LYMPHOCYTE TRANSFORMATION RESPONSES TO PULP ANTIGENS IN FORMOCRESOL PULPOTOMY PATIENTS

by

Dennis G. Longwill, D.D.S.

A Thesis

Presented to the Department of Endodontology
and the Advanced Education Committee of the
School of Dentistry
University of Oregon Health Sciences Center
in partial fulfillment
of the requirements for the degree of
Master of Science
June, 1979

APPROVED

[redacted]

F. James Marshall, D.M.D., M.S.
Professor and Chairman
Department of Endodontology

[redacted]

F. James Marshall, D.M.D., M.S.
Chairman,
Advanced Education Committee

ACKNOWLEDGMENTS

The author wishes to thank the following individuals for their part in this project.

My wife, Jeannette, whose understanding and loving encouragement were a constant source of strength.

The Air Force Institute of Technology and the Air Force Clinical Research Program, who provided the opportunity and necessary financial support.

Nellie Riggs, who not only tirelessly applied her skills in typing this manuscript, but was a constant source of encouragement.

Richard Tacke, for providing the services of the Clinical Pathology Laboratory and for his unwavering commitment to research.

Virginia Dunn and Norma Smith, who skillfully collected all of the blood samples used in this project.

William Harold, whose technical skills and dedication to scientific endeavor proved invaluable to the research.

Dr. Arthur Retzlaff, for allowing the author to work within the Department of Pedodontics, which cooperated with this endeavor in every way, and for his valued efforts as a member of the Thesis Committee.

Dr. Howard Creamer, whose input to this project provided every facet of the educational experience. Dr. Creamer has unselfishly shared his remarkable knowledge and constructive guidance from the inception of this project.

Dr. F. James Marshall who, as mentor, provided guidance, encouragement and valuable advice throughout this work. I am also grateful for his example and his sincere commitment to learning which will continue to influence my professional growth.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

INTRODUCTION

Rational therapeutics implies knowledge of the degree to which a treatment modality influences a biologic system. Many traditional methods of therapy have been accepted on the basis of clinical success regardless of the empirical nature of their introduction. There is considerable research effort in the health sciences to characterize the local and systemic responses to these procedures. Recent awareness of the complexities of the immune system has led to a re-evaluation of oral disease processes and treatment modalities as they relate to the immune response.

The use of Formocresol as an endodontic medication was introduced in the early part of this century. The rationale for its use was the alleged ability of the material to render decomposing tooth pulp tissue biologically inert through fixation. It is now a commonly used method for the treatment of deciduous teeth in which caries penetration requires extensive restorative procedures which will penetrate the pulp chamber.

A review of the literature reveals a variety of local effects from the treatment, but chronic inflammation usually predominates. The antigenic or haptenic potential of phenolic compounds has been established and recent animal studies have demonstrated the potential for Formocresol treated pulp tissue to induce an altered immune state. This potential, however, has not been adequately tested in humans in which the Formocresol material has been used in the pulpotomy procedure.

A study was designed to assay the level of immune responsiveness to endodontically related antigens in patients with a history of Formocresol pulpotomy. The lymphocyte transformation analysis, which is based on the ability of immunocompetent lymphocytes to undergo *in vitro* blastogenesis when exposed to a previously encountered antigen, was chosen as the method for analysis of immunologic reactivity. Formocresol treated and untreated human pulp tissue extracts were prepared and tested as stimulants for lymphocyte populations obtained from Formocresol pulpotomy patients. The levels of blastogenic response induced by the pulp extracts were measured by the incorporation of radiolabeled thymidine and were compared with responses induced by the mitogen phytohemagglutin as well as by known and putative antigens. These lymphocyte transformation responses were compared with those of a control population in which there was no history of Formocresol pulpotomy.

LITERATURE REVIEW

The Use of Formocresol in Dentistry

Formocresol was introduced by Buckley (1904) for the treatment of teeth with necrotic pulp tissue. The original formula, which consisted of equal parts of formalin and cresol, was reported to react chemically with the end products of necrosis "converting them into odorless and non-infectious compounds". This "mummification" process was reported to render the pulpal tissue biologically inert and to allow asymptomatic retention of the involved tooth.

