THE SIGNIFICANCE OF HUMAN LYMPHOCYTE TRANSFORMATION TO VARIOUS MICROBIAL STIMULANTS

by

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INTRODUCTION

Immunologic mechanisms provide the human with a measure of protection from materials present in the environment which may be potential pathogens. Classically, immunologic responses are divided into two categories: those mediated by humoral antibody, and those mediated by cells. However it is essential to remember that both types of responses are dependent upon the activity of the small lymphocyte. The small lymphocyte recognizes the antigen, undergoes transformation or blastogenesis, may recruit other nearby cells, may differentiate into antibody-producing cells, and may itself produce mediators or other soluble products such as migration inhibition factor, transfer factor, skin reactive factor, etc., which are commonly associated with delayed-type hypersensitivity.

Lymphocyte transformation is an established *in vitro* event whereby small lymphocytes transform morphologically into larger lymphoblasts. Such cells exhibit increased DNA synthesis prior to mitotic division and cell multiplication. Increased DNA synthesis can be assayed through the uptake of radioactive thymidine and serves as an accurate estimation of level of transformation. (1)

Various materials are capable of inducing lymphocyte transformation. Oppenheim (2) has divided these materials into four separate categories as follows: 1) non-specific stimulants such as phytohemagglutinin or PHA, pokeweed mitogen, streptolysin S, etc. These

mitogens are non-specific only in the sense of antigenic specificity; that is, they stimulate lymphocytes from fetal as well as from adult blood. They also characteristically lead to higher levels of *in vitro* transformation which are attained during relatively short incubation periods, 2) tissue antigens, as found on homologous lymphocytes in the form of histocompatibility antigens, 3) antisera against white cells, and 4) specific antigens including PPD, tetanus toxoid, penicillin, etc. Transformation induced by specific stimulants is dependent upon prior host sensitization to the particular antigen.

A generally accepted hypothesis states that the *in vitro* lymphocyte transformation test assays for the presence of the antigen-specific memory cells in a population of lymphocytes. (3) Thus it would seem that positive lymphocyte transformation responses to a particular antigen would indicate previous host sensitization by that antigen, at least in terms of the specific antigens as defined above. Such prior contact with the antigen would be essential in order to supply the host with the necessary numbers of antigen reactive or memory cells felt to be responsible for the *in vitro* response which we see as lymphocyte transformation. It would seem reasonable, therefore, to expect that the list of specific antigens cited above would be expanded with further testing to include all antigens with which most individuals have had sufficient contact for sensitization to occur.

Acceptance of this hypothesis leads to a series of important questions concerning the relationship between lymphocyte transformation and oral bacterial antigens. We must ask if oral bacterial

antigens act as specific antigens in stimulating lymphocyte transformation. Do these antigens in some way come in contact with the host's immune system, evoke a response in the local lymph node, and then generate production of specific defense cells and memory cells which may then at a later time, when examined in vitro, give a positive transformation response? If so, does this type of stimulation reflect normal antigenic contact, or is the response seen only to bacteria which have at a previous time been responsible for some clinically detectable disease state? Is response to plaque antigens the result of clinically apparent periodontal disease, or can individuals without any periodontal disease also become sensitized to plaque materials? Can the lymphocyte transformation test be used to reveal a susceptibility in a group of patients which would lead to future periodontal destruction? Would high levels of lymphocyte transformation to one or two plaque antigens in a group of patients allow us to make any conclusions about whether sensitivity to these materials was the initial cause, or only the result, of the inflammatory periodontal problem? Certainly we cannot expect a group of people picked at random to react identically to any specific antigen. It would only seem reasonable to expect a great deal of biologic variation in terms of the immune response of each individual to any naturally contacted bacterial antigen. What usefulness does lymphocyte transformation have in terms of helping us to understand the mechanisms of periodontal disease? In order to answer these questions a great deal of research and examination of ideas is yet to be done.

In an attempt to collect information which would aid in our

understanding of "normal" immune responses to microbes of the body flora, lymphocyte transformation studies were done on a representative sampling of the general population. The sampling included persons with varying degrees of periodontal disease who were otherwise medically healthy. The objectives of this study were threefold:

1) to assay lymphocyte transformation responses to a number of plaque and non-plaque bacterial antigens.

2) to begin to establish the range and normal limits of lymphocyte responses to these antigens within a group of people felt to be representative of the general population.

3) to attempt to more clearly define the role of the immune response in general, and lymphocyte transformation in particular, in the pathogenesis of periodontal disease.

Information gathered from this investigation was evaluated and interpreted with respect to other current concepts in the area of lymphocyte responses in medically healthy individuals, as well as in the area of the relationship of lymphocyte transformation responses in people with periodontal disease. It is hoped that this study will be a significant contribution to our understanding of the immune system and its functions in the pathogenesis of periodontal disease as well as its role in the broader aspects of host responses to any foreign material.

REVIEW OF THE LITERATURE

Transformation of human peripheral lymphocytes has been used by many, correctly or incorrectly, as an *in vitro* correlate of delayed-type hypersensitivity. The focus in most studies has been upon the response of lymphocytes to antigens of organisms known to be pathogenic (e.g. PPD), to antigens of haptens thought to be associated with various clinical allergies (e.g. penicillin), or to antigens associated with autologous tissue in cases of suspected autoimmune diseases. Little effort has been directed toward the examination of lymphocyte transformation responses toward antigens of the normal microbial flora in non-diseased individuals or in those individuals without allergic disorders. In some of the previously mentioned types of studies however, control subjects have been evaluated with results which are of interest to us in our consideration of the host's response to his own microbial flora or to other materials which may have come in contact with the immune system.

As early as 1934, Ratner and Gruehl (4) demonstrated that protein antigens were capable of passing through the gastrointestinal tract epithelium when ingested by mouth in the form of food. After feeding specific foods to individuals sensitized by subcutaneous injection of serum from allergic individuals, positive allergic skin responses could be noted, indicating passage of the antigenic food protein through the wall of the gastrointestinal tract and into the circulation. Likewise, May and Alberto (5) have reported significant lymphocyte

transformation responses in normal control children who were tested for sensitivity to milk proteins, ovalbumin, and wheat extract. Sensitivity to fetal calf serum has been described (6) and it was felt to be the result of ingestion of certain beef antigens via the diet which are similar to those antigenic determinants present in serum of unborn calves. Development of blood group antibodies is also felt to be closely related to and dependent upon ingestion of bacterial and plant polysaccharides in the diet of the infant (7), further supporting the idea that passage of antigenic material through the epithelium of the gastrointestinal tract is possible and quite common.

Lymphocyte responses of normal, non-diseased individuals to specific antigenic stimulants have been described as well. Reactivity to Streptococcal M proteins (8,9) has been described, as well as positive transformation responses to Staphylococcal protein A (10), Candida albicans antigen (11), and to a commercially available mixed bacterial antigenic material(12). In another experiment, Andersen (13) immunized a group of previously unsensitized individuals with Brucella abortus vaccine and tested their lymphocyte transformation responses to the antigen with the passage of time. Several patients sensitized in this manner developed positive lymphocyte transformation responses within as little as three days, thus demonstrating that immunizing doses of an antigen are sufficient for the production of specific antigen reactive memory cells in vivo. Similar transformation responses have been seen following immunization with Keyhole Limpet haemocyanin, again demonstrating that varying amounts of antigen are sufficient to elicit immune responses, measurable by the in vitro lymphocyte transformation test. Thus the

conclusion is reached that a clinical disease state induced by a specific organism is not a requirement for production of measurable lymphocyte transformation tests to a specific antigen of that organism.

Evaluation of lymphocyte transformation responses in patients with periodontal disease then becomes of interest in two ways. First, the use of medically normal subjects with varying degrees of periodontal disease would be a reflection of the "normal" pool of healthy people. As such, evaluations of their responses to a variety of microbial antigens would help to establish baseline data concerning normal responses to host flora. Second, by the proper choice of subjects it would be determined whether the responses seen in these medically healthy individuals were in fact related to the severity of clinically detectable gingivitis or periodontitis.

Very little has been done in the clinical area which would enable us to identify the bacterial product or products causative of gingivitis and/or periodontitis. Potentially damaging products of bacteria include enzymes, acids, exotoxins, endotoxins, and antigens. (14) It is safe to assume that a great number and variety of each is present in the sulcular area of the gingiva. Efforts have been made by many researchers to provide evidence which would substantiate the role of these materials in the etiology of periodontal disease. However, it is not pertinent to our study to include a comprehensive outline of these types of studies.

Although the presence of potential bacterial antigens in or near the gingival sulcus is felt to be contributory to the progression of periodontal disease, it was not until quite recently that any evidence existed to support the concept of antigen penetration of

the gingival barrier followed by the appropriate defensive immune response. Studies by Berglund (15), Ranney and Zander (16), and Ranney (17) indicated that artificial sensitization of animals via the gingival sulcus to foreign protein resulted in the production of specific antibody in the regional lymph nodes. It also seems that a similar sensitizing procedure could be occurring in human gingival areas if foreign proteins were penetrating the tissue, a possibility which seems likely in inflamed and often lacerated periodontally diseased gingival tissues and possibly in intact tissue as well.

Accordingly, investigations designed to show presence of circulating or tissue antibodies to naturally occurring oral bacterial antigens were successful. Mergenhagen, (18) showed low but significant levels of antibody present against Leptotrichia and Veillonella upon analysis of sera from non-periodontally diseased individuals. Nisengard (19) examined diseased and non-diseased individuals for sensitivity to Actinomyces antigens and observed both immediate and delayed-type responses in small but significant numbers of diseased individuals. Berglund (20) showed the presence of both local and circulating antibody to Fusobacterium, E. coli, and Veillonella when he examined periodontally diseased individuals. Therefore, the evidence seems conclusive that oral antigens do penetrate gingival epithelium, stimulating specific antibody production, and possibly influencing the progression of periodontal disease. Yet to be answered of course is the question of whether or not the antigen penetration occurs through a clinically healthy gingival sulcus, or whether antigen penetration in these instances is only secondary to the disease process, with penetration occurring

through an already inflamed epithelium.

Lymphocyte transformation studies in the periodontal literature are few to date, due primarily to relatively recent availability of the techniques involved. Ivanyi and Lehner have been responsible for the bulk of published material along these lines. In the first of a series of papers (21) Ivanyi and Lehner evaluated lymphocyte responses to various antigens in patients with varying degrees of periodontal disease. The patient's periodontal disease status was evaluated by means of Russell's Periodontal Index, and the patients were classed as controls (PI less than 0.2), those with gingivitis (PI of 0.2 to 0.9), those with periodontitis (PI of 1.0 to 4.0) and those with severe periodontitis (PI of greater than 4.0) Antigenic stimulants used were sonicates of Veillonella, Fusobacteria, Bacteroides, Odontomyces, Lactobacillus, and Proteus. Sonicates of Odontomyces, Fusobacteria, Bacteroides, and Veillonella induced specific transformation responses, with Odontomyces being the most potent stimulant. Good stimulation was seen in patients with gingivitis and mild to moderate periodontitis, but depressed transformation rates were seen in the severe periodontitis group. No response was seen in the controls. Observations of the depressed response in severely affected patients led to the next experiment (22) in which the same procedure was repeated using sonicated whole plaque, Veillonella, and Odontomyces. Duplication of the earlier results led them to propose that whole plaque shared antigenic determinants with the pure bacterial sonicates of Odontomyces and Veillonella. Additional work led them to conclude that the lymphocytes of the non-diseased controls were not sensitized to antigens

which were stimulatory to lymphocytes of the diseased groups. They felt that the depression of transformation responses seen in the severely involved group was somewhat more complicated, since these individuals would have been sensitized if we assume severe periodontitis is a continuation of moderate periodontitis. They postulated that this inhibition was due either to the presence of an antigen-specific serum inhibitory factor, or possibly due to feedback inhibition of some sort. Further work was done on this problem (23) in an attempt to further identify the serum factor responsible for the inhibition of transformation in the severely involved group. Evidence accumulated by Ivanyi and Lehner in this and a later publication (24) is not at all consistent with the postulated role of serum factors, but a general pattern has emerged from their work.

They have concluded that non-diseased control individuals do not respond to oral antigens (bacterial sonicates), theoretically due to a lack of contact and/or sensitization with those products. In contrast, patients with gingivitis and mild to moderate periodontitis had apparently been sensitized to the bacterial antigens tested, and gave the appropriate response when their lymphocytes were challenged with the various materials. Ivanyi and Lehner further concluded that in patients with severe periodontitis, an inhibitory serum factor was consistently present, which was capable of causing a depression in the lymphocyte response as compared with those of the GMP group. They postulated that this factor might be antibody, acting either as a blocking antibody, or if present in excess and complexed with antigen, acting as an inhibitor of <u>in vitro</u> transformation, a possibility explained by Oppenheim. (25)

Ivanyi and Lehner's work is impressive, by sheer effort and time involved. Unfortunately, some major complications arise in their series of papers. These complications center around their apparent evidence for the presence of inhibitory and/or stimulatory factors thought to be present in the sera of patients with varying degrees of periodontal disease. Ivanyi and Lehner propose that the variable effects of lymphocyte transformation rates are the result of the modulating effect of antibody. However, they could not detect the postulated antibody with conventional serological techniques. Their most recent publication centered around the use of fetal calf serum, rather than autologous serum, in an attempt to remove the serum factors. This attempt was apparently unsuccessful since lymphocyte responses in that study were unchanged in pattern when compared with their previous publications.

Some clarification of their work is necessary if we are to regard it as non-contradictory to the hypothesis of presence or absence of serum factors. One explanation may be that their serum factor is bound to the reacting cells' surface somewhat tightly, and is not removed in washing of the cells. If it remains bound, then it may exert its effect even if fetal calf serum is used, the fetal calf serum being free of such autologous serum factors. It could thereby resist identification by traditional serological techniques as well. Unfortunately, this theory does not explain why cells from severely affected periodontal patients, where the serum factor would be tightly bound to the cell, transform better when homologous serum from patients with mild to moderate periodontal disease is used in the culture. If the serum factor is bound to the cell in sufficient

quantity as to be inhibitory, why is an increase in relative stimulation seen when these cells are incubated in a system with homologous serum? Why is the blocking factor functional in fetal calf serum cultures, but not in the homologous cultures? Perhaps future work by these authors will clear up the apparent inconsistencies in their hypothesis. Until that time, their work can only be regarded as somewhat confusing to the reader who thoroughly examines their publications on the basis of their results as well as their conclusions.

Only one other publication in this area is available for examination, but it does not aid in interpretation or clarification of Ivanyi and Lehner's work. Horton (26) has examined unstimulated saliva and supra- and sub-gingival plaque for its ability to stimulate lymphocyte transformation. Subjects without periodontal disease did not generally respond to their own saliva and plaque. However, diseased individuals responded well to both saliva and plaque sonicates. This work indicates that material is present in both saliva and plaque to which periodontally diseased individuals are sensitive. Horton's work continues with emphasis on production of mediators by stimulated lymphocytes, and the biological functions of these mediators.

Overall evidence published until now by Horton and by Ivanyi and Lehner indicates that plaque materials have been present and have sensitized patients with periodontal disease, so that the *in vivo* sensitivity of these patients' lymphocytes is reflected in various significant levels of transformation. They conclude that this sensitivity is apparently not present in patients without

periodontal disease.

