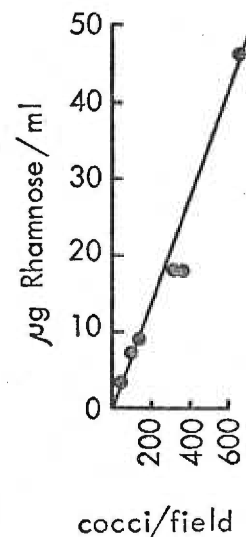
Streptococcal preparations		Rhamnose µg/ml	P/M
1970	"M"	12.7	
	"P"	19.7	1.55/1
	Sonicated "M"	12.8	
	Sonicated "P"	18.0	1.4/1
	Autoclaved "M"	9.4	
	Autoclaved "P"	16.4	1.7/1

TABLE IV. A monitoring experiment in which "M" and "P" streptococcal antigens are prepared. The rhamnose content of the samples is followed through the procedure. Results are the mean values of two or three tests.

Sample	µg/ml rhamnose, corrected to original volume
1 Initial, washed culture	85
2 "M" after incubation with saline	88
"P" after incubation with pepsin	86
3 Supernatants of first centrifugation	
"M" (1 ml of 33 mls)	45
"P" (1 ml of 33 mls)	15
4 "M" after completion of procedure	22.5
"P" after completion of procedure	35

TABLE V. Correlation between rhamnose content of a sample of whole streptococci and number of cocci present in that sample.

Sample	Rhamnose content µg/ml	Cocci per field
1 Pepsinized "M" cocci	3.75	38.6
	7.5	102
2 1970 "P" streptocci incubated in saline and rewashed	9.1	135
		138
	18.2	319
		367
3 1970 "P" cocci, used in transformation experiments	46.2	666.4



Pearson r Correlation Coefficient = + 0.98

rhamnose content. It appeared that the non-sticky nature of the pepsinized streptococci allowed them to sediment faster and more efficiently than the "M" protein-containing cocci. This would explain why "P" preparations had more rhamnose and protein in their final form. (It should be reiterated that the centrifugation speed used in this experiment was less than was used in the usual antigen preparation. Therefore, the loss of sample from removal of the supernatants was more marked than usual.)

A counting experiment was done in order to establish whether rhamnose content is correlated with number of individual cocci present. This data is presented on Table V. Since "M" bacteria could not be accurately counted because of their clumping characteristics, a batch of 1970 "M" cells was pepsinized and washed, and the rhamnose content of the final bacterial dilution was made. Two separate batches of "P" bacteria were used. One was a 1970 batch of "P" cells that was run in tandem with the "M" cells. It was washed but was not repepsinized. Another was a 1970 batch of "P" streptococci, diluted to 46.2 μg/ml for a subsequent experiment. All three preparations were evenly spread on 1 cm^2 areas of clean, glass microscopic slides in 5 microliter amounts. Nine evenly spaced, representative fields were counted on each slide. The mean number of cocci per field was determined. All slides were counted in duplicate or triplicate and two dilutions of each preparation were usually counted. Rhamnose assays, in triplicate, were

run on the three samples. A Pearson Product-Moment Correlation Coefficient (r) indicated that a positive correlation of 0.98 existed (62). This established that a positive correlation exists between rhamnose content of a given streptococcal dilution and numbers of streptococci that are present in that dilution.

The next transformation studies were run on equivalent batches of "M" and "P" bacterial dilutions, using rhamnose content as a measure of equivalence. Appropriate dilutions of "M" and "P" 1970 batches of streptococci were made to achieve this value, for the stock antigens to be used in future experiments. Samples were taken in siliconized pipettes. A rhamnose assay of each revealed that "M" dilutions had a mean value of 47.25 μg/ml rhamnose and "P" had a mean value of 43.5 μg/ml. These values were judged to be as close to equivalent as could be achieved, considering the accuracy of the assay methods. Aliquots of these same dilutions used for testing were frozen at -70°C for future lymphocyte transformation experiments. Stock antigens containing 90 μg/ml rhamnose were similarly made in saline; these samples were also frozen. To summarize, the whole streptococcal dilutions that were readied and later used for 1970 experiments were rhamnose equivalent batches of "M" and "P" streptococci at 90, 45, and 4.5 μg/ml. When used in 0.1 ml amounts in 2 ml cultures, final rhamnose values were 4.5, 2.25, and 0.225 μg rhamnose/ml culture.