Sweet (1923) advocated that Buckley's formula be used for the treatment of various types of pulpal involvement in deciduous teeth. He recommended multiple treatments of the root canal contents with Formocresol in both necrotic and vital cases. In 1955, Sweet modified the original recommendations by reducing the number of applications and limiting the treatment to teeth with vital radicular pulp tissue (pulpotomy). The procedure became popular and its success was established on a clinical basis with no consideration given to the local or systemic effect of the treatment.

The Formocresol pulpotomy is now a commonly accepted treatment for deciduous teeth in which caries removal and restorative procedures expose vital pulp tissue. The treatment is generally accomplished by the surgical removal of the coronal pulp tissue followed by a five-minute application of Formocresol to the radicular pulp. The material is

usually applied by a wetted cotton pellet with no attempt made to accurately limit the dose.

Chemical Properties of Formocresol

Formalin is a 37% solution by weight of formaldehyde gas in water. It is used extensively as a disinfectant, germicide, and preservative. It is also used in the production of textiles, phenolic resins, artificial silk, cellulose esters, dyes, organic chemicals, glass mirrors, explosives and waterproofing agents (Merck Index, 1968).

The primary reaction of a protein with a formaldehyde preparation is the formation of a methylol derivative from an amino, hydroxy, imino, sulfhydryl, amide, or carboxyl side group. The labile aminomethylol compounds can react in slow secondary reactions to form stabile intermolecular or intramolecular methylene cross-links. Optimal interaction of formaldehyde preparations and protein takes place in slightly alkaline conditions (pH 7.5 to 8). These properties of formaldehyde reactions have been reported by Wadsworth and Pangborn (1936), French and Edsall (1945), and Fraenkel-Conrat and Olcot (1974).

The ability of formaldehyde preparations to chemically block side groups is the key to their use as tissue fixatives. Structural alteration prevents enzymatic degradation and allows stabilization of the protein molecule. The efficiency of Formocresol as a fixative agent for the dental pulp was discussed by 's-Gravenmade (1975). He stated that the reaction time for fixation is relatively long and the ability of the material to form stabile compounds can be limited by dilution of the reaction mixture as it diffuses through tissue.

Cresol is an aqueous suspension of three isomers of methylphenol.

It was empirically added to formalin by Buckley (1904) to minimize the irritating properties of formalin. 's-Gravenmade (1975) suggested that a large hemiacetal molecule is formed by the addition of cresol which reduces the tissue diffusibility of Formocresol.

Biologic Effects of Formocresol

The biologic effect of Formocresol on a local level was not emphasized until the 1950's when extensive histologic evaluations were reported. Massler and Mansukhani (1959) described three zones of pulp tissue reaction in human primary and permanent teeth. An acidophilic zone adjacent to the pulp amputation site was described as fixation. This zone extended apically into a degenerative or atrophic zone which was followed by a zone showing a chronic inflammatory response.

Berger (1965) made similar observations in sections of 50 primary teeth with pulps that had been exposed to a five-minute application of Formocresol. An active inflammatory response was reported apical to an area of cellular necrosis. He described the presence of connective tissue extending from the periodontal ligament into the apical portion of the root canal in the seven-week specimens. Berger concluded that this "ingrowth" would replace the entire contents of the radicular canals and allow for the success of the treatment.

Loos et al (1973) applied different concentrations of Formocresol to implanted polyvinyl alcohol sponges. Histologic evaluation revealed an initial cytotoxicity and a slight inflammatory response which did not vary with the concentration of Formocresol. Using a histochemical analysis, they reported that the ability of fibroblasts to recover their cellular activities after various time periods depended upon the dilution

of Formocresol.

Langeland et al (1976) studied the histologic effects following a five-minute application of Formocresol to the pulps of monkey teeth. The animals were sacrificed at various time periods and block sections were examined. They reported areas of tissue disintegration with a generalized inflammation of the apical pulp and periapical osteitis in the 196-day specimens.