Thus, the literature cited leads us to the interesting and apparently conflicting conclusions that one group of medically normal subjects mount immune responses toward various antigens, both microbial and non-microbial, that come in contact with gastro-intestinal and/or skin surfaces, but that another group of medically normal subjects mount immune responses toward antigens of the oral bacterial flora 1) only if the gingival tissues are diseased, and 2) then in direct relation to the severity of the clinically observable inflammation. (26)

MATERIALS AND METHODS

Patient selection.--Subjects were chosen from among employees of the University of Oregon Dental School and from among patients receiving treatment at the Graduate Periodontology Clinic at that school. In order to evaluate periodontal disease severity, the patients were scored with the Löe Gingival Index (27), the Periodontal Index of Russell (28), and the Periodontal Disease Index of Ramfjord (29). Patients sampled using these criteria were medically healthy, and thus represent a selected sampling of the population further evaluated for one non-systemic disease state, periodontal disease.

Periodontal evaluation.--The Löe Index was chosen in order to allow proper evaluation of the gingival condition, without regard to the deeper periodontal problem. Gingival inflammation is felt to be a good correlate with the amount of plaque and debris present in any one area of the gingiva (30,31). Thus, the gingival index used here should be a reflection of the amount of debris present on the teeth, which theoretically may be related to the host response in terms of the immunologic response to challenge by foreign bacterial products.

The Ramfjord Index was chosen as the primary means of evaluating more extensive periodontal pathology. If in fact the severity of periodontal disease is related in some way to lymphocyte transformation responses, then some means of evaluating periodontal

disease is necessary. The Ramfjord Index fulfills this need by being a well-accepted method of scoring disease with the added advantage of distinguishing between small differences in disease severity, thus allowing the clinician to accurately assess gradations of periodontal disease on an individual basis.

The Russell Index is used solely as a supportive index in evaluating periodontal disease severity. Other investigations in this field of lymphocyte transformation in patients with periodontal disease have done all periodontal evaluations using the Russell Index. This index was intended to serve as a gross measure of periodontal disease in large populations where the large sample size examined would tend to minimize the inaccuracies present in the index. On smaller numbers of subjects, such as in our study, the Russell Index serves only as a crude approximation of disease severity. Nevertheless, we used it so as to be able to roughly compare our subject pool with reports of subjects sampled in the literature.

All subjects were evaluated in the Graduate Periodontology Clinic by the same examiner. The GI, PI, and PDI were done at the same time in most instances, and a brief questionnaire was also filled out in order to exclude persons with systemic disease or obvious allergic problems. Examinations were performed prior to the receiving of any formal periodontal care in our clinic so as to measure periodontal involvement at its pre-existent level. Length of time between the periodontal evaluation and collection of the lymphocyte sample did not exceed four to six weeks. All periodontal examinations were carried out using a standard dental light, mouth

mirror, and periodontal probe. GI and Ramfjord Index scores were recorded on a specially prepared form, and a Russell Index was calculated in addition with the use of a periodontal probe as an aid in determining the presence or absence of pockets.

Lymphocyte collection.--Between 150 and 180 ml. of whole venous blood was obtained from each patient by bleeding from the antecubital fossa. Immediately upon collection, the blood was defibrinated by use of a teflonized steel wool pad and constant agitation of the blood sample. After completion of defibrination, the pad was rinsed with TC 199 media in order to remove available cells, and the pad rinsings were added to an equal volume of the defibrinated blood. The whole blood- TC199 rinsings were layered onto sterile Ficoll/Hypaque and centrifuged so as to differentially separate the various cell types into definable and separable layers. The mononuclear-rich layer was collected with a sterile capillary pipette. These cells were washed twice in Hanks basal salt solution (GIBCO) and centrifuged following each washing. After final centrifugation and decanting, the cells were suspended in supplemented MEM-S (minimal essential media - Spinner, GIBCO) at a concentration of 5×10^6 mononuclears/ml. as determined by differential counting. The MEM-S was supplemented for support of lymphocytes by addition of 10% heat-inactivated sterile serum, 1% glutamine, and 0.1% penicillin-streptomycin solution (glutamine and penicillin-streptomycin available from GIBCO).

Antigens used.--Extracts of various microorganisms were used as antigenic stimulants in the evaluation of lymphocyte responses. Four concentrations of each antigen were used with each patient, with

triplicate tubes being used for each concentration. In addition to the bacterial extracts, two endotoxins and PHA were also evaluated at three concentrations with triplicate tubes used for each concentration. Six saline controls per individual were also used. Antigens were diluted to specific concentrations with MEM-S supplemented as previously described, the dilutions being made so as to allow us to approximate ranges of concentration which would give us maximum stimulation under the culture conditions used in our study.

Culture and harvest of lymphocytes.--A microculture system was used whereby 0.1 ml. of 5×10^6 mononuclear cells/ml. was added to 0.4 ml. of antigen diluted in MEM-S as previously described. Cultures were then incubated for the chosen period of time at 37° C. in 5% CO_2 and 95% air. (32) Cultures were incubated for five days under these conditions, and at the end of the day five 0.05 ml. of C-14 thymidine (New England Nuclear, 50 µC/0.5 ml., diluted to approximately 2 µC/ml. with saline and MEM-S) was added to each culture, with the cultures then being replaced in the incubator for another 24 hours to allow uptake of the label.

At the end of day six, cultures were removed from the incubator, cooled in an ice bath to stop cellular activity, and centrifuged at 1,500 x G for five minutes. The supernatant containing excess media and label was decanted, leaving a small cell button at the base of the tube. This button was suspended by agitation, washed onto GF/C glass fiber pads (Whatman) with ice cold physiologic saline (0.85%) and then rinsed repeatedly with a total of about 50 ml. of cold saline so as to remove all excess label not taken up by the cell.

The GF/C pads were then transferred into standard glass scintillation vials, and ten ml. of Insta-gel (Packard) added to each vial. After overnight equilibration at room temperature, the vials were placed in a scintillation counter, cooled, and counted using standard techniques. True counts were obtained, adjusted by the subtraction of background counts, and expressed as disintegrations per minute (d.p.m.'s). Stimulation indices were also calculated using the following formula which is standard:

$$S.I. = \frac{\text{d.p.m. of antigen stimulated culture less background}}{\text{d.p.m. of saline stimulated culture less background}}$$

Confidence intervals were determined by use of the t test on means of the values obtained using the triplicate tubes.

Serum used.--Several types of serum were used as supplements for the MEM-S at various stages of this investigation. All sera used were added at 10% concentrations following heat inactivation for thirty minutes at 56° C and filter sterilization. In most instances, autologous serum was used, obtained in the fresh state from the blood donor and prepared on the day of use. On other occasions, homologous serum was also used to study the effect of serum borne mediators which could theoretically affect transformation rates. Serum used in these instances was identical to the autologous serum, except that it was adsorbed with AB cells, and freeze stored until used. The other serum type which was used was fetal calf serum (commercially available from GIBCO) which also was filter sterilized and heat inactivated.

Antigen preparation.--Material extracted from a wide variety

of microorganisms was used as stimulant for our lymphocyte cultures. Protein-rich extracts were prepared from strains of the following organisms: Bacterionema matruchotii, Bacteroides melaninogenicus, Candida albicans, Streptococcus salivarius, Streptococcus mutans, Escherichia coli, Veillonella, Actinomyces naeslundii, Actinomyces israelii, Leptotrichia buccalis, Pseudomonas species, and Neisseria perflava. All organisms are common inhabitants in humans, most in the oral cavity (33). The strain of S. mutans used in this study was of hamster origin, but was believed to share common antigenic determinants with human strains. E. coli, Candida, and Pseudomonas were included in this study as "non-plaque" controls, with the rest being characteristically present on oral mucous membranes or in plaque. All extracts of these materials were prepared in an identical manner.

Pure cultures of the above-mentioned bacteria were grown under the appropriate optimal conditions and collected at or shortly following the log phase of growth. These cells were collected and washed thoroughly with physiologic saline. After washing and centrifugation with several volumes of saline, the cells were diluted to a slurry with saline in a ratio of one volume of cell pack to nine volumes of saline. This mixture was then adjusted to pH 12.7 with NaOH and stirred at room temperature for $1\frac{1}{2}$ to 2 hours. Upon completion of the extraction, the pH was readjusted to about 7.2 with HCl, and the material was then centrifuged at about 10,000 x G for 15 to 20 minutes. The supernatant was collected, filtered through a 0.45 micron filter, then dialyzed exhaustively in physiologic saline. When necessary in order to reduce volume to more convenient levels, the material was pervaporated and redialyzed.

Upon completion of dialysis, the supernatants were once again filtered in 0.45 micron filters for sterility and removal of non-soluble materials and were then freeze stored at -70° C until used in the lymphocyte transformation experiments. This technique was adapted from the methods of Fox and Dawes, (34) and was felt to be a technique which would yield maximum amounts of non-hydrolyzed protein. These extracts will subsequently be referred to as NaOH extracts of the various organisms in the following manner: B. mat.-N, B. mel.-N, Candida-N, SRi-N (S. salivarius), E-49-N (S. mutans), E. coli-N, V1-N, A. naes.-N, T6-N (A. israelii), Lepto-N, N. perf.-N, and Pseudo-N.

Our extracts of pooled plaque samples were prepared in a similar manner from a large pool of dental plaque. Plaque samples were obtained as a courtesy of the University of Oregon Dental Hygiene Department. The NaOH extract of plaque (NEP) was prepared by treatment of pooled plaque samples by the method described above. A second type of plaque extract was prepared in which no pH adjustment was employed. This extract is called SEP (saline extract of plaque), and was obtained by stirring pooled plaque samples in cold saline, collection of the soluble material, then pervaporating, dialyzing, and filter sterilizing the material.

The endotoxins used in our study were of two varieties, the first being a commercially available E. coli endotoxin prepared by Difco from E. coli 026B6, and diluted to a stock concentration of 486 µg/ml. in MEM-S. This material will be referred to as E. coli LPS. The second material was an endotoxin of Leptotrichia buccalis prepared in the manner described by Knox and Parker (35), and this

too was made up to a stock solution concentration of 486 µg/ml. This material will be referred to as Lepto LPS.

Another extract of Candida albicans (DM"O") was used. DM"O" is commercially available as Dermatophyton "O" (Hollister-Stier). For use it was dialyzed until phenol-free and then lyophylized and used on a dry weight basis diluted in MEM-S.

In order to assay for relative endotoxin content of our Lepto-N material, mouse lethality curves were run. Mice were injected with Actinomycin D followed by dilutions of the phenol soluble endotoxin of Leptotrichia and of the NaOH extract of Leptotrichia. From the LD_{50} plot derived by the deaths of mice, it was calculated that the endotoxin content of the Lepto-N was less than 10% of the dry weight.

Titration of antigens.--In order to avoid using sub-optimal or inhibitory concentrations of antigen in our cultures, titration curves were done using various concentrations of antigen in the lymphocyte cultures. This enabled us to use proper amounts of antigen without fear of toxicity or lack of stimulation due to improper amounts of antigenic stimulant. Four concentrations of each antigen were used on each patient tested in order to be able to plot a dose-response curve for the individual antigens. This dose-response curve served as a check against missing the optimal antigen concentrations, and also enabled us to determine the approximate maximal response to each antigen for a given patient in a given time period. This proved valuable in terms of interpreting the results of each lymphocyte run. Six days was chosen as the incubation period in our system. Although the six-day incubation period was past the

optimal stimulation time of PHA, the PHA was intended to serve only as a positive control for the system as a whole. Six days did seem to reflect with accuracy the lymphocyte sensitivity to the individual antigens in a qualitative manner. Incubation beyond that period of time revealed only a quantitative variation in terms of higher d.p.m.'s while the qualitative nature of the response seemed unchanged.

Summary.--Our system involved measuring a patient's lymphocyte response to thirteen NaOH extracts of bacterial cultures and materials and to two relatively pure endotoxins, with PHA and saline stimulated controls. The thirteen NaOH antigens were evaluated at four concentrations each and the endotoxins and PHA at three concentrations each, so as to provide a dose-response curve for each antigen. This made it possible for us to measure the peak response to any one antigen at the six day period. Transformation at each concentration was evaluated in triplicate and the mean result expressed as the measurement for that concentration of antigen. A microculture technique was utilized and incubation was carried out for six days. Cultures were labelled with C-14 thymidine at day five, and on day six, the cells were washed with saline and collected on GF/C pads. These pads were counted in a scintillation counter and the data collected as disintegrations per minute minus background. These techniques proved adequate and reliable for the assaying of *in vitro* lymphocyte transformation to selected bacterial antigens and other materials in patients evaluated in our study.

RESULTS AND DISCUSSION

Effects of serum source on transformation responses.--Our preliminary studies using the phenol-soluble endotoxin of Leptotrichia (Lepto LPS) indicated that fetal calf serum would not support transformation as readily as autologous serum. The question arose as to whether enhancers of transformation (23,36) might be lacking in fetal calf serum and a study was therefore designed to compare media containing either 10% autologous or 10% fetal calf serum. The Lepto LPS and the E. coli LPS were evaluated. Negative controls consisted of saline and antigen controls were DM"O", SEP, and Lepto-N. The data obtained in this study are presented in Table 1.

Interpretation of changes in transformation levels are difficult and open to question. First, what is to be considered as change in transformation, changes in d.p.m.'s, changes in S.I.'s, or both? Second, how much of a change in either is required before it can be considered to be significant. The central problem in answering these questions is the variation among control (saline) cultures of the same cells in media supplemented with different sera. An increased or decreased control count will markedly alter S.I.'s unless the antigen-induced stimulation is altered by the same numerical factor as was the control stimulation. We have tried several methods of evaluation in order to take these considerations into account, and have arrived at the following procedure. First, we must determine for a given antigen whether the antigen-induced level of stimulation (d.p.m.) in

Table 1

Comparison of Responses to Five Stimulants in Autologous and Fetal Calf Sera.

Patient	Stimulant	Autologous S.I.	Autologous d.p.m.	Fetal Calf S.I.	Fetal Calf d.p.m.
H.C.	Saline control		210		5073
	SEP	12.5	2620	1.0	5164
PI=2.2	Lepto LPS	8.4	1769	1.0	5281
	Lepto-N	32.2	6760	1.0	5078
	E. coli LPS	4.3	911	0.9	4547
	DM"O"	105.7	22202	2.1	10876
K.D.	Saline control		94		3960
	SEP	25.1	2333	1.2	4706
PI=3.9	Lepto LPS	16.3	1528	1.1	4420
	Lepto-N	75.8	7049	1.1	4417
	E. coli LPS	10.7	994	1.3	5114
	DM"O"	55.7	5189	1.9	7365
H.N.	Saline control		42		407
	SEP	8.6	362	0.7	275
PI=4.6	Lepto LPS	5.2	219	0.6	232
	Lepto-N	21.3	896	1.0	414
	E. coli LPS	5.2	217	0.7	266
	DM"O"	132.0	5501	2.3	938
L.F.	Saline control		7		35
	SEP	10.9	76	2.7	93
PI=5.4	Lepto LPS	12.4	87	2.6	89
	Lepto-N	72.6	508	3.8	131
	E. coli LPS	9.4	66	2.3	79
	DM"O"	52.0	364	9.7	341
R.L.	Saline control		26		53
	SEP	11.4	297	6.2	330
PI=5.8	Lepto LPS	3.8	99	1.3	68
	Lepto-N	34.2	889	5.2	277
	E. coli LPS	2.9	75	1.7	88
	DM"O"	110.2	2865	16.9	899
E.F.	Saline control		104		225
	SEP	0.8	76	0.9	208
PI=6.0	Lepto LPS	0.8	84	1.0	221
	Lepto-N	3.3	345	1.5	337
	E. coli LPS	0.7	78	0.8	171
	DM"O"	26.9	2691	6.0	1345
L.P.	Saline control		32		44
	SEP	6.4	204	5.0	221
PI=6.4	Lepto LPS	3.1	98	1.9	85
	Lepto-N	11.6	371	2.9	123
	E. coli LPS	2.3	74	1.8	81
	DM"O"	28.5	912	2.6	160

autologous serum is significantly different at the 95% level of confidence from that found in fetal calf serum, and second, we compute the S.I.'s for each antigen using as control the level of stimulation in saline in the respective media (autologous or fetal calf). Using the results of these two steps, we assign a plus sign (+) if the d.p.m.'s in autologous serum are at least double the d.p.m.'s found in fetal calf serum, and if the S.I. in autologous serum shows any increase over that in fetal calf serum. Or, we assign a negative sign (-) for the comparison if the autologous d.p.m. value is one half or less of the d.p.m. value in fetal calf serum and if the S.I. in autologous serum shows any decrease as compared to the S.I. in fetal calf serum. We assign a zero (0) in all other instances.