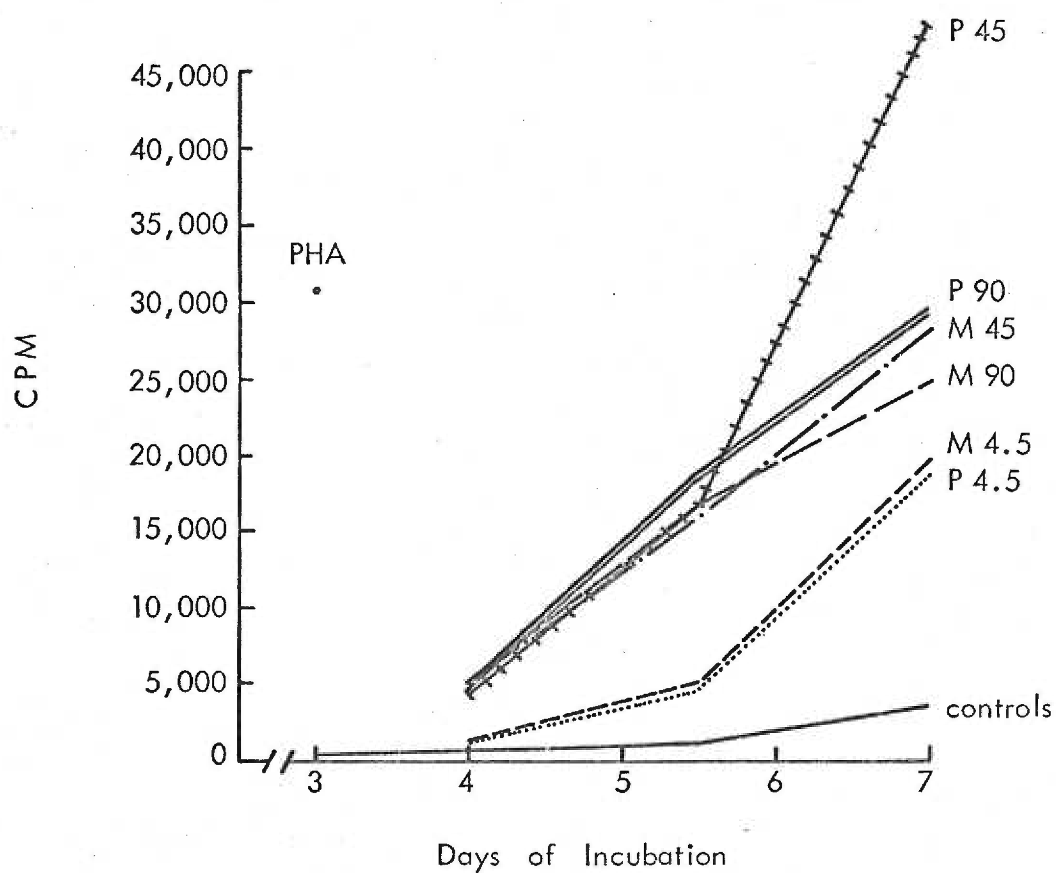
1970 Transformation Results

It was evident in the first transformation experiment on

Fig. 4. First transformation experiment on Wardlaw, 1970. Results of harvest at 7 days.

Stock Antigen µg/ml rhamnose	CPM	$\bar{X}$
P 45	49,406	45,687
	41,968	
P 90	30,083	28,870
	27,657	
M 45	38,497	28,346
	18,195	
M 90	19,910	25,077
	30,244	
M 4.5	19,911	
P 4.5	18,884	
Controls	3,902	2,592
	1,281	

Figure 4 - Transformation experiment of Wardlaw (I), 1970, using unshaken "M" and "P" whole streptococci. Stock 1970 antigen values: 90, 45, 4.5 μg/ml. These are diluted to final concentrations of 4.5, 2.25, and 0.225 μg/ml culture medium.



Wardlaw that "P" bacteria were more stimulatory than "M". Figure 4 shows that, at 7 days, "P" dilutions gave maximum incorporation of tritiated thymidine. At 45 μg/ml "M" stock antigen was less stimulatory than "P". A microscopic examination of the two antigens revealed that there was a difference in dispersion of the streptococci; "M" cells were often clumped; as well as being chained or in smaller units. "P" bacteria were more rarely clumped and were more dispersed, in general.

In order to make the two antigens more equivalent in a physical sense, "M" antigens were shaken vigorously on a Vortex at top speed for 2 min. Nelson's first transformation (Figure 5) used "M" antigens prepared in this manner. Pepsinized streptococci were not shaken. A microscopic examination after shaking revealed that "M" cells suspensions were much more dispersed than they had been before agitation, but still contained many cocci that were in clumps. Nelson's experimental results again showed the pattern of more stimulation by "P" antigens (Figure 5) with a maximum response at 7.8 days.

In the next transformation experiment on Hosack, both shaken and unshaken antigens were used in an effort to determine if agitation had an effect. The effect of shaking was not of significance, as seen in Figure 6. (It should be noted that in Hosack's experiment, the optimum responses were recorded at day 4. However, data in the box on the effect of shaking was taken later, at 7.6 days,

Fig. 5. Transformation experiment on Nelson, 1970. Results of harvest at 7.8 days.

Stock Antigen μg/ml rhamnose	CPM	X̄
P 90	23,560	22,130
	20,700	
P 45	23,500	22,100
	20,700	
M 90	10,500	13,600
	16,700	
M 45	15,910	13,215
	10,520	
M 4.5	11,761	
P 4.5	5,218	
Controls	10,600	7,335
	4,070	

Figure 5 - Transformation experiment of Nelson, 1970. "M" antigens were shaken. "P" antigens were not shaken.