Variations in response to the Formocresol pulpotomy were illustrated by the study of Rolling et al (1976). They studied 27 cases of primary teeth with cresol pulpotomies which were considered to be successful based on clinical evaluation at three to five years following treatment. Histologic and histochemical evaluation showed a range of pulpal conditions from minimal inflammation to total necrosis. Severe chronic inflammation with active resorption and apposition of hard tissues within the root canals was seen in most cases.

Some general observations can be made concerning the local response to Formocresol even though there were obvious variations in the research results reported. Formocresol does not simply render pulpal tissue biologically inert as Buckley proposed. Local destruction of tissue is apparent as well as is an inflammatory reaction that may last long after the application of Formocresol.

The Effect of Formocresol on the Immune Response

The ability of formaldehyde preparations to stimulate the immune system has been established. Epstein and Maibach (1966) mentioned the high incidence of allergy to formaldehyde and reported a positive response to patch tests in 5% of eczematous patients. Formaldehyde

induced sensitivity to facial tissues was reported by Peck and Palitz (1956), to nail hardeners by Lazar (1966), and to clothing by O'Quinn and Kennedy (1965). A publication by Fisher (1976) gives additional information on a wide range of potentially allergenic products which contain formaldehyde.

For the Formocresol pulpotomy procedure to induce an immune response, antigens must affect small lymphocytes either at the local level or systemically by diffusion from the root canal into the circulation. A study by Myers et al (1978) reported on the uptake of ^{14}C-formaldehyde following pulpotomies in rhesus monkeys. Approximately one percent of the dose was absorbed systemically after a five-minute exposure to the drug at the pulpotomy site.

The ability of the root canal to act as a site for immunization has been verified in a number of animal studies. Rosengren (1962) used streptococci to innoculate the root canals of cats and found a significant antibody titer in those animals in which periapical lesions developed. A control group in which an identical dose of microbes was administered submucosally showed limited inflammation and no antibody titer. This suggests that the anatomical uniqueness of the root canal may provide a sequestered environment which leads to antigen release into the apical tissue over a long period of time.

Similar animal studies using potent non-microbial antigens have also demonstrated the potential for immune stimulation through the root canal. Barnes and Langeland (1966) used a single application of bovine serum albumin or packed sheep erythrocytes which were introduced into monkey teeth following pulpotomy. The subsequent presence of circulating antibody to these antigens was demonstrated by two-dimensional agar gel

diffusion and direct hemagglutination methods.

Okada et al (1967) used lyophilized horse serum and administered it at repeated intervals into the root canals of rabbits. Significant antibody titers as measured by agar diffusion and hemagglutination were reported. Local destruction and chronic inflammation was seen histologically in adjacent periapical tissues. Histologic evaluation of heart, lung, liver, kidney and spleen demonstrated various degrees of inflammatory degeneration which was related to the number of times the animals were sensitized.

Steffen et al (1976) introduced ovalbumin agar gel at weekly intervals into the pulpectomized teeth of rabbits. Hemagglutinating antibody titers developed which were identical to those of control animals which had been sensitized by subcutaneous injection. Positive arthus skin tests were reported as evidence of immune complex formation.

It is obvious from these animal studies that a potent antigen administered into the root canal at repeated intervals is capable of stimulating the immune system.

There are few human studies which relate to the antigenic potential of Formocresol as it is used clinically. A case report by Al-Nashi and Al-Rubayi (1977) described a patient who developed localized swelling and itching one hour after application of Formocresol in the root canal of a pulpless tooth. Patch tests confirmed that formalin was the agent responsible for the reaction. This report verifies the potential for immune challenge of an already hypersensitive individual following an endodontic application of Formocresol.

A report by Rolling and Thulin (1976) represents the only study in humans which has attempted to determine the antigenic potential of

Formocresol as it is used clinically. One hundred and twenty-eight children who had received Formocresol pulpotomies on deciduous teeth were tested for sensitivity to formaldehyde, eugenol and cresol. The number of treated teeth in each child varied from one to six and the time between the pulpotomy and patch tests varied from two months to eight years. Patch tests using pulpotomy materials (2% formaldehyde in water, 5% eugenol in petrolatum, 2% cresol in water) showed no positive results. The authors concluded that contact sensitivity to the components of Formocresol does not generally occur as a result of their use in pulpotomy procedures.