In this evaluation, a positive is considered evidence for the presence of serum-borne enhancer of transformation in autologous serum, and a negative is considered as indicative of a serum-borne inhibitor of transformation in autologous serum. This analysis takes into account the drastic effect of changing control values on S.I.'s and it demands a difference between the transformation levels (d.p.m.) that is statistically significant.

The described procedure was used in evaluating the data in Table 1. It should be emphasized that the procedure is an evaluation of the possible presence of enhancers or inhibitors in autologous sera, and cannot be looked upon as simply a comparison to two media with respect to their capacities to support significant transformation levels. The results of the evaluation are shown in Table 2.

Table 2

An Estimation of the Presence of Enhancers or Inhibitors of Transformation in Autologous Serum.

Antigen	Cell Source						
	HC	KD	HN	LF	RL	EF	LP
Lepto LPS	0	0	0	0	0	0	0
E. coli LPS	0	0	0	0	0	0	0
SEP	0	0	0	0	0	0	0
Lepto-N	0	0	+	+	+	0	+
DM"O"	+	0	+	0	+	+	+

This study produced no evidence to support the contention that endotoxin-specific or SEP-specific enhancers or inhibitors of transformation exist in the autologous sera tested. It is seen in Table 1 that S.I.'s were generally lower in fetal calf than in autologous sera, but the analysis depicted in Table 2 indicates that the shifts in S.I.'s are most frequently due to increased control values in media containing fetal calf serum, and are not due to the presence of specific enhancers of transformation in autologous sera. In contrast, the analysis indicates that enhancers specific for DM"O" existed in five of seven autologous sera and that enhancers specific for Lepto-N existed in four of seven autologous sera. In these nine situations where enhancer seemed to be present, the analysis indicated that the lowered S.I.'s seen in fetal calf serum could not be explained on the basis of elevated control levels of stimulation in fetal calf serum. Rather, the analysis indicated that the presence of antigen-specific enhancers of transformation in autologous sera were responsible for the increased stimulation seen in the autologous system.

Stimulation by endotoxin.--The results of transformation to the phenol soluble endotoxin of L. buccalis and to the endotoxin of E. coli were obtained by testing these materials in culture with the lymphocytes from four control individuals and from eighteen patients with periodontal disease ranging from mild gingivitis to moderate periodontitis. All cultures were run with autologous serum as the MEM-S supplement.

Table 3

Patient Response to Endotoxin and NaOH Preparations of E. coli, L. buccalis.

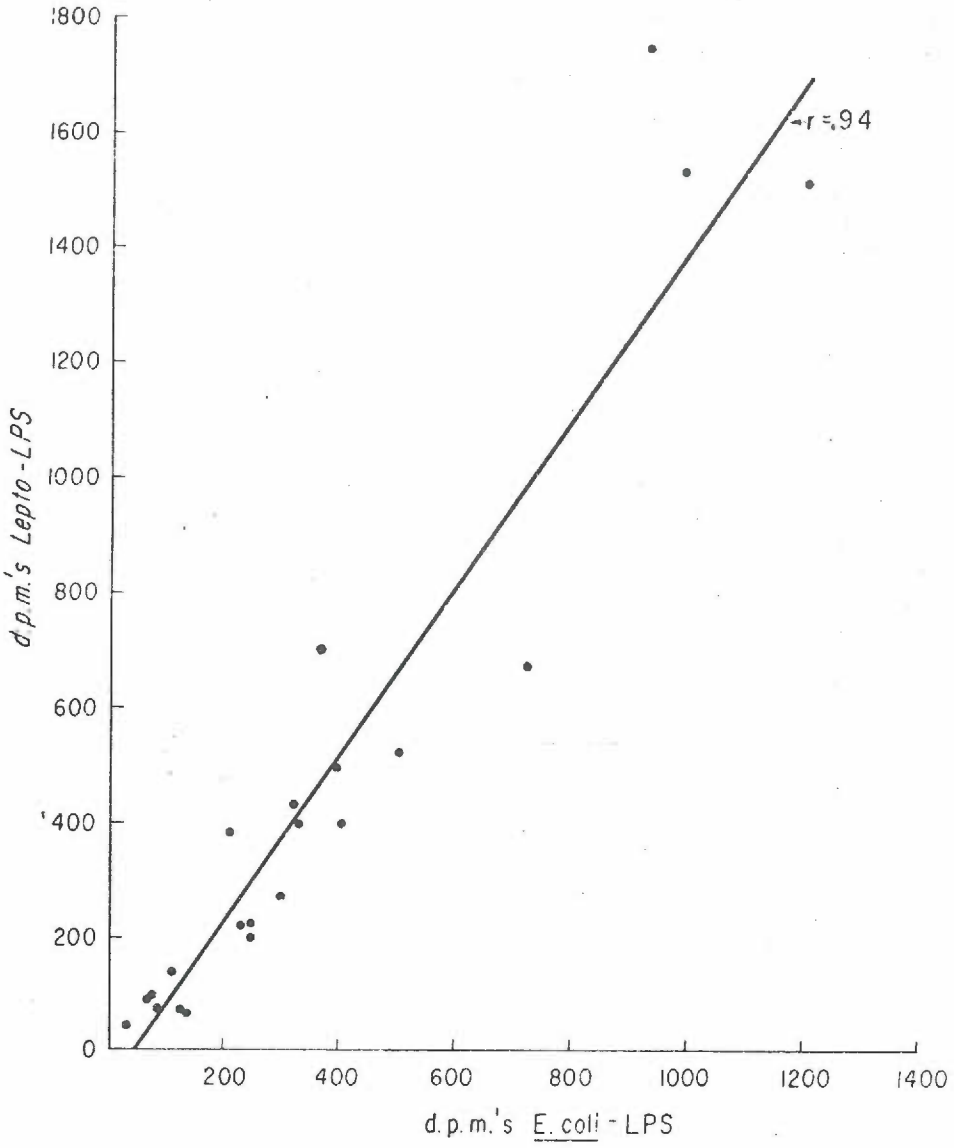
Patient (*Control)	Control d.p.m.	Lepto-N d.p.m.	(SI)	Lepto-LPS d.p.m.	(SI)	E. coli-LPS d.p.m.	(SI)	E. coli-N d.p.m.	(SI)
H.N.	130	2169	(25.3)	391	(4.1)	402	(4.2)	----------	
E.F.	104	345	(3.3)	84	(0.8)	78	(0.7)	----------	
H.C.	210	6760	(32.2)	1769	(8.4)	911	(4.3)	----------	
K.D.	94	7049	(75.8)	1528	(16.3)	994	(10.7)	----------	
H.N.	42	896	(21.3)	219	(5.2)	217	(5.2)	----------	
R.L.	26	889	(34.2)	153	(3.7)	75	(2.9)	----------	
L.F.	7	508	(72.6)	87	(12.4)	66	(9.4)	----------	
L.P.	32	371	(11.6)	98	(3.1)	74	(2.3)	----------	
W.W.	46	3429	(74.5)	380	(8.3)	207	(4.5)	3443	(74.8)
E.C.	34	1783	(52.4)	431	(12.7)	335	(9.9)	6024	(177.2)
O.C.	87	2503	(28.8)	272	(3.1)	301	(3.5)	3186	(36.6)
*B.O.	44	4098	(93.1)	221	(5.0)	253	(5.8)	6806	(154.7)
H.C.	30	752	(25.1)	139	(4.6)	110	(3.7)	1387	(46.2)
E.M.	18	797	(44.3)	74	(4.1)	140	(7.8)	2283	(126.8)
R.K.	193	5686	(29.4)	1496	(7.8)	1209	(6.3)	11530	(59.7)
D.R.	51	2176	(42.7)	396	(7.8)	341	(6.7)	9360	(183.5)
P.C.	99	5528	(55.8)	200	(2.0)	245	(2.5)	6541	(66.1)
S.S.	124	3087	(24.9)	675	(5.4)	730	(5.9)	5320	(42.9)
W.G.	477	2039	(4.3)	491	(1.0)	398	(0.8)	4323	(9.1)
*C.S.	28	279	(10.0)	53	(1.9)	36	(1.3)	1412	(50.4)
*F.M.	51	831	(16.3)	665	(13.0)	395	(7.7)	1189	(23.3)
*J.S.	28	868	(31.0)	520	(18.6)	505	(18.0)	1196	(42.7)

In sixteen of the eighteen patients and in three of the four controls, stimulation was seen to significantly exceed the control

levels with S.I.'s of two or greater. The stimulation indices ranged from 0.7 to 18.6 with only three subjects below a S.I. of two, and the raw counts varied from 36 to 1769 d.p.m.'s. It is also important to note that in eighteen of the twenty-two patients tested, the total counts were seen to fall below 500 d.p.m.'s, which represented rather weak stimulation when compared to the related NaOH antigen of the respective organisms.

Closer examination of these data reveal two important findings. First, a definite relationship was seen to exist between the endotoxins of E. coli and L. buccalis in terms of their respective abilities to stimulate peripheral lymphocytes. As can be seen on Figure 1, a high level of correlation exists between the level of transformation induced by the two endotoxins in any given patient. This strongly suggests that there is no difference between these two materials antigenically, and may therefore allow us to speculate that endotoxin may be a type of selective "non-specific" stimulator for a certain group of cells within the population of circulating lymphocytes. It may be possible that recognition of any endotoxin, whether of E. coli, L. buccalis, or of any other gram-negative organisms, may be based upon some common configuration shared by all endotoxins. Further examination of this possibility might be of considerable interest. Secondly, stimulation of lymphocytes by endotoxins from E. coli and L. buccalis was consistently weaker than stimulation by the corresponding NaOH extract of the same organism. This difference was marked in all instances, and the relatively low level of stimulation with LPS was a consistent finding. Additionally, there was no correlation between stimulatory effects of the E. coli-N and

Figure 1. Correlation Between Transformation Responses to *E. coli* LPS and *L. buccalis* LPS.


1800
1600
1400
1200
1000
800
600
400
200
0
d.p.m.'s Lepto-LPS
r=.94
200
400
600
800
1000
1200
1400
d.p.m.'s E. coli - LPS

Lepto-N such as was seen between the endotoxins of these two species. The low level of stimulation by endotoxin may be due to one of several factors which will be discussed later. It must also be noted that a dose-response curve was not established over a wide range of endotoxin concentration. Stimulation seemed to be present at a given level, with both the E. coli and L. buccalis materials,

Table 4

Representative Dose-Response Relationships with Endotoxin.

Patient (Control d.p.m.)	Endotoxin-Concentration		d.p.m.
	Lepto LPS	5 µg/ml.	406
	Lepto LPS	1 µg/ml.	431
E.C.	Lepto LPS	0.1 µg/ml.	222
	E. coli LPS	5 µg/ml.	335
(34)	E. coli LPS	1 µg/ml.	238
	E. coli LPS	0.1 µg/ml.	218
	Lepto LPS	5 µg/ml.	139
	Lepto LPS	1 µg/ml.	112
H.C.	Lepto LPS	0.1 µg/ml.	87
	E. coli LPS	5 µg/ml.	101
(30)	E. coli LPS	1 µg/ml.	110
	E. coli LPS	0.1 µg/ml.	98
	Lepto LPS	5 µg/ml.	1170
	Lepto LPS	1 µg/ml.	1496
R.K.	Lepto LPS	0.1 µg/ml.	1461
	E. coli LPS	5 µg/ml.	842
(193)	E. coli LPS	1 µg/ml.	1193
	E. coli LPS	0.1 µg/ml.	1209
	Lepto LPS	5 µg/ml.	142
	Lepto LPS	1 µg/ml.	168
P.C.	Lepto LPS	0.1 µg/ml.	200
	E. coli LPS	5 µg/ml.	245
(99)	E. coli LPS	1 µg/ml.	210
	E. coli LPS	0.1 µg/ml.	230

or within a given range regardless of the quantity of material used. Attempts to find a high inhibitory concentration or a low non-stimulatory concentration were unsuccessful. Concentrations of endotoxin from 200 µg/ml. to 0.05 µg/ml. failed to show any effect

other than slightly lower transformation rates which were not significantly depressed, but no absolute inhibition or lack of response was ever seen. Table 4 gives a representative example of ranges of endotoxin used as a stimulant and the resulting d.p.m.'s. This type of finding also lends support to the possibility that stimulation via endotoxin is somehow different than stimulation with specific antigen.

The fact that our endotoxin preparations stimulated human peripheral lymphocytes at low but significant levels is of considerable interest in terms of the literature on this subject. Endotoxins in general are known to be extremely stimulatory to mouse spleen cells. More recent work in the mouse system indicates that endotoxin can be considered a non-specific type of stimulator of that animal's B cells. (36). That is, certain known endotoxins have the capability of selectively stimulating that sub-population of lymphocytes without a requirement of prior host sensitization. This is apparently due to some structural specificity within the endotoxin moiety which has the capability of stimulating mouse B cells at significant levels.

While this phenomenon is readily accepted in terms of the mouse system, no comparable response has of yet been identified with use of human peripheral lymphocytes. Peavy (37) incubated human lymphocytes with Salmonella endotoxin, and found no significant response. A serum source consisting of either 10% autologous or 20% fetal calf serum was used in an attempt to supply necessary serum components, but stimulation remained slight and insignificant. On the basis of this and other information, Greaves and Janossy (38)

recently concluded that attempts to stimulate human peripheral lymphocytes with bacterial endotoxins have thus far been unsuccessful.

However, the matter is not that well-defined. As early as 1965, Oppenheim and Perry claimed some measure of success in the area of endotoxin stimulation of human lymphocytes. (6) Using plasma concentrations of 25-30% and incubating for five days, these investigators assayed responses to five endotoxins. Direct microscopic evaluation of blast transformation as well as uptake of tritiated thymidine were used to measure lymphocyte responses. Endotoxins of S. typhosa, S. enteritides, and E. coli were seen to induce significant transformation. Another publication by Heilman showed similar results. (39) Using peripheral leukocytes from twelve healthy donors, and measuring blast transformation microscopically, she found low but positive responses to both S. typhosa and E. coli endotoxins in several patients. Responses were quite low however, and the author at that time did not feel that these responses were representative of the type of stimulation that should be seen with specific stimulants.