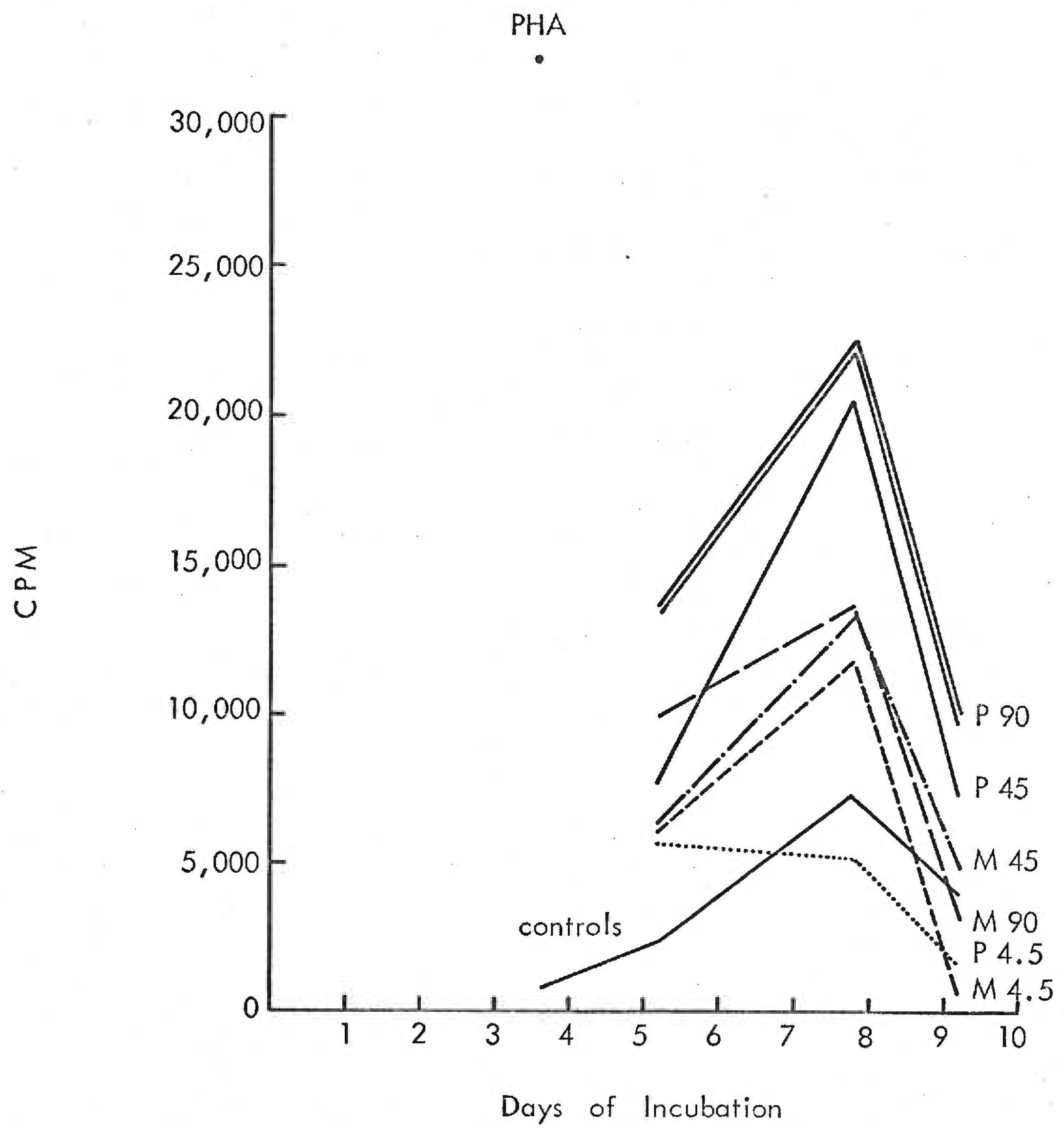
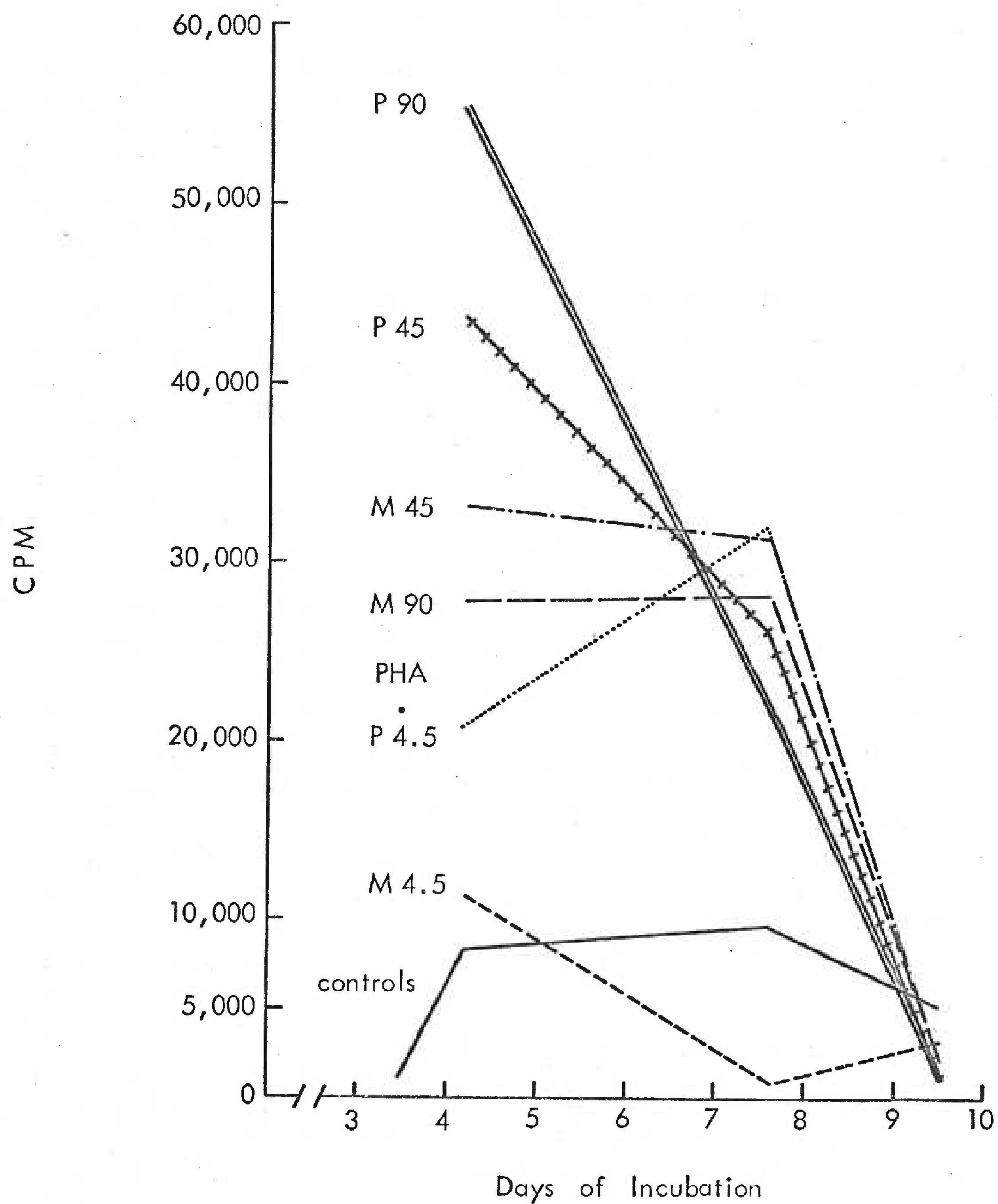