This study by Rolling and Thulin evaluated the ability of the Formocresol components to induce a cutaneous delayed hypersensitivity response. Another area of concern, however, is the potential for Formocresol to alter tissue components of the pulps in such a way that these altered host tissues could be rendered antigenic and cross-reactive with normal tissues.

The Antigenicity and Cross-Reactivity of Formocresol Altered Tissues

Benacerraf (1965) and Milgrom (1965) have demonstrated that modified tissue components can be antigenically active to the host. In each of these studies, denatured autologous gamma globulin was shown to be antigenic by its ability to induce antibody formation in rabbits and guinea pigs. Horsfall (1934) used the same animal models to demonstrate antibody formation to formalized homologous and heterologous sera. The results of this study led him to the following conclusion: "The changes produced in the protein molecule by the action of formaldehyde are sufficient to modify very considerably the immunological characteristics

of such proteins."

In a more recent publication, Dennert and Tucker (1972) demonstrated in mice the antigenicity of formaldehyde fixed erythrocytes. Cell surface antigens were altered by the fixation process and T-cell recognition was shown to be the primary immunogenic response.

Thoden van Velzen and Feltkamp-Vroom (1977) studied formaldehyde fixed autologous connective tissue implants in rabbits. Histologic analysis revealed a dense lymphocytic infiltrate with enlargement of regional lymph nodes, primarily associated with the thymus dependent areas. Serum antibody titers remained low throughout the implant period. This study gives additional support to the role of the cell-mediated immune response to altered autologous tissue. The authors criticized formalin fixation for therapeutic purposes on the basis of its ability to induce systemic immunization.

The ability of formalin fixed dental pulp to induce an immune response was first reported in rabbits by Nishida et al (1971). Hemagglutinating antibodies were formed to the treated autologous rabbit pulp extracts which were intravenously administered two or three times a week for 100-140 days. This prolonged systemic sensitization also induced autoantibodies to untreated autologous pulp tissue. The observation that immune responsiveness to damaged host tissue may be cross-reactive to unaltered (native) tissue supports one of the current theories for the etiology of autoimmune disease.

Block et al (1977) evaluated cell-mediated immune responses to Formocresol treated dog pulp tissue. Primary immunization of the animals was accomplished by the intramuscular injection of suspensions of autologous Formocresol treated pulp tissue. Subsequent challenge was

provided via the root canals at seven-day intervals for 28 days. Lymphocyte transformation responses were significantly higher for the Formocresol treated pulp tissue as compared to the responses obtained with suspension of untreated pulp tissue. Skin tests in these animals demonstrated that pulp treated with Formocresol induced more demonstrable swelling, necrosis, induration, and erythema than when Formocresol was injected alone. The authors concluded that Formocresol renders dog pulp tissue antigenically active and that a specific cell-mediated immune response to dog pulp altered by Formocresol was obtained.

It is apparent that there is a potential for formalin-altered autologous pulp tissue to induce an immune response through stimulation of T-cell recognition. This potential, however, has only been demonstrated in animals where intense sensitization methods were employed. The application of Formocresol as it is used clinically in humans has not been studied in animals. The only study in which a human population of Formocresol pulpotomy patients was used revealed no evidence of hypersensitivity to the chemical components of Formocresol as determined by patch testing.

The question of whether Formocresol-treated tissue induces an immune response by the host to the treated tissue or, more significantly, to his own untreated pulp tissue has not been evaluated in humans. An evaluation was thus designed to determine the level of lymphocyte blastogenesis to prepared human pulpal extracts and to relate these responses to the clinical Formocresol pulpotomy experience. Of secondary interest was the identification of other variables which might be statistically associated with a history of Formocresol pulpotomy. The results of this study should add to the knowledge of the biologic consequences

of this commonly used dental treatment.