Correlation of these findings with one another is difficult, but our results seem to be in agreement with those researchers who have shown positive lymphocyte responses to endotoxins. Perhaps Peavy's negative results can be attributed to serum problems. Fetal calf serum is not the best support of human lymphocyte cultures in our experience, and therefore his cells may not have had the support from the media necessary to give adequate responses. Use of 10% autologous serum instead of 20% fetal calf serum raised the S.I. of his transformation to 1.4, which while low, is not different than

that seen with some of our patients. Another criticism of Peavy's work would be that he used only 5×10^6 cells in a 4 ml. culture tube, a concentration of 1.25×10^6 cells/ml. Standard concentrations in use currently are closer to 5×10^6 cells/ml., so the possibility exists that insufficient cells were present in his system to give measurable responses at 5 days of incubation.

Analysis of Oppenheim's and Heilman's work is of more interest. Heilman makes reference to the unusual ability of endotoxin to stimulate at low levels in a manner unrelated to previous vaccination. Reference is also made to the ability of endotoxin to give the same level of transformation regardless of 10-fold to 200-fold differences in concentration used as stimulant. Their work also led them to believe that the low levels of stimulation by endotoxin were not due to the variable amounts of protein present in the preparations. Oppenheim also found the response to endotoxin to be elicited over a wide range of concentrations, with responses seen to amounts of endotoxin as low as 50 μμg/ml.

Our results support and extend the findings of these investigators. Our Lepto LPS and the commercial preparation of E. coli endotoxin give low levels of stimulation in most patients, and less than significant stimulation in a few patients. Our endotoxins are also found to be stimulatory over a very wide range of concentration, from 200 μg/ml. to 0.05 μg/ml. in our hands. Our endotoxins are also found to be less stimulatory than common protein-rich extracts of the same species of bacteria, indicating that protein content does not play an important role in the pattern of response observed. The differences in stimulatory capacity between the two endotoxins

examined are minimal when compared with protein extracts of the same bacteria, indicating a possible common effect of endotoxin. These findings seem to point towards the possibility that endotoxin may be a "non-specific" stimulant for a certain sub-population of human lymphocytes, as is seen in the mouse system. Greaves and Janossy (38) have stated that with the possible exception of anti-immunoglobulins, no currently available mitogen for human B cells is known. Further more definitive work investigating the effect of endotoxins on purified populations of human B cells will be necessary in order to confirm a hypothesis which suggests that endotoxins are selective stimulators of human B cells. However, in the absence of such direct evidence, this possibility seems to be strongly supported by the nature of our findings.

Ours is not the first evidence of endotoxin stimulation of human peripheral lymphocytes. Yet our findings come at a time when the possibility of such stimulation is questioned, and therefore is of some importance. It seems reasonable to expect positive lymphocyte transformation responses to endotoxin for a number of reasons. It has been shown that antibody titers to endotoxin are detectable in the serum of medically healthy individuals. (6,18) Therefore it seems likely that somatic antigens of gram-negative bacteria come into contact with the human immune system in the absence of clinically detectable disease. If antibody titers are detectable, one would expect to find further evidence of immune contact by using the <u>in vitro</u> methods of lymphocyte transformation.

We feel that lymphocyte transformation, whether it be the result of non-specific endotoxin effect, or whether it be the result

of prior sensitization by some portion of the endotoxin complex seems to be commonly seen in the general population, regardless of any periodontal disease state, or of any medical illness. It therefore seems to be completely dissociated from any disease state. It also seems possible that this pattern of stimulation is consistent with the role of bacterial endotoxin as a non-specific stimulant of human B cells.

Transformation responses to NaOH extracts.--Responses to thirteen NaOH extracts of various bacteria were made. The results of lymphocyte transformation to these various extracts are very straightforward. A summary of all the information gathered is seen in Table 5. Initials of the patients examined, along with their respective ages, gingival indices, and periodontal indices are given. Stimulation is expressed as disintegrations per minute less background, and the S.I. is computed in each instance. These values represent maximum stimulation values seen for each antigen. Figure 2 is a representation which shows the manner by which maximum stimulation was recorded. Dose responses curves were set up for each antigen, as was previously explained, and the value for each concentration was plotted against the concentration of antigen in ul./ml. In this way, we felt that we could accurately estimate the maximum response at six days without fear of our antigen concentration being toxic or sub-stimulatory.

As can readily be seen from Table 5, all patients responded at levels significantly above saline stimulated control values in response to the various materials tested. No distinctions or differences are apparent among the responses on the basis of age, periodontal disease severity, or any other obvious parameter.

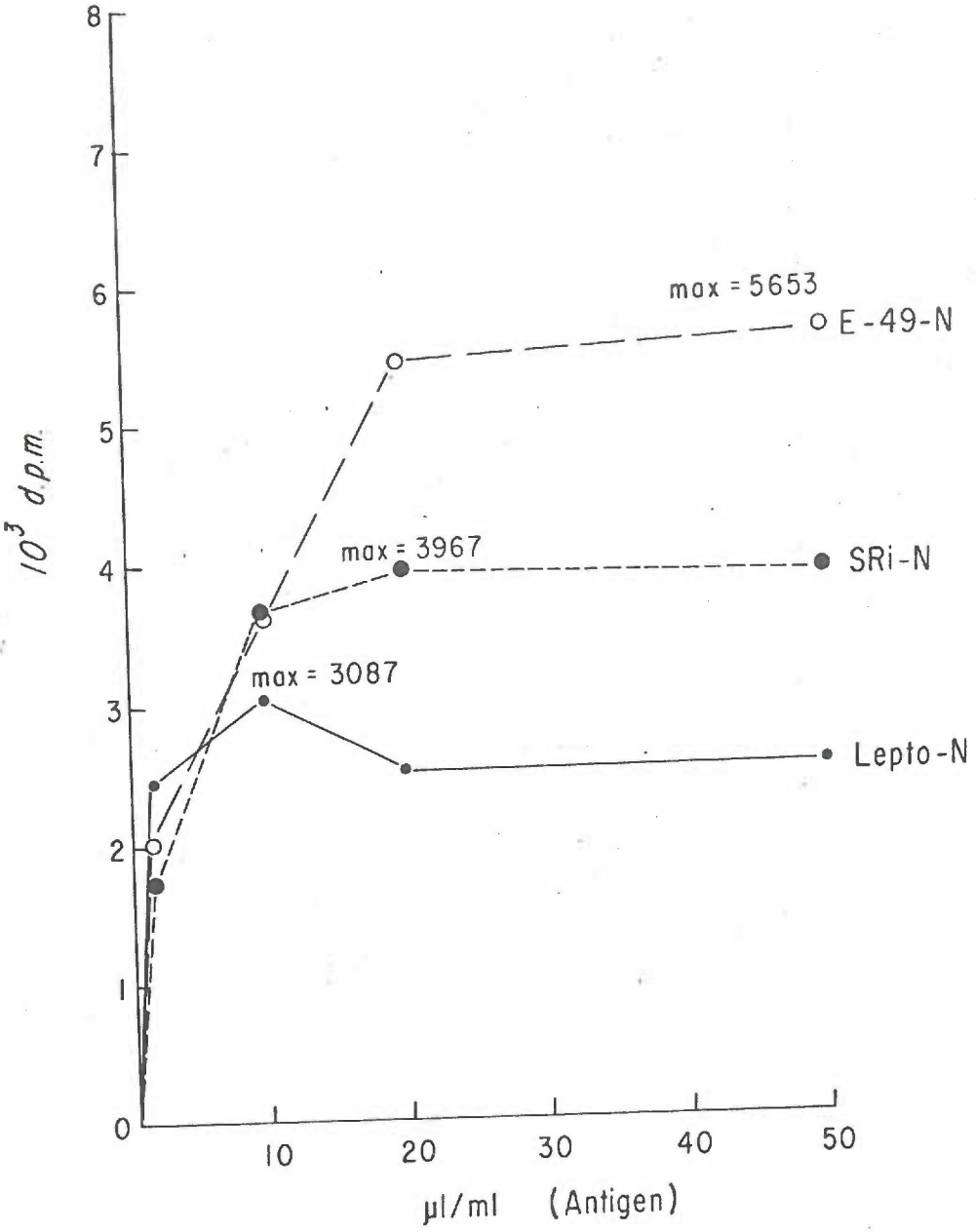
Closer examination of the raw data shows quite a wide variation of peak responses by the patients to any single antigen, both in terms of S.I. and d.p.m.'s. In most instances, Candida-N was the best stimulator of peripheral lymphocytes while NEP gave the weakest stimulation. But even in these two antigens, the range of patient responsiveness was quite large. The other antigens seemed to elicit

TABLE 5

Patient Responses to NaOH Antigens

PATIENT	AGE	PI	PDI	GI	B-mat.-N.	B-mel.-N	Cand.-N	SRi-N	E-49-N	E.coli-N	N.perf.-N	Lepto-N	NEP	A.naes.-N	VI-N	Pseudo-N	T-6-N	PHA	CONTROL
B.O.	22	0.1	2.0	0.05	1582 36.0	1061 24.1	16117 366.3	1187 27.0	1580 35.9	6806 154.7	1570 35.7	4098 93.1	576 13.0	1677 38.1	1138 25.1	4939 112.3	967 22.0	11591 263.4	44
C.S.	24	0.15	3.7	0.04	1433 29.9	252 9.0	1810 64.6	672 24.0	1192 42.6	1412 50.4	759 27.1	279 10.0	316 11.3	541 19.3	276 9.9	156 5.6	309 11.0	43950 1570.0	28
F.M.	27	0.17	1.5	0.1	1714 33.6	2325 45.6	2542 49.8	1676 32.9	1536 30.1	1189 23.3	1910 37.5	831 16.3	1282 25.1	1227 24.0	617 12.1	1348 26.4	1193 23.3	8899 174.5	51
J.S.	37	0.19	2.7	0.1	1583 56.5	2014 71.9	4119 147.1	1062 37.9	1498 53.5	1196 42.7	983 35.1	2400 85.7	868 31.0	685 24.5	1334 47.6	988 35.3	1534 55.1	11775 420.5	28
R.K.	27	0.8	3.0	0.3	6602 34.2	2958 15.3	14490 75.1	4206 21.8	7682 39.8	11530 59.7	8413 43.6	5686 29.5	3036 15.7	6102 31.6	6351 32.9	8465 43.9	7076 36.7	68128 353.0	193
W.W.	40	1.0	4.3	~~0.6~~ 0.06	2379 51.7	1797 39.9	5936 129.0	3122 67.9	3677 79.9	3443 74.8	4458 96.9	3429 74.5	1186 25.8	2069 45.0	1653 35.9	4272 92.9	1586 34.5	19301 419.6	46
D.R.	37	1.3	2.7	0.7	6631 130.0	3609 70.7	21151 414.7	7458 146.2	7386 144.8	9360 183.5	3534 69.3	2176 42.7	943 18.5	3153 61.8	1861 30.5	3523 69.1	2641 51.8	13393 262.6	51
S.S.	28	1.5	4.0	0.3	6411 51.7	7852 63.3	13556 109.3	3967 32.0	5653 45.6	5248 42.3	6860 55.3	3087 24.9	3022 24.4	2288 18.5	2154 17.4	6947 56.0	3499 28.2	16585 133.8	124
W.C.	41	2.0	4.0	0.7	4351 9.1	2671 5.6	5462 11.5	2103 4.4	3625 7.6	4323 9.1	2898 6.1	2039 4.4	629 1.3	2398 5.0	1308 2.7	2045 4.3	1937 4.1	17432 36.5	477
M.C.	42	2.2	4.2	0.4	972 32.4	365 12.2	2422 80.7	389 13.0	468 15.6	1387 46.2	428 14.2	752 25.1	202 6.7	332 11.1	286 9.5	1138 37.9	363 12.1	32900 109.7	30
E....	57	4.2	4.7	1.3	1655 91.9	357 19.8	2504 139.1	424 23.6	682 37.9	2283 126.8	714 39.7	797 44.3	162 9.0	484 26.9	216 12.0	246 13.7	933 51.8	23997 1333.2	18
D.C.	50	4.6	5.0	1.0	600 7.4	1544 17.7	13176 151.4	2053 23.6	3185 36.6	3186 36.6	2770 31.8	2503 28.8	1532 17.6	1206 13.9	1397 16.1	6364 73.1	1358 15.6	13264 152.5	87
F.C.	33	4.9	5.3	1.4	6977 70.4	7122 71.9	16224 163.9	2403 24.3	5114 51.7	6541 66.1	5083 51.3	5528 55.8	2523 25.5	9466 95.6	2481 25.1	3787 38.3	2174 22.0	15930 160.9	99
E.C.	46	5.8	5.8	1.2	3503 103.0	1882 55.4	12310 361.2	3709 109.1	6395 188.1	6024 177.2	3220 94.7	1783 52.4	780 22.9	1093 32.1	1671 49.3	9322 274.2	1596 46.9	8650 254.4	34

Figure 2. Representative Dose-Response Curves for Three Antigens.


8
7
6
5
4
3
2
1
0
10^3 d.p.m.
max = 5653
E-49-N
max = 3967
SRi-N
max = 3087
Lepto-N
10
20
30
40
50
µl/ml (Antigen)

responses somewhere between the level of Candida-N and NEP, but no real consistency of effect was apparent. Some patients responded at greater levels than did others, but there was no obvious relationship with any of the parameters measured. Oral and non-oral bacterial products alike gave good stimulation, thus allowing us to speculate that plaque antigenic products as well as non-plaque antigenic products have both induced production of specific memory cells through some previous contact with the immune system in each individual tested.

A variety of attempts were made to find some relationship between the stimulation values obtained and periodontal disease severity, since the dental literature cited seems to indicate that such a relationship exists. Figures 3 through 8 represent an examination of lymphocyte responses in patients with varying degrees of periodontal disease severity in response to stimulation by selected representative antigenic materials. Patients were classed as having no disease (PI of 0 to 0.2), gingivitis, (PI of 0.2 to 2.2) and periodontitis (PI of 4.2 to 5.8). Each point on the graph represents one patient responding to the antigen indicated with respect to the d.p.m.'s and S.I.'s. The random scattering of points observed here suggests that there is no clear relationship existing between lymphocyte stimulation and periodontal disease severity. It would appear that control patients, for instance, respond within a range that is within the range of response seen in the gingivitis group, as well as the periodontitis group. This suggests that possibly all of the individuals tested are really members of the same sample, and that distinguishing them as different on the basis

Figure 3. Response to B. mat.-N According to Groups of Patients.