Fig. 6. Transformation experiment on Hosack. Comparison of shaken and unshaken antigens. Results of harvest at 7.6 days

Stock Antigen μg/ml rhamnose		CPM
M 90	unshaken	28,409
	shaken	28,084
M 45	unshaken	33,156
	unshaken	26,793
	shaken	34,278
P 45	unshaken	23,821
		31,165
	shaken	23,422

Figure 6 - Transformation experiment of Hosack, 1970. All "M" antigens were shaken except those indicated on preceeding page. None of "P" antigens were shaken except those indicated.



when the "P" response had dropped.) In the second experiment on Wardlaw, Figure 7, the effect of shaking was not of significance. In both experiments, pepsinized streptococci were more mitogenic than "M" streptococci.

To be sure "M" and "P" streptococci were equivalent, a re-run of rhamnoses on left-over tubes of diluted antigens was done with non-siliconized pipettes. Samples of 0.25 ml showed that "M" was about 0.6 the value of "P" antigens. In a later experiment, new dilutions of approximately 45 μg/ml stock antigens were made up and refrozen. "M" measured 39.7 μg/ml and "P" was 46.2 μg/ml. When later assayed in 0.2 ml amounts, "M" dilutions were again 1/3 less than corresponding"P" values. When larger volumes (0.75 ml) were tested, rhamnose values between "M" and "P" dilutions were not significantly different ($P = < .05$). It was concluded that small volumes of dilute antigens cannot be accurately measured by the rhamnose assay method used. However, equivalent "M" and "P" dilutions, in small volumes, were delivered to lymphocyte cultures.

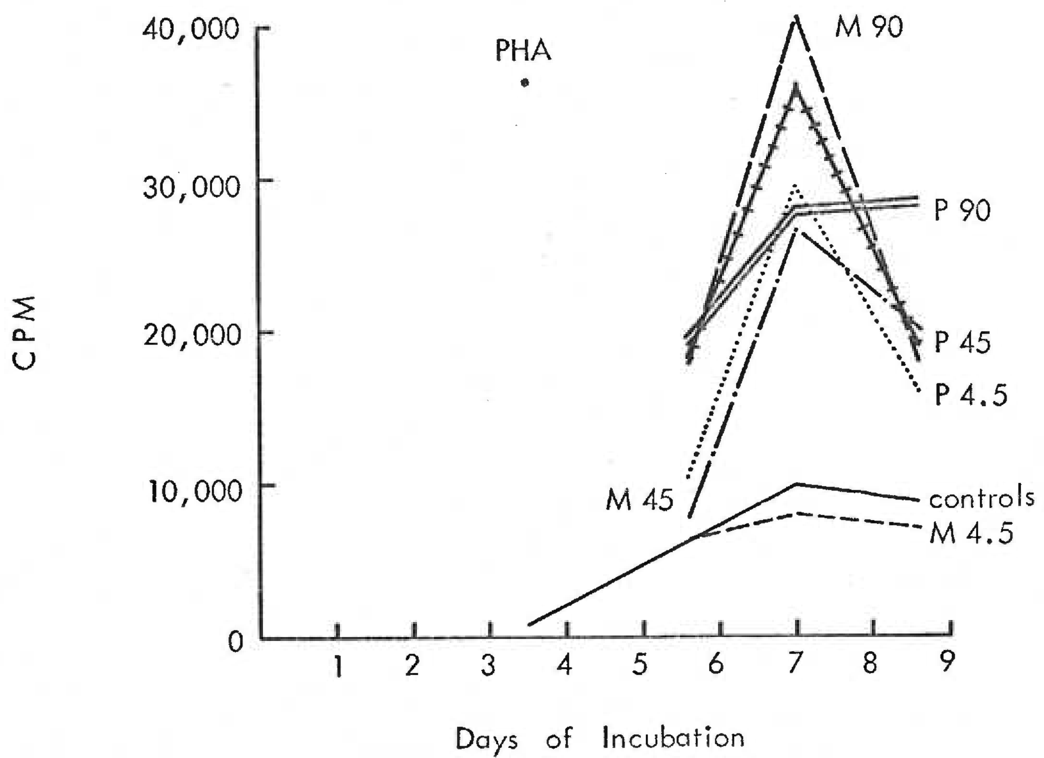
Bactericidal Tests

The transformation assays indicated that the pepsinized form of Type 6, Group A streptococci were equally or more stimulatory than the M protein-containing form. A hypothesis to explain this phenomenon is that Type 6 is a "foreign" M Type to the lymphocytes used in transformation tests. When the Type 6 protein is removed with pepsin, the underlying cell wall is exposed. This structure

Fig. 7. Second transformation experiment on Wardlaw, 1970. Comparison of shaken and unshaken antigens. Results of harvest at 7.2 days.