RATIONALE FOR EXPERIMENTAL DESIGN

The Lymphocyte Transformation Analysis

The lymphocyte transformation analysis was chosen as the assay best able to detect evidence of the antigenic effect of past experience with Formocresol pulpotomy. This is based on the role of the small lymphocyte in the primary recognition of antigen (Gowans and McGregor 1965), and the identification of two main populations of lymphocytes as functionally different, immunologically specific, effector cells (Davis et al 1971, Owen 1972). Thus, humoral immunity which is a function of B-cell response through antibody production is distinguished from cell-mediated immunity (CMI) which is a function of T-cell response through the activation of other mononuclear cells (Lawrence and Landy 1969).

The lymphocyte transformation analysis was developed as an *in vitro* correlate of immune responsiveness following the discovery by Nowell (1960) that a kidney bean extract, phytohemagglutin (PHA), transformed small lymphocytes into lymphoblasts in tissue culture. Substances such as PHA, which induce blastogenesis in a large number of lymphocytes, are called *mitogens*, and have been used to demonstrate the correlation between lymphocyte transformation and deficiencies in CMI (Lischner et al 1967). A study by Bach et al (1968), in which B-cell deficient patients were shown to have normal lymphocyte transformation responses, supports the functional role of T-cells in the response. Some mitogens, however, demonstrate activation of pure cultures of B-cells (Greaves et al 1974)

and additional evidence shows that the transformation response can be detected in association with *in vivo* antibody production (Dutton and Eady 1964).

The role of antigens in the lymphocyte transformation was first reported by Pearmain et al (1963) when they established that lymphocytes of tuberculin-positive individuals transformed in the presence of the purified protein derivative (PPD) obtained from tuberculin while no transformation was demonstrated in tuberculin-negative patients. Curtis et al (1970) demonstrated that increases in human lymphocyte transformation could be observed at seven days following primary immunization using Keyhold Limpet hemocyanin and O'Reilly and Lopez (1975) reported that patients with a history of repeated herpetic infections had a positive lymphocyte transformation response to extracts of the herpes simplex virus. Antigens are thus distinguished from mitogens in that a prior exposure, i.e., immunization, is required to induce proliferation of a specifically responsive clone of cells. Lymphocyte transformation can be used to assay secondary B or T-cell responsiveness and is thus an *in vitro* correlate of immune memory (Oppenheim et al 1970).

The technical aspect of the analysis requires the isolation of lymphocytes from whole peripheral blood. A method of centrifugal density gradient separation is commonly used to provide a mononuclear cell population consisting of approximately 90% lymphocytes and 10% monocytes (Eisen et al 1973) which can then be added to a supportive medium containing a specific concentration of the test stimulant. Cultures are incubated for a period of time sufficient to allow the development of a peak transformation response. This is limited by the efficiency of the stimulant and the ability of the culture environment to support

logarithmic growth of lymphocytes. The mitogenic substances are known to induce peak responses in two or three days while the antigenic stimulants typically require a six-day incubation period or longer. Quantitation of lymphocyte transformation is performed by pulse-labeling the cells with a radioactive DNA precursor prior to the conclusion of the culture period. The cells are harvested, washed to remove excess label, and the degree of isotope incorporation is measured by liquid scintillation counting (Oppenheim and Schecter 1976).

Mitogenic or antigenic materials are the main types of stimulants used in the lymphocyte transformation analysis. A mitogen is usually chosen to evaluate the immune potential of the individual as well as to serve as a positive control for the ability of the culture system to support cell growth. Potentially antigenic stimulants are selected to determine a prior sensitization to the material and to quantitate the level of transformation capability in an individual or group. The ability of a stimulant to induce the *in vitro* transformation response is concentration dependant. High and low dose inhibition exists which appears to be regulated by a specific cell surface signal (Hadden et al 1975). The optimal concentration of a given stimulant will vary among individuals and within the same individual relative to time. A range of concentrations which theoretically includes the optimal dose is thus utilized whenever possible.

There are many other factors which can influence the reactivity of lymphocytes *in vitro*, including: the mononuclear cell concentration, size and shape of the culture chamber, volume and depth of media, O_2 tension, length of incubation, concentration of label and degree of cell manipulation (Moorhead et al, 1976). These variables must be taken into

account when comparing the results obtained from different laboratories.