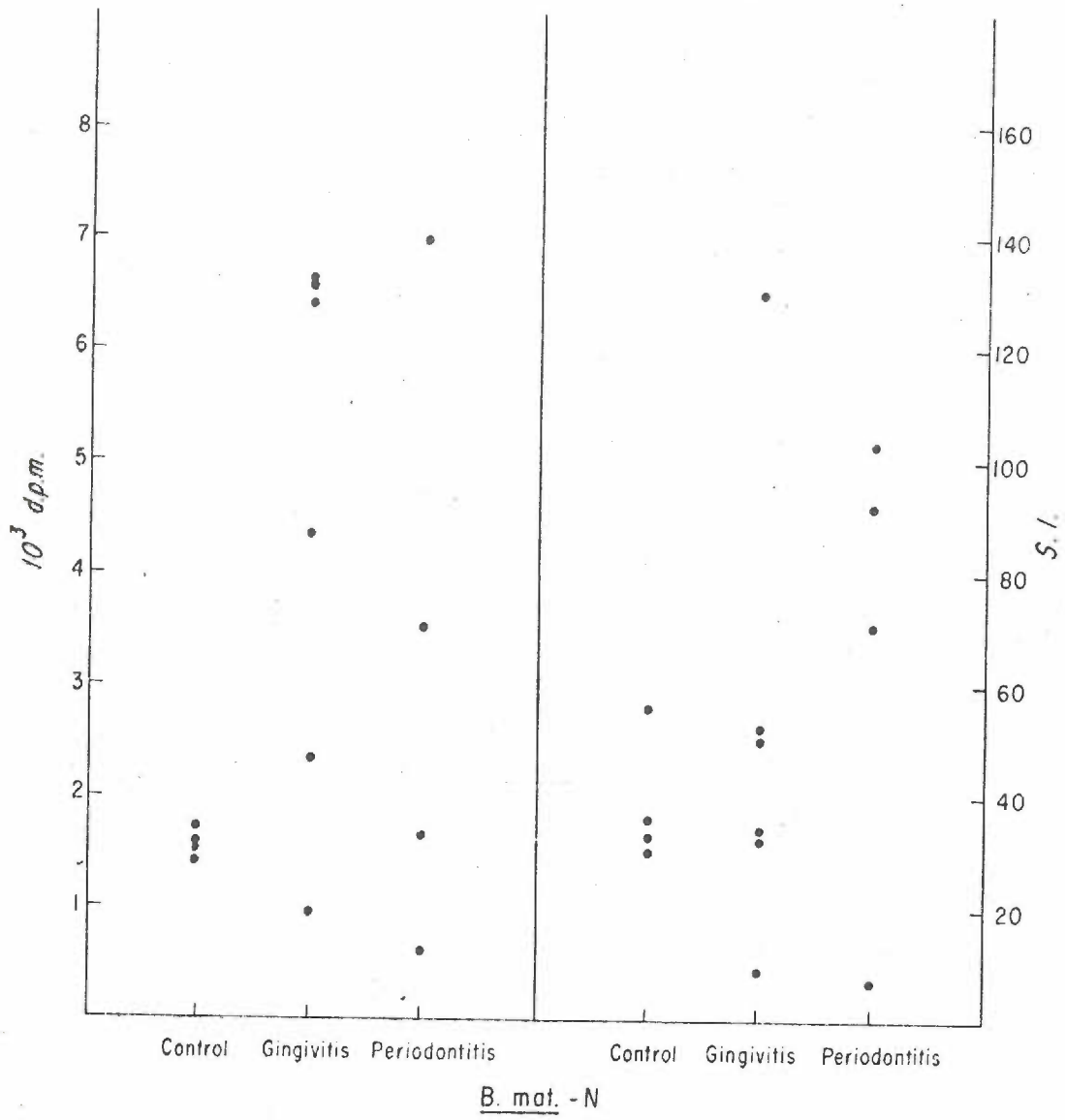

10^3 d.p.m.
S.I.
8
7
6
5
4
3
2
1
160
140
120
100
80
60
40
20
Control
Gingivitis
Periodontitis
Control
Gingivitis
Periodontitis
B. mat. - N

Figure 4. Response to SRi-N According to Groups of Patients.

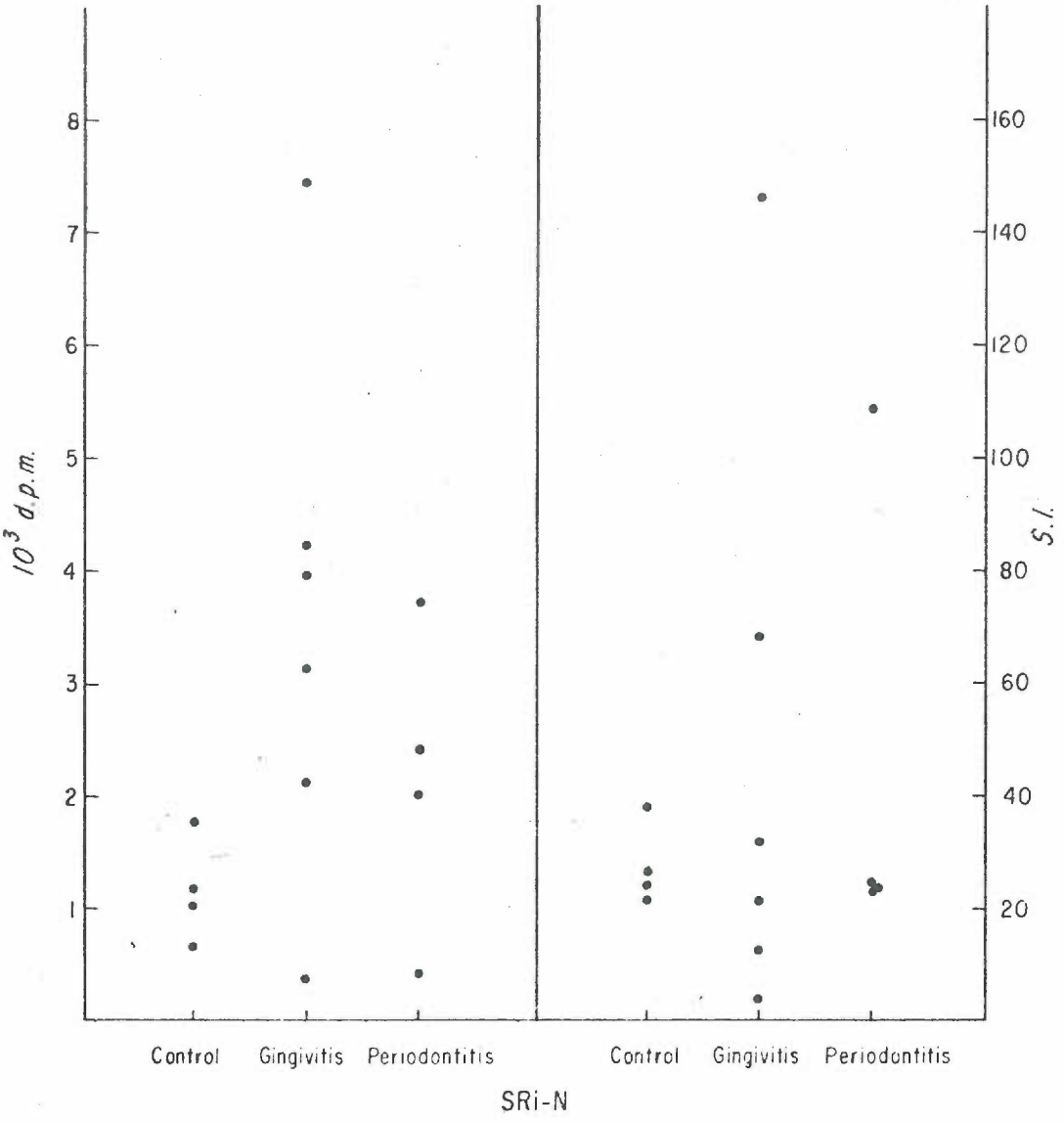
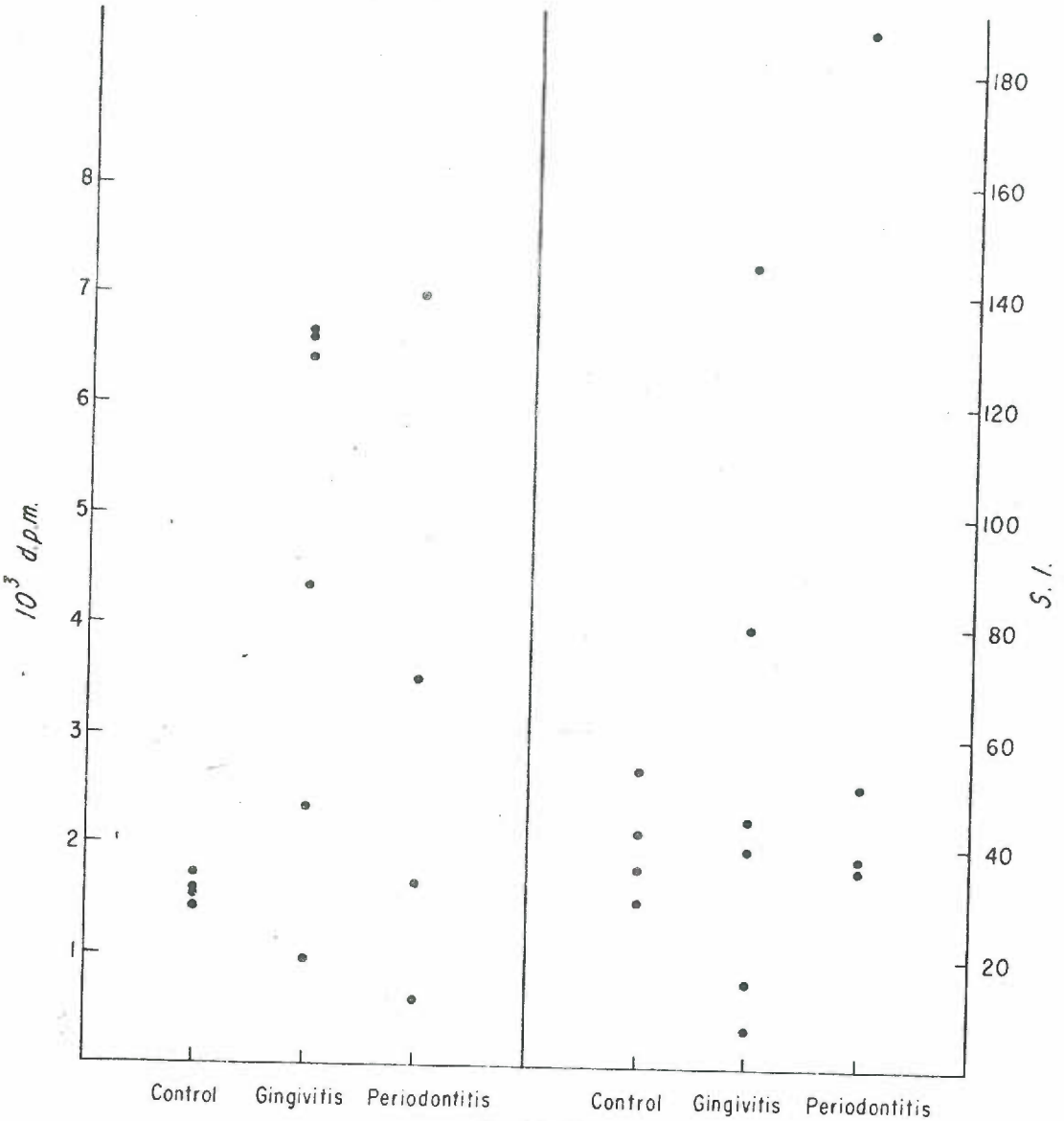

8
7
6
5
4
3
2
1
10^3 d.p.m.
160
140
120
100
80
60
40
20
S.I.
Control
Gingivitis
Periodontitis
Control
Gingivitis
Periodontitis
SRi-N

Figure 5. Response to E-49-N According to Groups of Patients.


10^3 d.p.m.
S. I.
8
7
6
5
4
3
2
1
180
160
140
120
100
80
60
40
20
Control
Gingivitis
Periodontitis
Control
Gingivitis
Periodontitis

E-49-N

Figure 6. Response to NEP According to Groups of Patients.