Stock Antigen µg/rhamnose		CPM	
M 45	unshaken	11,390	26,726
		42,067	
	shaken	36,919	26,519
		16,118	
P 45	unshaken	36,638	35,993
		35,348	
	shaken	32,778	35,826
		38,873	
M 90	shaken	52,810	40,259
		27,707	
P 90	unshaken	33,760	27,847
		21,933	

Figure 7 - Second transformation experiment on Wardlaw, 1970, using shaken and unshaken antigens. All "M" antigens were shaken except those indicated on preceeding page. None of "P" antigens were shaken except those indicated.



is common to all Group A streptococci and would be expected to be familiar to the mature human hosts's immune apparatus.

To test this hypothesis it was necessary to determine if antibodies to Type 6 M protein were present in the three student bloods. Opsonic antibody was measured by bactericidal tests. If opsonic antibody were found, an indication of previous (*in vivo*) contact would be present.

Table VI outlines some typical experiments. In the direct test, freshly drawn blood from each of the three student donors was incubated with three dilutions of Type 6 streptococci. After a 3 hr incubation period, neither Nelson, nor Hosack's blood was found to be inhibitory toward the bacteria. Wardlaw's index was slightly elevated in one of the two cases. A Kuttner Index of .025 or greater is considered inhibitory (64). Indirect tests use freshly drawn normal human blood (without Type 6 antibody), serum from one of the students, and a dilution of streptococci. After a 3 hr incubation period, none of the student serums had demonstrable opsonic antibody toward Type 6 M protein. Pooled human serum (HS), from individuals in the same age bracket as the three student donors, had slightly more opsonic antibody against this M type in this experiment. In several other indirect bactericidal experiments, HS was slightly less effective than one of the student serums. None of the three students consistently exhibited more opsonic antibody than HS, and usually less.

Streptococci that were recovered from the throats of Nelson

TABLE VI. Direct and indirect bactericidal tests with whole blood and serum of humans and Group A, Type 6 streptococci.

Direct Test: Phagocytes and antibodies in same human blood		No. colonies from 0.1 ml culture dilution ($\bar{X}$) 10-5 1-4	1-16	1-64	Kuttner Index[ε] = inoculum/3 hr count
1. Hosack	Inoculum	13	5	0	
	After 3 hr	Tntc*	524	0	5/524 = .007
2. Nelson	Inoculum	8	2	2	2/196 = .01 or
	After 3 hr	Tntc	196	168	2/168 = .012
3. Wardlaw	Inoculum	43	16	-	
	After 3 hr	Tntc	512	-	16/512 = .031
4. Wardlaw	Inoculum	3.5	2.5	1	
	After 3 hr	Tntc	Tntc	125	1/125 = .008
Indirect Test: Phagocytes in normal human blood[&], serum from sources as noted					
	Inoculum	38	10	0	
1. Hosack serum		Tntc	900	-	10/900 = .01
2. Nelson serum		Tntc	625	-	10/625 = .016
3. Wardlaw serum		Tntc	1040	-	10/1040 = .096
4. Pooled human serum		Tntc	473	-	10/473 = .021

[ε] Kuttner Index (60) is $\frac{\text{number of streptococcal chains in the inoculum}}{\text{number of streptococcal chains after incubation}}$

* Tntc denotes innumerable colonies

[&] Normal refers to blood or serum in which type 6 antibody is not demonstrable.

Hosack, and Wardlaw were cultured, passed through at least three mice, and were used in bactericidal testing. All student streptococci were readily phagocytized by normal human blood, an indication that an M protein was not present. This was affirmed by the Communicable Disease Center's finding that all three student streptococci were negative for M proteins of types 1-60.

It was unfortunate that M protein was not present on the student streptococci, because it would have been of interest to measure opsonic antibody against these homologous bacteria. Transformation experiments could have been performed in which mitogenesis produced by "M" containing bacteria, to which the lymphocyte donors had contact, *in vivo*, was contrasted with pepsinized forms.

To summarize the bactericidal results that were found: Tests were run for opsonic antibodies, directed against Type 6 M protein. Blood from the three student donors of lymphocytes showed low levels of opsonic antibody; the levels were usually lower than those of HS, pooled serum from individuals in the same age group. This indicated that Type 6 M contact *in vivo* was absent or minimal.

Antistreptolysin O Titers

Antistreptolysin O titers were run on several samples of blood from Hosack, Nelson, and Wardlaw. The pooled human blood that was used in indirect bactericidal tests was also titered, as was the blood used as phagocyte source in the indirect bactericidal tests. All were within normal limits except Wardlaw, whose anti-SLO level is

TABLE VII. Antistreptolysin O (anti-SLO) titers of various individuals whose blood were used in transformation experiments or bactericidal tests.

Source of serum	Date sample was drawn	Anti-SLO titer in Todd Units (TU)
Wardlaw	10/68*	166
	4/69	166
	1/70	166
	4/70	166
Hosack	4/69	12
	4/70	12
Nelson	5/69	12
	2/70	12
	4/70	12
Blood donor I	10/69	50
	4/70	12
Blood donor II	5/70	12
Pooled human serum	10/69	50

* Sample was taken before or during streptococcal infection

considered by some authorities to be slightly elevated (65). The normal limits for anti-SLO titers are set by some authors at 125 Todd Units (66) and by others at 250 units (67).