Cultures containing specific concentrations of each stimulant are generally established in triplicate and the arithmetic mean of the radioisotope uptake is expressed in counts per minute (CPM). The CPM values can be corrected for background radiation and variation in the ability of the liquid scintillation analizer to measure the total photon output of the system. Any factor such as absorption of light by the sample itself which alters the light output of the system is called quenching. A "quench curve" can be determined for a series of standardized radioactivities and an efficiency factor can be used to convert CPM values into absolute units of disintegrations per minute (DPM) (Kobayoshi and Maudsley, 1974).

The data are often interpreted by dividing the mean value of the experimental culture by the mean value of the control (E/C) in which no stimulants were added. The resulting value is known as the stimulation index (SI) and is considered significant if the experimental culture has at least a two-fold increase in counts over the control. The problem with the SI value is that control values may vary to a great degree depending upon transfer of *in vivo* stimulants or proliferation due to handling. Cunninham-Rundles et al (1976) emphasized the potential variation in control values caused by other factors than those which influence the test cultures. They advised caution in using the SI when control culture DPM are excessively high or low. The best evaluation of published results is possible when control results as well as experimental results are presented.

Thymidine incorporation by unstimulated cultures is usually low because only stimulated lymphocytes go on to synthesize significant

amounts of DNA. The low counts of these control cultures are responsible for the sensitivity of the assay. Oppenheim and Rosenstreich (1976) have estimated that "repeated division of an initial clone with fewer than 10 cells in a population of 10^6 lymphocytes theoretically can result in significant increases (E/C) in thymidine uptake after 5-7 days of incubation". Since this growth rate is exponential, small differences in the number of responding cells may become magnified with time and result in non-normal distribution of the data. Normalization of the data can be achieved by logarithmic conversion of the DPM which can then be analyzed by student's t test or analysis of variance.

In summary, the lymphocyte transformation analysis was selected because of the following reasons.

1. It is the broadest and most sensitive correlate of immune memory. It is a more effective indicator of previous distant sensitization than the commonly used measurement of antibody levels which are not maintained if the challenge is not repeated. The half life of the longest lasting class of immunoglobulin is only 23 days.
2. It is safer and more efficient than *in vivo* skin testing which measures only the effector limb of hypersensitivity and challenges the subject with potentially antigenic materials.
3. It includes the influence of enhancing and inhibiting factors in the immune response which may be provided by serum components or other subpopulation of mononuclear cells.
4. It permits the evaluation of levels of immune response

based on the quantitative nature of the assay.

Stimulants

Several types of materials, pulp related and unrelated, were evaluated for their ability to induce *in vitro* lymphocyte transformation responses. The test stimulants of primary interest were those which would express the potentially antigenic properties of untreated and Formocresol-treated pulp tissue as they exist *in vivo*. A soluble extract of sonicated, pooled, human pulp was prepared in the hope of obtaining tissue specific antigens without including the histocompatibility antigens associated with non-autologous (allogenic) tissue. The solubility of the material also allowed for filter sterilization and for the quantitation of protein. Half of the obtained extract was incubated with Formocresol which comprised 2% of the final extract volume. The formation of a particulate suspension indicated that a chemical modification had taken place.

In addition to the pulp extracts tested, a sonicated preparation of pooled human tooth plaque samples was also prepared and tested. This allowed the evaluation of the lymphocyte transformation responses to immunologic challenge by components derived from ubiquitous oral microorganisms.

Other materials tested consisted of the mitogen, PHA, and three microbial extracts which were known stimulants according to previous studies. These provided control values which confirmed the ability of the culture environment to support cell growth and allowed comparison with the previously untested materials.

Patient Selection

The experimental design called for the evaluation of lymphocyte transformation responses to Formocresol-treated and untreated pulp extracts relative to the clinical Formocresol pulpotomy experience. The selection was thus limited to children in which the pulpotomy procedure is most commonly used.

Two options were considered for patient selection with provisions for control. The first possibility was to perform pre- and post-treatment bleedings on patients requiring Formocresol pulpotomy. This approach would have allowed internal controls for comparison of lymphocyte transformation responses, since a direct test of the possible conversion of individuals from a nonresponsive to a responsive state would have been made. However, it was considered important to evaluate the presence of long-term immune sensitization and this choice would have required too lengthy a follow-up period with multiple bleedings of the same children. In addition, the use of internal controls was not considered essential since uncontrollable variables, such as age and childhood diseases, might alter the responsiveness of the immune system over a period of time.