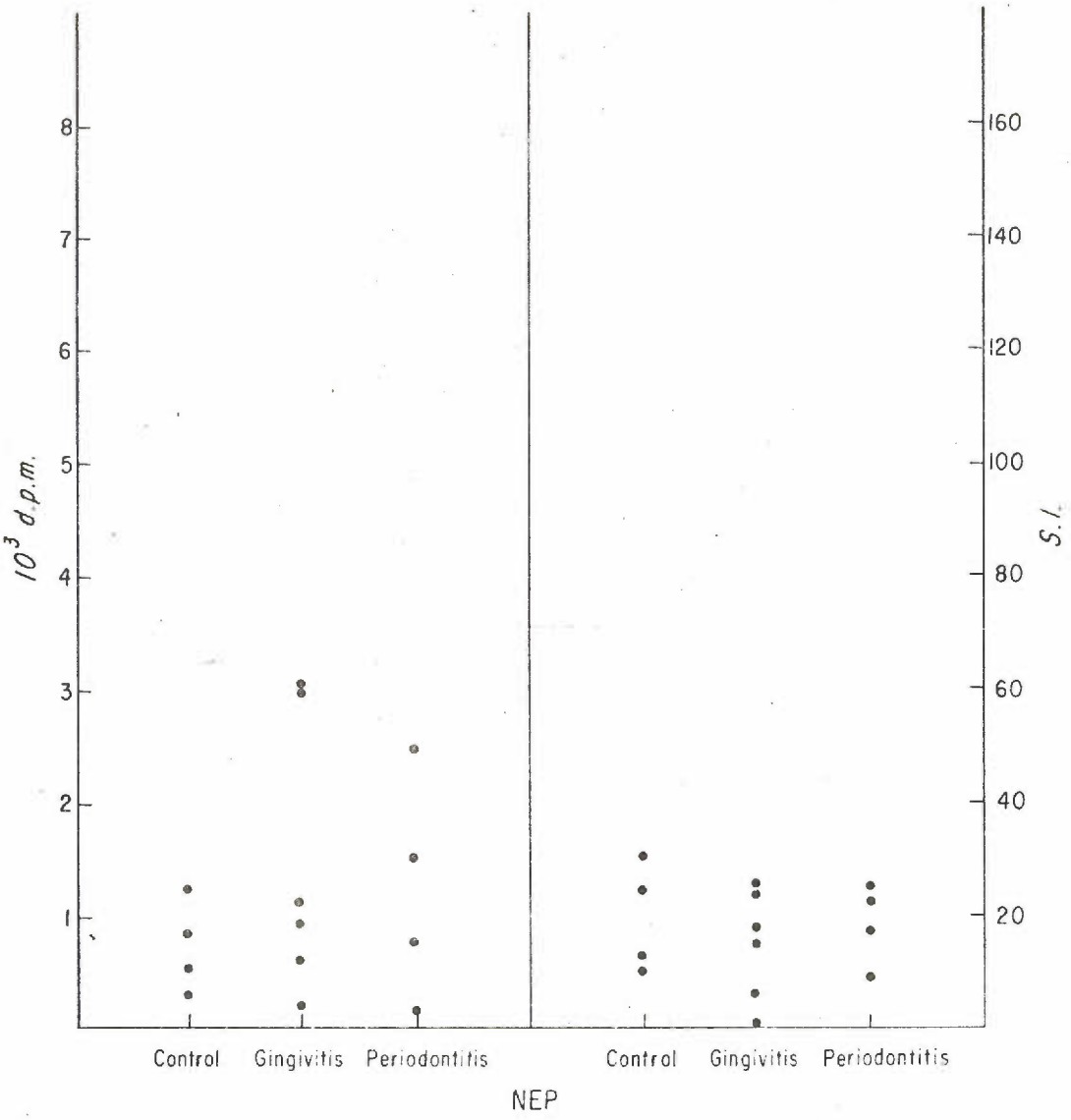
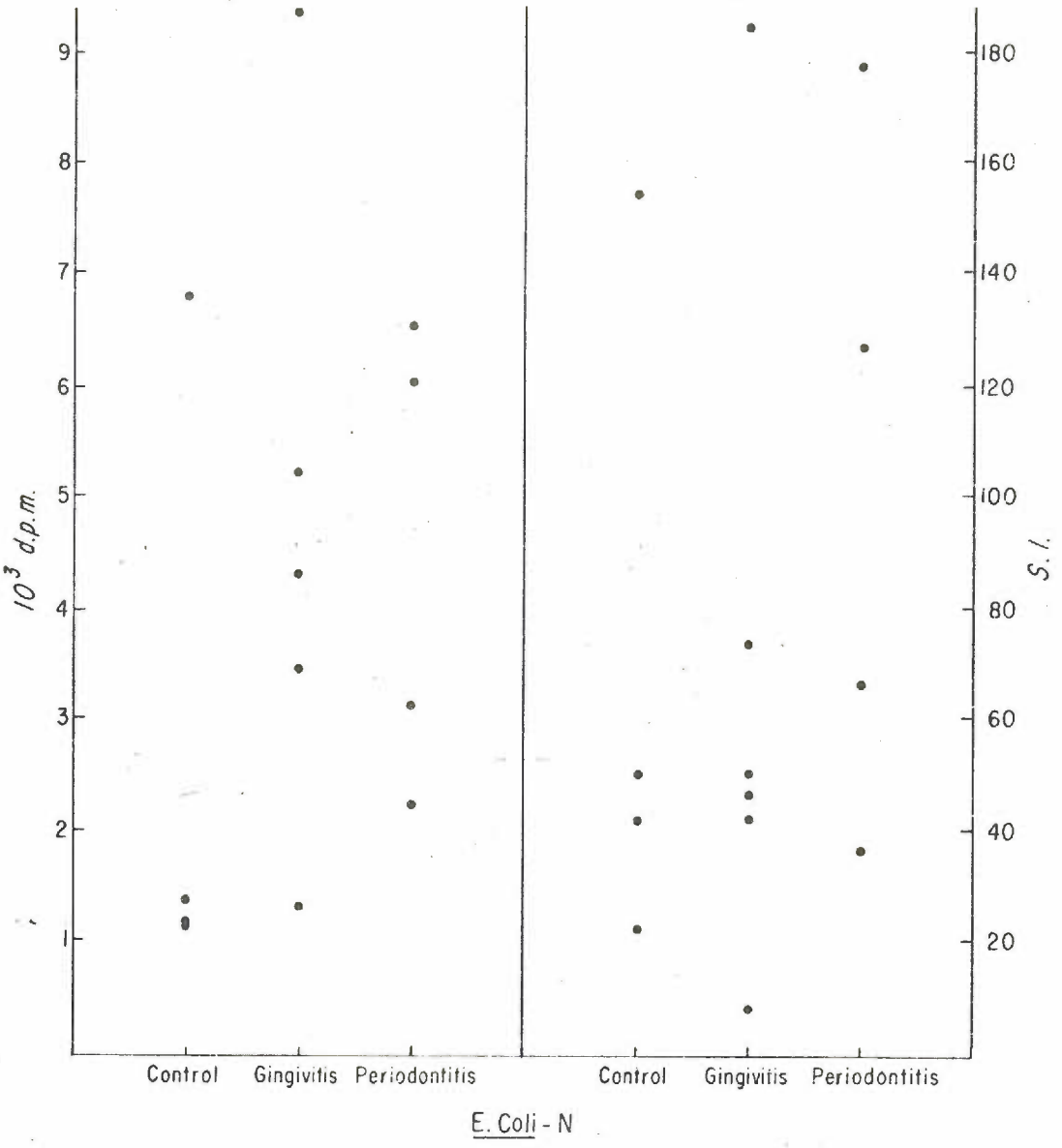
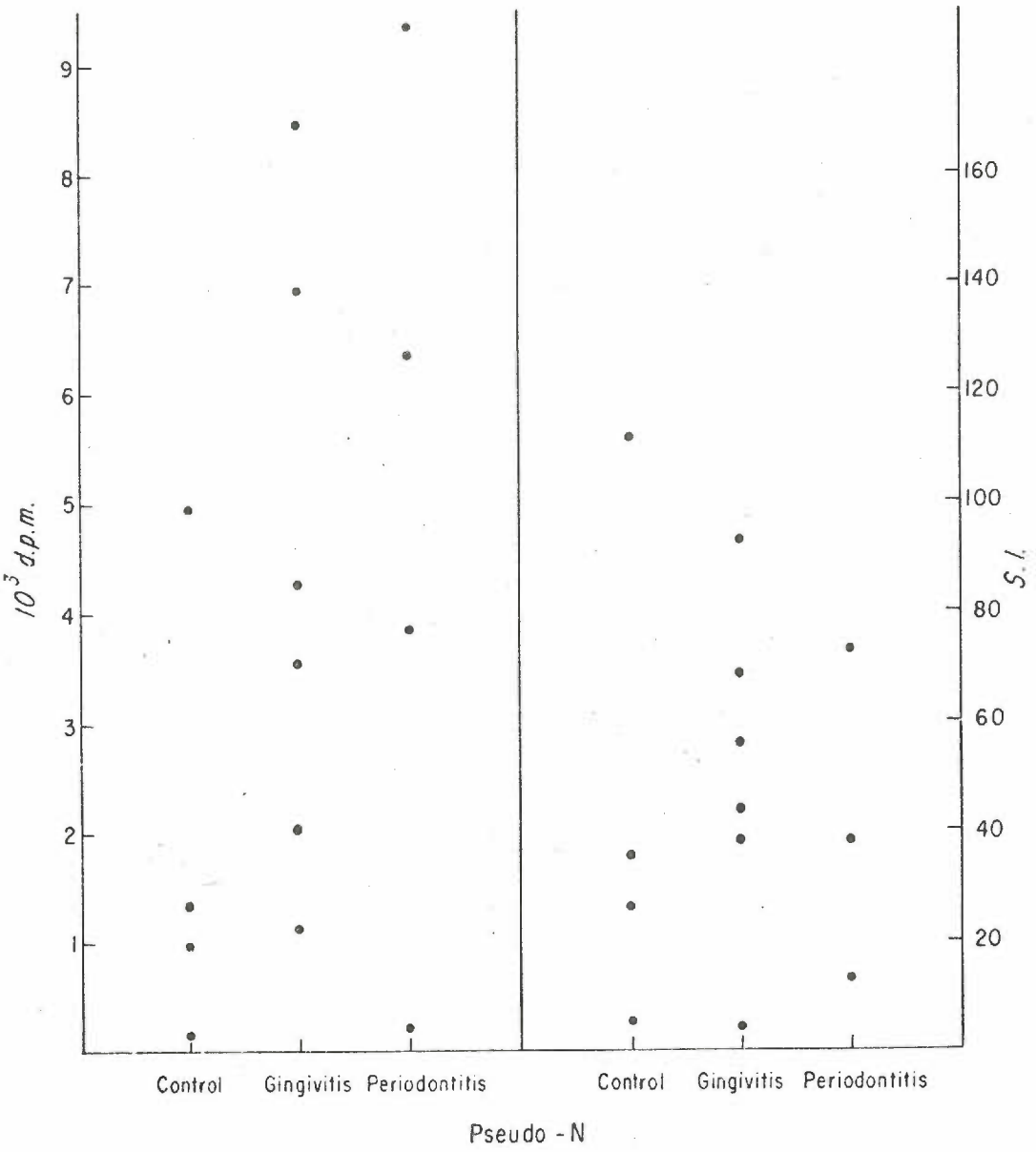

8
7
6
5
4
3
2
1
10^3 d.p.m.
160
140
120
100
80
60
40
20
S.I.
Control
Gingivitis
Periodontitis
Control
Gingivitis
Periodontitis
NEP

Figure 7. Response to E. coli-N According to Groups of Patients.


10^3 d.p.m.
S.I.
9
8
7
6
5
4
3
2
1
180
160
140
120
100
80
60
40
20
Control
Gingivitis
Periodontitis
Control
Gingivitis
Periodontitis

E. Coli - N

Figure 8. Response to Pseudo-N According to Groups of Patients.


10^3 d.p.m.
S.I.
9
8
7
6
5
4
3
2
1
160
140
120
100
80
60
40
20
Control
Gingivitis
Periodontitis
Control
Gingivitis
Periodontitis
Pseudo - N

of periodontal disease severity is a completely artificial separation.

Figure 9 represents stimulation levels to all of the antigens tested in three individuals, one from each of the control, gingivitis, and periodontitis groups. As can be seen from this diagram, no consistent level of response is seen within the three individuals. The control individual does not consistently respond to all antigens at levels below the diseased individuals, nor does the severity of disease seem to exert a consistent effect in terms of lymphocyte response to the antigens in any way. Analysis of this type of data statistically showed that no relationship existed between transformation responses to any single antigen or groups of antigens and periodontal disease severity at the 95% level of confidence.

Figure 10 is a plot of the mean d.p.m.'s of the plaque antigens (B. mat-N, B. mel.-N, SRi-N, E-49-N, N. perf.-N, Lepto-N, NEP, A. naes.-N, V1-N, and T-6-N) against severity of periodontal disease as measured by the Russell Index. Mean d.p.m. values were obtained by averaging one patient's response to the above mentioned stimulants, then using that mean value as the value plotted against disease severity. Figure 11 is a plot of the mean d.p.m.'s of the non-plaque antigens (Candida-N, E. coli-N, and Pseudo-N) against severity of periodontal disease. Mean values were again obtained in the same way described above. Figure 12 is a plot of the means of all the antigens (plaque and non-plaque) against periodontal disease severity. Figures 13,14 and 15 are plots of mean S.I.'s for non-plaque, plaque and all antigens plotted against disease severity. It therefore becomes apparent that there is absolutely no relationship between

Figure 9. Comparison of Antigenic Stimulation in Three Individuals, One from Each of the Three Periodontal Disease Groups.

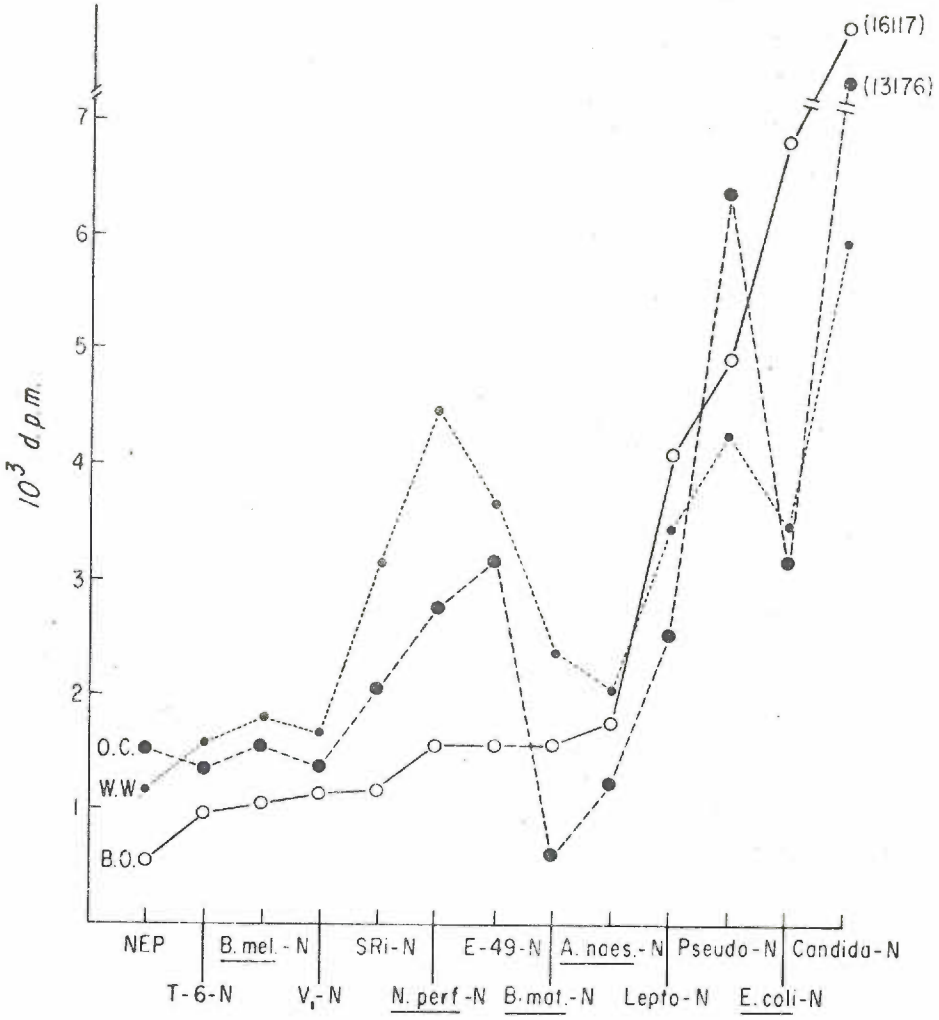

(16117)
(13176)
7
6
5
4
3
2
1
10^3 d.p.m.
O.C.
W.W
B.O.
NEP
B. mel. - N
T-6-N
SRi-N
V_1-N
N. perf.-N
E-49-N
B. mat.-N
A. naes.-N
Lepto-N
Pseudo-N
E. coli-N
Candida-N

Figure 10. Array of Mean d.p.m.'s of Plaque Antigens in Each Patient Against Russell Index.

Figure 11. Array of Mean d.p.m.'s of Non-Plaque Antigens in Each Patient Against Russell Index.

Figure 12. Array of Mean d.p.m.'s of All Antigens in Each Patient Against Russell Index.