Final Transformation Experiment

After the results of the transformation studies with "M" and "P" streptococci were tabulated, another experiment was done in order to try to understand the nature of the mitogenic stimulus on the pepsinized bacteria. This experiment used rabbit antiserum to "P", the basic Group A structure, as revealed by pepsinization. The pepsinized bacteria were used as positive control in this test. The lymphocyte donor was Nelson; the established optimum time of harvest and 2.25 μg rhamnose/ml culture (from 45 μg/ml stock) streptococcal dosage were used.

Table VIII show that Tube 1 is the positive control. The effect of rabbit antiserum to the "P" streptococci was tested in Tube 2 in which antigen and antiserum are preincubated together before being added to the tissue culture system. Alternatively, in Tube 3, lymphocytes were preincubated with the rabbit antiserum and the streptococci were then added. Normal rabbit serum controls, Tubes 4 and 5, were included. Tubes 9-16 use rabbit antiserum to which had been added 10 mg (high dose) or 160 μg (low dose) of rhamnose or N-acetyl glucosamine. These two haptens make up the major determinants in the group-specific carbohydrate of Group A streptococci. The absorbed antiserum was then incubated with the

TABLE VIII. Final transformation experiment, using peripheral blood lymphocytes of Nelson, optimum "P" streptococcal stimulus and rabbit antiserum to the same bacteria. Harvest was at 7.2 days.

Tube No.	Contents	CPM	$\bar{X}$
1	0.25 ml MEM* + 0.1 ml "P" streptococci + lymphocytes	20,335 20,196 25,856	22,129
2	0.25 ml rabbit antiserum + 0.1 ml "P" streptococci 1 hr / 37° + lymphocytes	32,088 27,098 36,780	31,989
3	0.25 ml rabbit antiserum + lymphocytes 1 hr / 37° + 0.1 ml "P" streptococci	10,292 10,153	10,223
4	0.25 ml normal rabbit serum (NRS) + 0.1 ml "P" streptococci 1 hr / 37° + lymphocytes	1,585 1,663	1,624
5	0.25 ml NRS + lymphocytes 1 hr / 37° + 0.1 ml "P" streptococci	556 523	540
6	0.25 ml rabbit antiserum + lymphocytes + 0.1 ml saline	8,010 3,688	5,849
7	0.25 ml NRS + lymphocytes + 0.1 ml saline	699 153	426
8	0.25 ml MEM + lymphocytes + 0.1 ml saline	2,242 2,659 3,241	2,714

TABLE VIII. (Continued)

Tube No.	Contents	CPM	$\bar{X}$
9	Rhamnose, low dose (160 µg) + 0.25 ml rabbit antiserum 1 hr ------ 37° + lymphocytes + 0.1 ml saline	9,500 16,923	13,212
10	Rhamnose, high dose (10 mg) + 0.25 ml rabbit antiserum 1 hr ------ 37° + lymphocytes + 0.1 ml	6,975 5,449	6,212
11	N-acetyl glucosamine (NAG) low dose + 0.25 ml rabbit antiserum 1 hr ------ 37° + lymphocytes + 0.1 ml saline	946 4,973	2,960
12	NAG, high dose + 0.25 ml rabbit antiserum 1 hr ----- 37° + lymphocytes + 0.1 ml saline	3,412 2,757	3,085
13	Rhamnose, low dose + 0.25 ml rabbit antiserum 1 hr ------ 37° + 0.1 ml "P" Streptococci 1 hr ------ 37° + lymphocytes	37,800 36,197	36,999
14	Rhamnose, high dose + 0.25 ml rabbit antiserum 1 hr ------ 37° + 0.1 ml "P" Streptococci 1 hr ------ 37° + lymphocytes	26,584 38,873 26,170	30,542
15	NAG, low dose + 0.25 ml rabbit antiserum 1 hr ------ 37° + 0.1 ml "P" Streptococci 1 hr ------ 37° + lymphocytes	12,153 19,739	15,946
16	NAG, high dose + 0.25 ml rabbit antiserum 1 hr ------ 37° + 0.1 ml "P" Streptococci 1 hr ------ 37° + lymphocytes	11,836 16,850	14,343

* Minimum Essential Medium, Eagle

streptococci; later both are added to the tissue culture system. Controls for this group of experiments in which the haptens are present and bacteria are absent are found in Tubes 9-12. Further controls were added in which serum alone, or medium, alone, were present. Results obtained at harvest at 7 days are also given on Table VIII.

It was noted from the results that the normal rabbit serum appeared inhibitory. A test was run, in which rhesus lymphocytes at 1 x 10^6/ml in 2 ml amounts in tissue culture medium were incubated with 0.25 ml of this normal rabbit serum. Two other rabbit serums were also tested with the same rhesus lymphocytes. Tubes were incubated at 37° for 90 min. A trypan blue dye exclusion test (56) revealed a loss of viability of 12% in rhesus lymphocytes incubated in this serum. Lymphocytes incubated in another normal rabbit serum or in the rabbit antiserum that was used in the transformation experiment, in contrast, lost only 2% or 6% of their viability, respectively. Since the first normal rabbit serum was cytotoxic to the 12% level at 90 min, it was not a useful control in the transformation experiment.