An alternate decision was made to choose a group of patients with a history of previous Formocresol pulpotomy and compare them with a group of age- and sex-matched patients with no previous history of Formocresol treatment. This allowed the selection of post-treatment time periods which would permit the evaluation of a biologically significant immune memory. In addition, it was possible to select individuals who had multiple treatments to insure that the test population had the greatest potential for sensitization.

The use of dental records was essential in determining the past Formocresol treatment of the patients. Individuals whose dental records were incomplete or did not provide sufficient information to establish a history of all past dental treatment were excluded from the study.

The selection of patients was made from a single source, which insured that standard criteria and procedure, as outlined in Appendix I, were employed in all pulpotomies. It was not considered desirable to control the potential variability of the pulpotomy procedure which existed within these standards. This variability was thought to be representative of the universal treatment modality which the study hoped to evaluate.

The decision to compare two separate populations of patients required that other parameters of biological status be recorded for each group. The DMFS index, as described by Klein et al (1938) was modified for the existence of mixed dentition to establish the past caries history of each subject. A PMA index, as described by Massler et al (1950), was used to establish the current gingival health of each subject. This was done to determine if significant differences in gingival health existed between groups and to allow evaluation of possible correlations between gingival health and other variables in the data analysis.

Clinical hematological data were obtained for each subject in order to establish systemic parameters which may have differentiated the two groups of patients. This also provided a health screening service for the subjects as well as establishing a set of data base standards for a group of young, medically healthy dental patients.

MATERIALS AND METHODS

Subjects

Subjects were chosen from a population of patients receiving treatment in the undergraduate and graduate Pedodontic Clinics of the School of Dentistry, University of Oregon Health Sciences Center. Group 1 consisted of 20 individuals, ages 5-9, with a history of dental treatment which did not include Formocresol pulpotomy. Group 2 consisted of age- and sex-matched patients with a history of two or more pulpotomies in anterior or posterior primary teeth which were rendered 5 to 18 months prior to the time of blood sampling. A signed consent form was obtained from the parent or guardian of each patient in compliance with the Human Rights and Welfare Policy of the University of Oregon Health Sciences Center (Appendix II).

The following variables were recorded for each patient:

1. Pulpotomy History

   On the day of blood sampling the patient's age, sex, history of allergies and information regarding the number and dates of previous Formocresol treatment were obtained from the dental record.

2. Caries History

   A DMFS index was recorded on the basis of a clinical examination and the dental record. The sum of all decayed, missing or filled dental surfaces was totaled for primary and

permanent teeth in each individual. A tooth which had been normally exfoliated was not counted as missing, but a tooth extracted because of caries involvement was counted as five missing surfaces.

3. Gingival Health

   A PMA index was recorded on the basis of clinical observation by a single observer utilizing a dental light and an air dry field. The buccal gingiva of the maxillary and mandibular anterior teeth was divided into ten units and the total number of papillary, marginal and attached gingival segments which showed signs of inflammation were recorded. Clinical color photographs were taken of the anterior teeth and associated gingiva, using standard settings for each subject.

4. Systemic Health

   Clinical hematological data consisting of blood type, a complete blood count and differential, hemoglobin and hematocrit values, protein electrophoresis and a multi-channel serum analysis (SMAC*) were recorded by the clinical laboratory service at the School of Dentistry, University of Oregon Health Sciences Center.