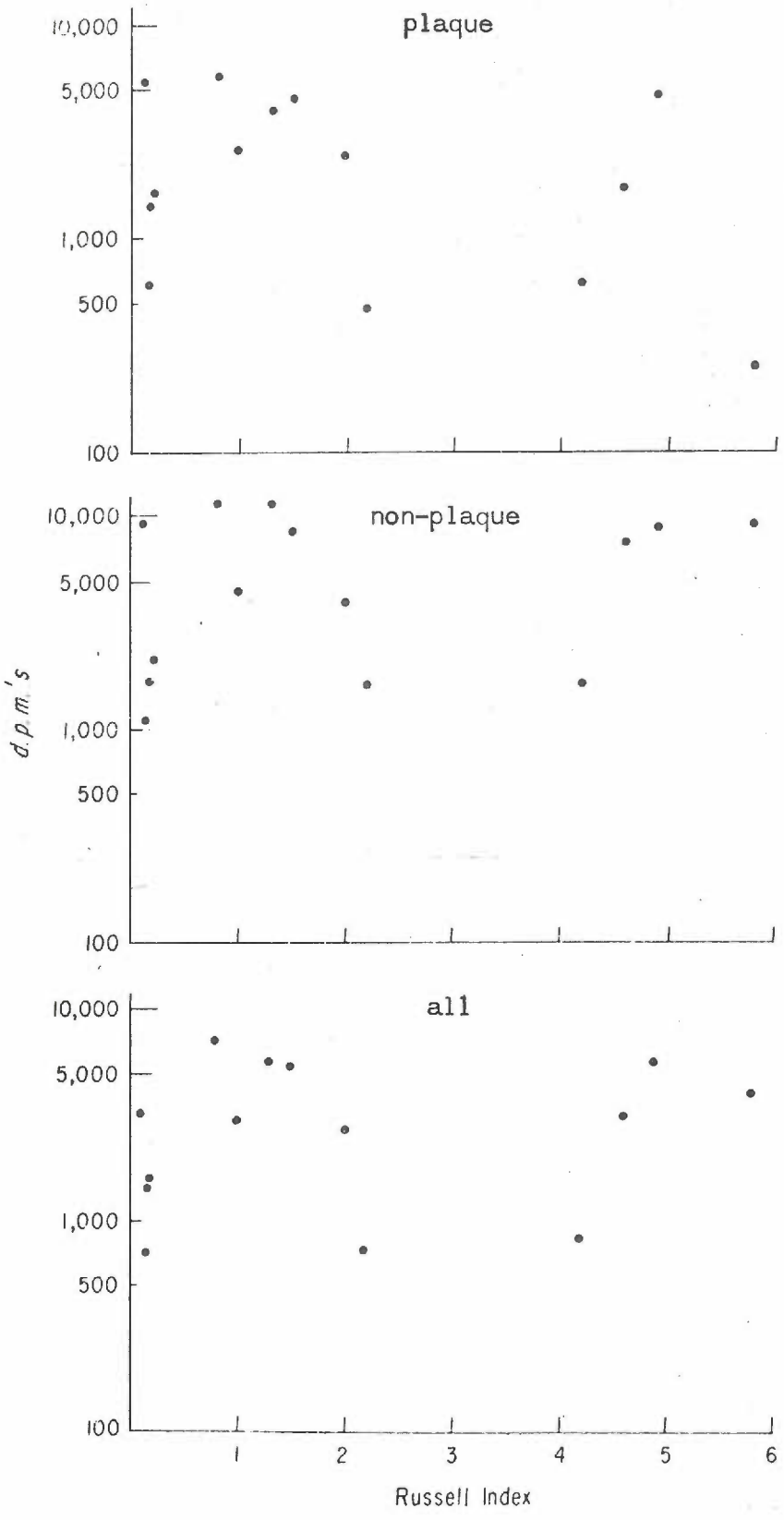
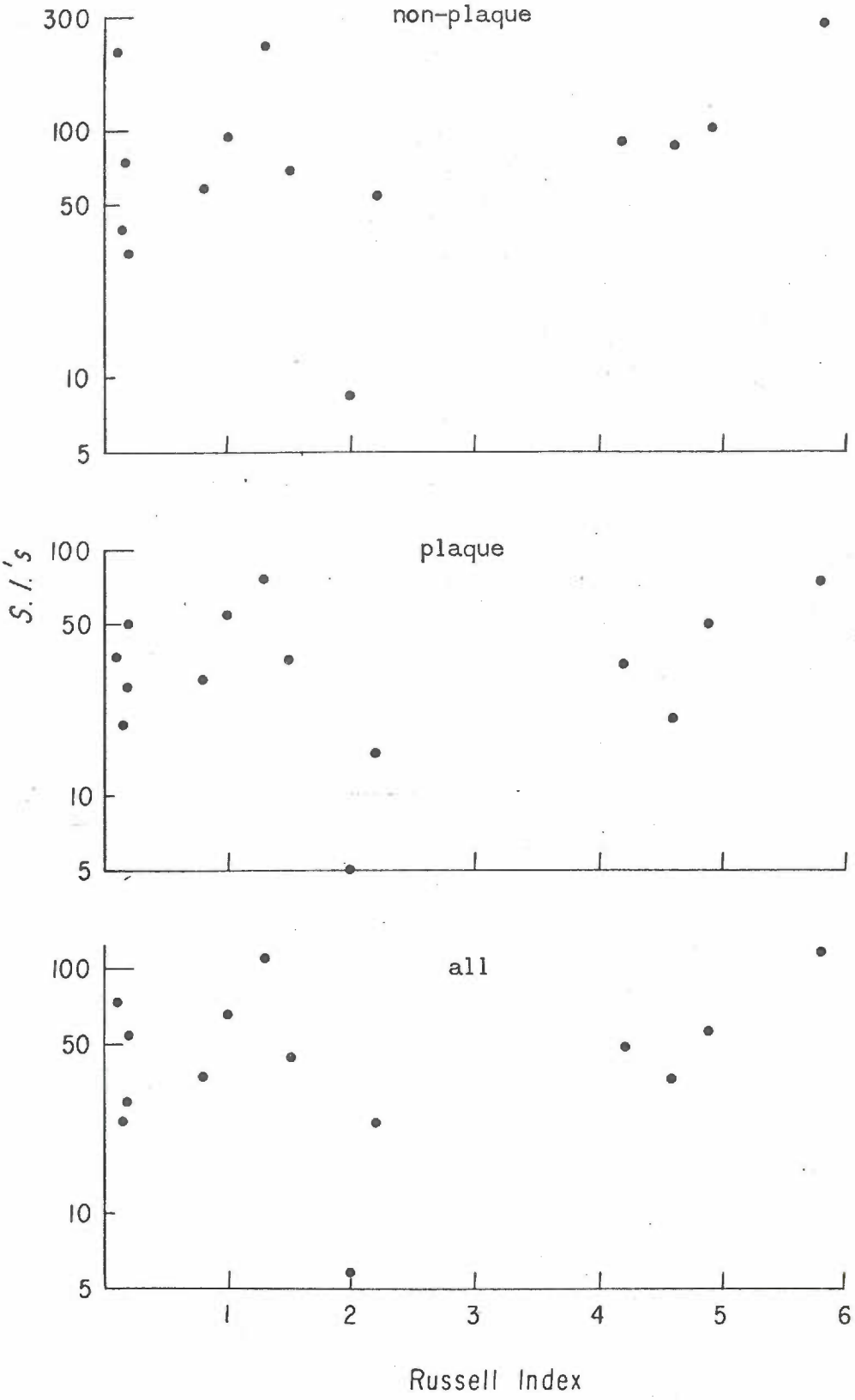

plaque
non-plaque
all
10,000
5,000
1,000
500
100
d.p.m.'s
1
2
3
4
5
6
Russell Index

Figure 13. Array of Mean S.I.'s of Non-Plaque Antigens in Each Patient Against Russell Index.

Figure 14. Array of Mean S.I.'s of Plaque Antigens in Each Patient Against Russell Index.

Figure 15. Array of Mean S.I.'s of All Antigens in Each Patient Against Russell Index.


non-plaque
plaque
all
300
100
50
10
5
S. I.'s
1
2
3
4
5
6
Russell Index

the ability of one individuals lymphocyte transformation responses and the severity of the periodontal disease of that individual, whether we choose to use d.p.m.'s or S.I.'s as measures of that response. Figure 16 shows that when comparing the ratio of responses from plaque to non-plaque stimulants, no relationship is seen between those responses and periodontal disease. This would indicate that individuals with little or no periodontal disease respond equally well to both plaque and non-plaque antigens as do people with periodontal disease. Thus our data does not support the theory that patients with periodontal disease are, or become, more sensitized to the agents thought to be involved in progression of periodontal disease.

Table 6 represents efforts to find correlations of any kind when evaluating lymphocyte transformation responses. Lymphocyte transformation responses to each antigen as measured by both S.I.'s and d.p.m.'s were compared for plaque and non-plaque antigens with the age of the patient, the PI, the GI, and the PDI of the patient. Thus 112 correlation coefficients were calculated. These values represent the highest correlation coefficients found when evaluating these parameters. The highest correlation seen anywhere is r= .432 for the comparison between response to Pseudo-N and the severity of disease as measured by the Russell Index. All correlations are low so as to be considered statistically non-significant.

Figure 16. Ratio of Plaque Antigens to Non-Plaque Antigens Against Russell Index.

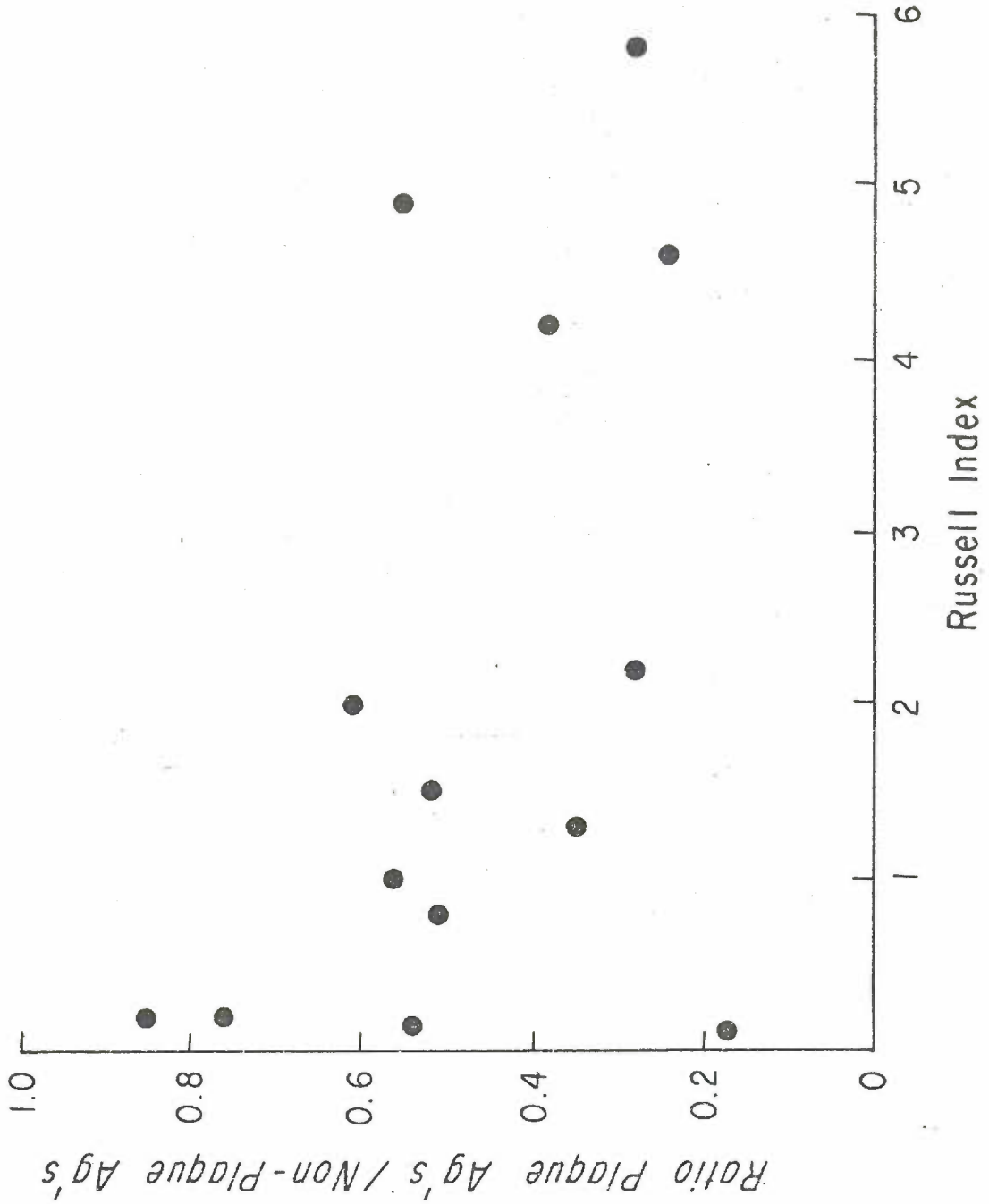

Ratio Plaque Ag's / Non-Plaque Ag's
1.0
0.8
0.6
0.4
0.2
0
1
2
3
4
5
6
Russell Index

Table 6

Highest Correlations between Various Parameters.

	PI	PDI	GI	Age
Non-plaque d.p.m.'s	Pseudo-N r= .330	Pseudo-N r= .280	*E. coli*-N r= .381	*E. coli*-N r= .262
Plaque	E-49-N r= .217	E-49-N r= .180	*A. naes.*-N r= .226	*A. naes.*-N r= .360
Non-plaque S.I.'s	Pseudo-N r= .432	Pseudo-N r= .343	*E. coli*-N r= .310	Pseudo-N r= .163
Plaque	E-49-N r= .344	*N. perf.*-N r= .270	*A. naes.*-N r= .412	T6-N r= .281

Our results show that significant lymphocyte transformation responses to extracts of various bacteria occur routinely in a sample of the general population. It is therefore assumed that these individuals' immune systems have previously come in contact with materials which have induced stimulation of antigen specific cells, which are a part of the population of peripheral lymphocytes examined by our *in vitro* techniques. It is believed that these antigen specific cells, when again challenged by our antigens in our test culture system, are stimulated into blast formation and are thus responsible for the uptake of C-14 thymidine measured.

We chose to represent uptake of C-14 thymidine in two ways: by actual d.p.m.'s minus background, and by use of S.I.'s. It is felt that the best measure of lymphocyte stimulation within a single patient is the S.I., which takes the control value of saline stimulated cultures into account and measures antigen stimulation against

that selected value. Within a single patient, a 10-fold difference in S.I. must represent a difference in stimulation, but the significance of such differences is not fully understood. The problem of significance becomes even more severe when comparison of S.I.'s between different patients is attempted because the S.I. becomes dependant on the control values of the various patients. If six patients all respond to B. mat.-N with an S.I. of five, we might be led to believe that all six patients responded alike to that material. This is the value of the S.I.; it equalizes responses among patients by compensating for the different control values. But if the actual d.p.m.'s for those six patients varied between 500 and 5,000, we might wonder if the S.I. of five in each person accurately reflected response to B. mat.-N. It becomes apparent that large differences of response in terms of d.p.m.'s may actually become hidden within the calculation of the S.I. It must also be stated that we do not at this point in time fully understand the significance of differences in stimulation expressed as either d.p.m.'s or S.I.'s. Does an increased response in one of two patients reflect an increased sensitization in the one patient toward that antigen? Or does it simply reflect a more recent in vivo exposure to that antigen in that individual? Does it mean that his immune system is more sensitive to antigenic challenge? We do not yet know the answers to these questions, and it is important that we realize the limitations inherent in attempts to quantitate the lymphocyte responses by use of either d.p.m.'s or S.I.'s.

Our present investigations have shown that there are a number of variables present in lymphocyte transformation techniques which will affect lymphocyte transformation as expressed as either d.p.m.'s

or S.I.'s. A short discussion of these variables will further support the feeling that quantitative evaluations of these responses are unreliable. Table 7 is a list of some of the variables evaluated in our study.

Table 7

Variables Evaluated for Lymphocyte Transformation Results.

1. Time of incubation of lymphocyte cultures.
2. Serum concentration used in supplementing MEM-S.
3. Use of dose-response curves in each antigen concentration and in each patient.
4. Use of triplicate tubes for each antigen concentration.
5. Method of preparation of sample antigen: sonication vs. saline extraction, vs. NaOH extraction.
6. Method of collection of lymphocyte cultures: saline washing vs. TCA precipitation methods.
7. Attempts to stimulate lymphocytes using growth media in which organisms were cultured.