The results on Table VIII also show that an enhancing effect has been achieved by preincubation of the streptococci with the rabbit antiserum (Tube 2). When the same dilution of bacteria and the same antiserum were later viewed, microscopically, agglutination of the bacteria could be seen. On the other hand, when the lymphocytes are preincubated with the antiserum (Tube 3), and the strepto-

cocci are added later, decreased incorporation occurs. Rhamnose added to Tubes 13 and 14 did not decrease the stimulatory capacity of the antiserum, as seen in Tube 2, but N-acetyl glucosamine in both dosages did decrease stimulatory effect of the antiserum. Only the rhamnose in low dosage (Tube 9) gave increased counts over the control for Tubes 9-12, Tube 6. The results obtained for Tube 9 are very divergent.

It appears that N-acetyl glucosamine, in high or low dosage, inhibits an enhancement of mitogenesis effected by antiserum to "P" streptococci and its homologous antigen. Rhamnose has little or no effect.

DISCUSSION

The results presented in this thesis show that equivalent dilutions of streptococci coated with Type 6 M protein were less stimulatory than pepsinized streptococci to peripheral blood lymphocytes of the three individuals tested. Results already given indicate that "P" bacteria are 1.3 to 1.9 times more mitogenic than are "M" with optimum doses of antigen at the optimum day of harvest in the systems tested. This means that the lymphocytes in culture recognized differences in the chemical composition of the exposed streptococcal wall antigens, and found the pepsinized cocci more stimulatory than those with Type 6M protein because of previous _in vivo_ contact.

Serum antibody studies on the three students indicated that minimal opsonic antibody was present toward Type 6 M protein. Therefore this M type is "foreign" to the three, or else some low-level cross-reactivity occurred with antibody to other M types. The T and R antigens may also be "foreign." It is not surprising, then, that more transformation occurs when the stimulus is from degraded streptococci. This structure, common to all Group A streptococci, is recognized by the lymphocytes if degradation occurs _in vivo_, as has been theorized (25). Anti-SLO levels indicate that Nelson and Hosack, who were probably infected with the same streptococcal

strain, are normal. Wardlaw has a slightly elevated anti-SLO level. He has had this titer since he was first cultured; it has not changed. Anti-SLO results indicate that significant streptococcal infection probably did not occur during the period when transformation studies were being carried out.

Results obtained with the last transformation experiment indicate that enhancement occurs when "P" streptococci and rabbit antiserum to the same bacteria are present in culture with lymphocytes from one of the three students. This enhancement can be blocked by preincubation of the antiserum with NAG. Enhancement is also blocked by preincubation of the antiserum with lymphocytes, prior to the addition of the bacterial stimulus.

Results that were reported for this last experiment are disappointing from two standpoints. First, the normal rabbit serum, used as a control for the rabbit antiserum, was shown to be cytotoxic. A good control was not present with which to check the specificity of results with the rabbit antiserum. Secondly, in some of the duplicate results, counts are very far apart. Nevertheless, the results furnish some new ideas concerning the role of carbohydrate haptens in streptococcal disease.

Enhancement of transformation levels that occurred when "P" antigen and its antibody were present was probably due to the formation of antigen-antibody complexes which adhere to lymphocytes and trigger blastogenesis. It has been found that these complexes adhere to the surface of lymphocytes, whereas nonspecific antibody does not (69). Möller (70) found that Salmonella bacteria, cultured

with lymphocytes from immune patients, caused marked stimulation of DNA synthesis, which was enhanced by the addition of Salmonella-specific antibody. The species origin of the antibody did not affect enhancement, but the physical state of the antigen was important. Particulate antigens were more efficient, but soluble antigens were also stimulatory. The addition of fresh complement to the tested systems did not further increase stimulation. Bloch-Shtacher et al. (40) observed enhancement with antigen-antibody complexes and non-immune human lymphocytes. The stimulation was complement dependent.

Alternately, the enhancement could mean that the rabbit anti-serum recognizes different determinants on the "P" streptococci than the human lymphocyte does. If this is true, the rabbit anti-serum serves to align the antigenic cocci in such a manner that other, more mitogenic, determinants are accessible to the lymphocytes than before.

To test whether this effect is occurring, human antiserum with high streptococcal Group A carbohydrate antibody titer could be run in parallel with the rabbit antiserum, each preincubated with "P" streptococci before addition of lymphocytes, to see if species differences exist. If they do, changed results should occur with the human antiserum.

The latter possibility seems less likely than the first, since agglutination of "P" streptococci in the presence of rabbit antiserum does occur. All the requirements for Möller's system are present: bacteria, immune noninactivated antiserum in excess,

and lymphocytes from an individual who had *in vivo* exposure to the same bacterial species.

A lowered response occurred when lymphocytes were preincubated with antiserum before streptococci were added. This suggests a coating of the lymphoid cells. Since nonspecific antibody does not adhere to lymphocytes, specific antibody must be involved which does not trigger mitosis. Coulson (44) hypothesizes that this type of coating by specific antibody is directed against determinants in the *vicinity*, but not at, the mitogenic site on the lymphocyte surface. A steric hindrance of the mitogenic site results. When antigen is later added, little or no mitosis results, depending on how complete the hindrance is.

A microscopic inspection of Wright's stains of cultures should reveal differences if this hypothesis is correct. Where blocking has occurred, little or no evidence of lymphocytes with adherent streptococci should be seen. In contrast, when enhancement is seen in results, adherent, aggregated cocci should be present at lymphocyte surfaces.

If the antiserum does, indeed, recognize determinants on the lymphocyte surfaces, the chemical nature of these determinants cannot be deduced from the present experiment. If haptenic blocking had been tried and a less depressed response had been obtained, then the nature of the sites could be identified. (In this experiment, a hapten would be preincubated with antiserum, next lymphocytes would be added; further incubation would be done. Lastly,

"P" streptococci would be added.)