Table 8 shows that the optimal length of incubation in our system was seen to be close to eight days, the time when our examinations showed maximal response in terms of d.p.m.'s. It is noted that at three days of time antigen stimulation seems to be just beginning, and if measured at that time definitely would not accurately reflect total response such as is seen at six or eight days. Table 8 also reveals that the non-specific stimulation by PHA has apparently peaked around three days. Daily or more frequent measurements could also be taken in order to strictly pinpoint the time of peak response, and this could be repeated in every patient

Table 8

Variation in Antigen Stimulation with Time.

Antigen	Day 3	Day 6	Day 8	Day 10
Control (saline)	28	27	12	38
PHA	23030	12299	5370	2949
Pseudo-N	356	4113	9384	8262
B. mat.-N	200	3422	10935	7231

tested to eliminate any inter-patient variability in this respect if desired. The point is made that the time of incubation with antigen is capable of influencing d.p.m. measurement. Our cultures were arbitrarily limited to six days, and while this may be within the range of peak d.p.m.'s, it does not accurately represent the maximum possible response to antigen in any patient in our study.

Another variable capable of causing changes in d.p.m.'s is that of the concentration of serum used to supplement the culture media. Table 9 shows the comparative responses of one antigen incubated in systems using 10%, 15%, and 20% autologous serum. This table shows that while d.p.m.'s increased across the board, serum concentration had no effect on the concentration of antigen required for maximum stimulation. It would appear that 20% autologous serum gives the best support for transformation responses. Our system

Table 9

Effect of Serum Concentration on Cell Responses.

Antigen	maximum six day d.p.m.'s 10%	15%	20% (serum)
Control (saline)	27	46	42
B. mat.-N 50 ul/ml.	4252	7150	10979
B. mat.-N 20 ul/ml.	3414	8062	10601
B. mat.-N 10 ul/ml.	3539	6846	9192
B. mat.-N 2 ul/ml.	1195	2026	2840

again arbitrarily used supplementation at the 10% level and may therefore not represent maximal stimulation with respect to this variable.

The use of dose-response curves was discussed previously as a method by which we might determine what the maximal stimulatory concentration of antigen was for each patient at a given point in time. Had we not used a range of concentration with our stimulants, we would have been unable to determine peak responsiveness to those materials tested at six days. If an investigator were to consistently use one antigen at an optimal range and one other at a sub-stimulatory range, relative potency of the two stimulants would be seriously misjudged. Use of optimal concentrations in our system enables us to accurately assess the relative potency of the extracts. The use of triplicate tubes for each concentration also served as a valuable aid in estimating stimulation with more confidence. There are undoubtedly other factors as well which will affect the quantitative measure of lymphocyte transformation, such as method of collection and handling of the cells, culture conditions, and variations in the type of support media used. We have not felt it necessary to examine all of these variables. The fact that variables such as the ones mentioned are capable of exerting measurable changes in both d.p.m.'s and S.I.'s should require that we accept comparisons of results of lymphocyte transformation between two separate systems only after careful analysis of the methods and materials unique to each investigation. Comparison of S.I.'s and d.p.m.'s between patients within one investigative system is more easily done, since the number of variables in this type of comparison has been considerably

reduced.

Some criticism may also be directed toward our preparation of antigen or harvesting techniques. Whereas other investigators in the dental field used sonicates of various plaque and bacterial collections as stimulants (21,26), we used NaOH extracts of pure cultures grown in artificial media. It is our feeling that the NaOH extract successfully presents the antigenic protein to the lymphocyte in a purified, concentrated, and non-hydrolyzed form (34). While sonication will undoubtedly be complete in terms of antigens present, disruption and fractionation of cell components will likely occur as well. The NaOH extraction procedure is a successful method of collecting a large amount of non-hydrolyzed protein from the bacteria with only minimal alteration of that protein. Table 10 shows comparative effects of lymphocyte stimulation by a NaOH extract, a saline soluble extract, and a sonicate of SRi (S. salivarius) in cells obtained from one of our control patients (F.M.). This evidence indicates that all forms of the bacterial preparation contain stimulatory material, probably antigen. It may be possible that the NaOH extraction concentrates or more neatly presents the antigen to the lymphocyte, but this is just speculation. The ratio of d.p.m.'s to µg/ml. of protein in this table might lead us to believe that the NaOH material was actually less antigenic. However, production of the SRi-N material actually allowed us to use small increments (2-50 µl.) of the material as stimulant, without loss of effective stimulation. This data also supports the idea that the stimulation by NaOH extracts is not due to some selective effect of NaOH on the cell, but that any method of extraction will suffice

to demonstrate the antigenicity of S. salivarius.

Table 10

Comparison of Stimulation by Three Preparations of a Strain of S. salivarius.

	d.p.m.	concentration
Control	51	
SRI-N	1676	50 µl/ml.
	1312	20 µl/ml.
	1241	10 µl/ml.
	976	2 µl/ml.
Sonicate	866	200 µg/ml.
	610	80 µg/ml.
	509	40 µg/ml.
	618	8 µg/ml.
	563	2 µg/ml.
Saline	671	60 µg/ml.
	657	24 µg/ml.
	539	12 µg/ml.
	381	2.5 µg/ml.

Another departure from standard procedure in our system involved the use of repeated rinses with ice cold saline at harvest rather than the more generally accepted rinsing with TCA. Although our procedure is not generally in use, it is not without precedent. (40, 41) We found the use of saline to be without hazard and without any apparent effect on the counts obtained as compared to the TCA collection techniques. Table 11 demonstrates a comparison that was made between saline and TCA harvests to assure us that the use of the saline rinsing technique was an allowable method of assessing uptake of C-14 thymidine into the DNA of the lymphocyte. The differences noted were not significant at the 95% level of confidence. We concluded that our system accurately measured uptake of C-14

Table 11

Comparison of Saline and TCA Harvesting.

	3 days		6 days		8 days		10 days	
Antigen	saline	TCA	saline	TCA	saline	TCA	saline	TCA
Control	28	19	27	5	12	4	38	15
PHA	23030	19795	12299	10499	5370	4999	2949	3101
Pseudo-N	356	315	4113	4328	9384	8863	8262	6485
B. mat.-N	200	177	3422	3630	10935	12393	7321	7921

thymidine by peripheral lymphocytes which were stimulated by optimal concentrations of antigen, with the peak response in our system being measured at the six-day mark in a manner reflecting in vivo lymphocyte sensitivity.

We also examined the possibility that something within the growth media used to cultivate the pure cultures of bacteria might be contaminating our system and giving us false responses. Therefore, lymphocyte cultures were set up using dilutions of full strength media as stimulant. Results showed that media was not able significantly to stimulate lymphocytes above control levels, and we therefore concluded that any contaminant present in the media was not responsible for the stimulation observed in our system.

While we have discussed some of the problems inherent in use of quantitative measurements of lymphocyte transformation, we feel that qualitative comparisons can be made. It is obviously useless to compare our S.I.'s, which are a measure of lymphocyte responses at six days, to optimal concentration of antigen incubated in 10% autologous serum, with the results in a system where S.I.'s were

obtained from incubation of lymphocytes for three days to an arbitrary single level of stimulant in a media supplemented with 20% autologous serum. However qualitative responses can be compared. Do patients in both systems respond at significant levels to stimulation by Lepto-N, or endotoxins? Do people without any periodontal disease fail to give lymphocyte responses when their cells are stimulated by oral bacterial antigens? These types of questions can be answered by qualitative analysis and comparison of results when quantitative comparisons are meaningless.

How then do our results compare with previous reports published in the literature by Ivanyi and Lehner, and by Horton? (21,22,23,24,26). Ivanyi and Lehner have stated that lymphocyte responses in controls and in patients with periodontal disease follow a definite pattern: cells from controls whom they consider to be non-sensitized to oral bacterial antigens do not respond, while cells from persons with gingivitis and mild to moderate periodontitis respond well. Cells from persons with severe periodontitis give depressed responses. We found no result in control patients which is comparable to responses in the controls examined by those investigators. All of our controls with no disease responded well to all of the materials tested, with no evidence of any inhibition. Our gingivitis and periodontitis groups also responded well, as did the patients in studies by Ivanyi and Lehner. Our patients with severe periodontal disease showed no depression of response, and in fact gave responses indistinguishable from those of our other patients. Possible differences in evaluation of periodontal disease severity may exist

between our assessments using the Russell Index and those methods employed by Ivanyi and Lehner. It may be that, as a result of such differences, we did not evaluate any patients comparable to those seen in their severe periodontitis group. But one striking difference remains, that being the responses seen in the control individuals.

Horton (26) has stated, "The degree of lymphocyte reactivity to these substances (plaque and salivary sonicates) was found to be directly proportional to the severity of periodontal disease of the lymphocyte donor." In our system, no support for any conclusion of this type was found. In fact, the overall impression gained from our findings is that within the limits of biologic variation all people responded approximately equally to all of the stimulants examined. No differences relating to the incidence of periodontal disease were found, and no selectivity of effect was seen in comparison of plaque and non-plaque bacterial antigens.

We feel that such responses as those observed in our study are evidence that at some prior time in each patient's life the immune system had contacted and responded to antigens which were also present in our bacterial extracts. This initial contact allowed the patient to develop memory cells and to become sensitized to these materials. These memory cells were then demonstrated to be present by the *in vitro* transformation of these peripheral lymphocytes when incubated with the specific antigen.

Since responses were seen to all of the stimulants, it seems unlikely to assume that sensitization in every instance was the result of some pathologic disease state involving the bacteria in

question. Pathogenic and non-pathogenic bacteria alike gave positive stimulation. Plaque and non-plaque bacteria served as good stimulants without regard to periodontal disease severity. It is most likely that the sensitization to these materials is the result of normal contact between the immune system of the patients tested and their normal indigenous flora, and as such, lymphocyte transformation in the individuals examined is merely a reflection of their total immune experience with regard to the various bacteria tested.

These observations are consistent with the literature cited (5,7,8,9,10) which would indicate that the antigens of food and of the environment seem capable of permeating intact mucous membranes and other surface areas on the body, and once entering the body, of being antigenic to the hosts immune system with the resultant production of antigen specific memory cells. It is obvious that people can become sensitized to various materials without anything more than small non-damaging exposures to antigen. The only requirement for sensitization would be a discrete contact between the host's immune system and bacterial antigens. Such contact should be regarded as normal in that it would occur regularly without a resultant clinically-detectable disease state.

Our results indicate that oral and non-oral bacterial antigens serve as specific antigen. When present in the host as indigenous flora, their antigens often come in contact with the host immune system by passage through the epithelial barrier of the mouth, gut, or respiratory tract. This is a normal event and is not a severe enough insult so as to produce any clinical pathologic state. Use of the lymphocyte transformation test then gives evidence that the

host indeed has become normally sensitized to the bacterial products of the indigenous flora, but absolutely no correlation with any disease state can be attributed to the sensitization procedure, nor should it be expected that significant transformation levels can only be found if a disease state has been previously established. Lymphocytes sensitized to plaque antigens are not necessarily produced only as a result of periodontal inflammation. They can be present in the absence of any periodontal pathology, as responses in our control people indicate. But the presence of lymphocytes sensitized to plaque antigens in all individuals tested indicates very strongly that the lymphocytoid system is a major participant in the inflammatory periodontal lesion. Evidence indicates that the immune system of the periodontal patient functions in a normal manner by recognizing antigen, responding in the appropriate manner, and functioning as a viable system in protecting the host from potential insult. Periodontal patients have a normal, responding, intact immune system which is capable of recognizing and responding to plaque insult. Periodontal patients have that disease because they have allowed accumulation of materials on the teeth, which are capable of triggering the immune response with subsequent development of inflammation. The non-periodontally involved patient has the same immune capacity as does the periodontal patient. Both have functioning immune systems, both have circulating antigen-specific memory cells to plaque and non-plaque antigens. But the non-diseased individual has not allowed the accumulation of plaque on the teeth to develop, and the immune response is not activated to the extent that clinically detectable inflammation occurs. However,

the potential is still there, and within individuals, if the plaque build-up is able to reach the point where sufficient challenge is present, the immune system will trigger the inflammatory response in that individual, and gingivitis and/or periodontitis will eventually become clinically evident. This capability is common to both the periodontally-diseased as well as the non-diseased individual, and with regard to the responses observable in lymphocyte transformation tests, there is no difference between them. All individuals examined in our study are part of a medically healthy population, and to differentiate between them on the basis of lymphocyte transformation responses or periodontal disease severity is to make an artificial distinction between them with respect to their immune history and capabilities.

CONCLUSIONS

1) Stimulation of human peripheral lymphocytes by endotoxin occurs in an unusual and unique manner. Usually low and sometimes statistically insignificant levels of stimulation are seen at an extremely wide range of concentration of the stimulant, with an absence of the characteristic dose-response relationship.

2) The nature of lymphocyte transformation to endotoxin in human lymphocyte systems has certain parallels in the mouse system. It can be hypothesized that endotoxin is a selective but non-specific stimulator of B cells in the human system. This seems more likely than accepting endotoxin as a specific antigenic stimulant, on the basis of the data collected.

3) Lymphocyte transformation to endotoxin can be considered a normal finding in a normal population of individuals. This is true whether we accept endotoxin in the role of either a specific or a non-specific type of stimulant.

4) All individuals examined by means of <u>in vitro</u> lymphocyte transformation techniques responded at significant levels when their peripheral lymphocytes were challenged by various NaOH extracts of bacterial cultures.

5) The range of response in terms of d.p.m.'s and S.I.'s varied widely. No significant correlation was found between transformation responses and age or disease severity.

6) Variables in culture techniques such as antigen concentration, serum content, time of incubation, and antigen preparation may alter cell responses in a quantitative way. Therefore, although quantitative comparison of the results of different investigators may be meaningless, qualitative comparisons can be made successfully.

7) Lymphocyte responses to the antigens tested in our system were felt to be the result of stimulation of antigen specific memory cells whose presence in the peripheral circulation was the result of normal contact between the host's immune system and his normal indigenous flora.

8) The immune system in patients with periodontal disease is functional in a normal manner. Both periodontally involved and healthy control individuals have similar immune capacities, as well as specific memory cells which have been sensitized to members of the plaque and non-plaque flora.

9) Periodontal disease may therefore be the result of *in vivo* insult by plaque materials which results in activation of the immune system for defense of the host. Our findings indicate that these immunopathologic potentials are shared by both diseased and non-diseased individuals.

10) If, as some suggest, transformation were a correlate of sensitization, our results would show that sensitization is unrelated to either the absence of or severity of clinical disease. However, transformation levels are best related to immunological memory and our results show that the level of past immune experience also is unrelated to the absence of or severity of clinical disease. Therefore, frequency of antigenic contact modulated by the host responses most probably determines the severity of the gingival diseases.

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APPENDIX A

ANTIGEN CONCENTRATIONS

B. mat.-N	0.970 mg/ml.
B. mel.-N	0.934 mg/ml.
Candida-N	3.240 mg/ml.
SRi-N	2.764 mg/ml.
E-49-N	2.570 mg/ml.
E. coli-N	3.805 mg/ml.
N. perf.-N	3.315 mg/ml.
Lepto-N	8.530 mg/ml.
NEP	0.400 mg/ml.
A. naes.-N	0.960 mg/ml.
V1-N	3.935 mg/ml.
Pseudo-N	9.600 mg/ml.
T-6-N	1.433 mg/ml.
E. coli LPS	used on dry wt. basis
Lepto LPS	used on dry wt. basis
PHA	used on dry wt. basis