The blocking effect of enhancement that occurred when NAG was preincubated with the antiserum, before the streptococci were added, could be explained as follows: The hapten combines with antibody and the combination remains soluble. When streptococci are later added, the antibodies directed against NAG are blocked. Any antigen-antibody reactions that do take place are those between other determinants and antibodies of other specificities. Since less insoluble complexes are present, less transformation occurs than would take place without the NAG. Therefore, NAG and its antibody are responsible for a major part of the enhancement effect, probably due to the formation of antigen-antibody complexes that are present when NAG is attached to a carrier molecule. In the streptococcus, the NAG is attached to a rhamnose backbone (71).

Rhamnose monomers do not block enhancement. Rhamnose polymers might participate in blocking if used in a similar experiment. Or, rhamnose attached to NAG might prove to be the ideal inhibitor. McCarty (71) blocked a precipitin reaction up to 50% with NAG, but could not demonstrate blocking with rhamnose monomer.

Ayoub and Schmidt (68) tested the susceptibility of varying sizes of Group A carbohydrate fragments to rabbit macrophage degradative enzymes. The susceptibility was found to correlate with the capacity of the fragments to block Group A antibody precipitation with A carbohydrate antigen. The specific action of the

macrophage enzyme was the release of NAG. There are enzymes in the rabbit macrophage, and presumably, also in the human, that degrade Group A carbohydrate to NAG (25). In an *in vivo* situation, it could be hypothesized that when the hapten is released into the environment that also contains group-specific carbohydrate antibody, blocking of agglutination occurs between antibody and whole bacteria which have exposed carbohydrate layers. This blocking action is very similar to what was seen in the last transformation experiment reported. Here the NAG partially blocked transformation, presumably by preventing the formation of antigen-antibody complexes with surface carbohydrate on pepsinized streptococci. *In vivo*, if lymphocyte transformation were held to a minimum, the release of toxic products from stimulated lymphocytes would also be held to a minimum. Dumonde *et al.* (72) and others have shown that a variety of products are released from stimulated lymphocytes, parallel to their morphological transformation. These products include mitogenic factor, permeability factor, macrophage inhibitory factor, and nonspecific lymphotoxins. Dumonde terms these products "lymphokines." Their release into the environment should adversely affect live streptococci in the vicinity. In fact, studies have shown that Group A streptococci, devoid of M protein, can be cultured from the throats of apparently healthy humans who have recovered from streptococcal pharyngitis (11,12). Or, convalescents may carry cocci with M protein until opsonic

antibody level becomes high (73).

Hirata and Terasaki (34) found that Type 1 M protein blocks recognition of histocompatibility antigens on human lymphocytes by human antiserum to seven HL-A specificities. When Type 1 M protein was preincubated with allogeneic antiserum and then lymphocytes were added, blocking of expected cytotoxic reactions occurred. This means that Type 1 M protein is structurally similar to human histocompatibility antigens. Theoretically, if Type 1 M protein were covering a streptococcus, it might be recognized as "self." Whether Type 1 M protein would block lymphocyte mitogenic sites as well as histocompatibility sites should be tested. Type 6 M protein and Group A polysaccharide were tested by Hirata and Terasaki, but were not found to block reactions with allogeneic antiserum. In the experiments described in this thesis, Type 6 M did not completely block lymphocyte transformation; it was definitely stimulatory. However, pepsinized streptococci were more stimulatory. Therefore, Type 6 M masked some recognition sites that were more mitogenic to the lymphocytes tested. This was explainable because Type 6 was shown to be "foreign" or minimally recognized by the lymphocyte donors. If the experiments described in this thesis had used Type 1 instead of Type 6 streptococci, some information about the applicability of the Hirata and Terasaki work to lymphocyte transformation would be available.

SUMMARY AND CONCLUSIONS

How can the last transformation experiment suggest how Group A streptococci without M protein can live in the lymphoid tissue of the pharyngeal regions or tonsils of apparently healthy humans? Antibodies to the Group A streptococcal carbohydrate are present in most adults. Furthermore, phagocytes are present which should engulf and kill the bacteria. In addition, lymphocyte transformation should be triggered, according to the experimental data presented. With transformation, lymphokines would be released that would spell the end for the cocci.

Partial immunosuppression, on a local level, must be in operation. The manner in which this might take place would be that antibody to the basic Group A structure is neutralized by haptenic pieces from macrophage degradation of some of the streptococci. Bacteria-antibody complexing is thereby prevented. Lymphocyte transformation is held to a low level by the prevention of antigen-antibody complexing; few lymphokines are elaborated. Some of the phagocytes and lymphocytes that are attracted to the area may be killed by extracellular enzymes of the streptococci. Thus, a stalemate is set up and maintained. The streptococci do not multiply to any great extent, because they are decimated by local phagocytes which escape extracellular streptococcal enzymes.

In a like manner, it can be speculated that degradation of cocci with M protein can release haptenic M fragments which can block low-level opsonic antibodies and prevent phagocytosis of the cocci. Lymphocyte transformation is minimized, due to lack of antibody participation and to cytotoxic effects of wall fragments or extracellular products on the leucocytes. Later opsonic antibody levels become high enough to eliminate the M-containing streptococci, or else to select for cocci without M protein.

It will be recalled that less than normal lymphocyte transformation, in the presence of stimuli from streptococci, has been found in patients with certain sequelae to streptococcal disease, including rheumatic fever (54,55). Further experimentation to establish the nature of the suppression of transformation in the presence of these pathogens is indicated.

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