Preparation of Mononuclear Cell Population

A single 28 ml sample of whole venous blood was drawn from the antecubital fossa of each subject by a licensed phlebotomist. The

---

*SMAC - Sequential, Multiple, Analyzer, Computerized

technique described by Creamer and Harold (in preparation) was used to obtain mononuclear cells for the transformation analysis. The whole blood was defibrinated, centrifuged and the serum layer drawn off. The "buffy-coat" of leukocytes was transferred into test tubes containing a suspension of MEM-S (Minimal Essential Media, Eagle, GIBCO) and cells which had been rinsed from the defibrination pad. Two concentrations of Ficol/Hypaque were layered into the tubes to establish a discontinuous gradient for centrifugal separation of mononuclear and granulocyte populations. Following centrifugation, the mononuclear cell layer was collected, suspended in MEM-S, centrifuged twice and resuspended in supplemented MEM-S. The MEM-S was supplemented for support of mononuclear cells by the addition of 1% Glutamine (GIBCO), 0.1% penicillin-streptomycin (GIBCO) and 10% fresh autologous serum which had been filter sterilized and heat inactivated at 57°C for thirty minutes. Following differential counting, a concentration of 3×10^6 mononuclear/ml was prepared for culturing.

Lymphocyte Transformation Technique

A volume of 0.1 ml of 3×10^6 mononuclear cells/ml was added to 0.4 ml of the test material in supplemented MEM-S contained in 10 x 75 mm disposable glass culture tubes (#T-1285-2, SCIENTIFIC PRODUCTS) employing stainless steel closures. A range of test material concentrations, as outlined in Appendix III, was employed to insure an optimal lymphocyte response under the culture conditions used in the study. Incubation was accomplished at 37°C in 5% CO_2 and water saturated air (Model 30 Incubator, SHELDON) for three days (PHA) or six days (other stimulants) according to previously determined optimal response periods. All concentrations

of the materials were tested in triplicate. Controls were provided by three- and six-day triplicate cultures of mononuclear cells in supplemented MEM-S.

One day prior to harvesting, the cultures were removed from the incubator, placed in a water bath at 37°C and a 0.01 ml volume of ^{14}C-Thymidine (50 µC/0.5 ml, diluted to 10 µC/Ml with MEM-S (NEW ENGLAND NUCLEAR) was pipetted into each culture. Sterile manipulation of all materials was maintained throughout the preparation, incubation and labelling period to prevent microbial contamination of the cultures.

At the end of the incubation period, cultures were removed from the incubator and cooled in an ice bath to stop cellular activity. The button of mononuclear cells present in the bottom of each tube was suspended by agitation and vacuum collected onto glass fiber pads (Grade 934 AH, WHATMAN) through the use of a multiple automated harvester. The pads were washed with distilled water to remove excess label and then transferred into standard glass scintillation vials containing 10 ml of scintillation fluid (Insta-gel, PACKARD). The vials were allowed to stabilize at room temperature overnight, and counted in a Searle Delta 300 Scintillation Analyzer (SEARLE ANALYTIC, INC.) using the channel ratio method. Counts per minute (CPM) were corrected for quenching, efficiency of the counter, and subtraction of background radiation and expressed as disintegrations per minute (DPM). The mean DPM for each triplicate culture was recorded and the peak level of transformation for each stimulant was taken to be the mean of the triplicate cultures giving the highest count regardless of the concentration of the stimulant at which the highest count occurred.

Preparation of Stimulants

1. Pulp extracts

   Unerupted human molar teeth, which had been frozen following extraction, were split longitudinally and a pool of human dental pulp tissue was obtained. The pooled sample was diced, washed, freeze-thawed, sonicated, centrifuged and clarified by filtration to provide a soluble extract. The soluble extract was divided equally and one portion was incubated for seven days with a 2% by volume concentration of Formocresol (50% formalin, 50% cresol, ROTH DRUG COMPANY), thoroughly dialized to remove excess drug, and resonicated. The protein concentration of the Formocresol treated and untreated extracts was determined by the Lowry method (Campbell 1970). The untreated dental pulp extract was designated PS-A while the Formocresol treated extract was designated PS-B. A detailed description of the preparation of the pulp extracts is provided in Appendix IV.

2. Plaque extracts

   Individual plaque samples which had been collected from tooth surfaces of adult patients by the Dental Hygiene students at the School of Dentistry, University of Oregon Health Sciences Center, were available in one dram screw cap vials, frozen in 3 ml saline. The contents of the vials were thawed and individually vortexed for five seconds before being spilled into a 700 ml beaker contained in an ice bath. The pooled sample was sonicated

at 100 watts for one minute intervals with a cooling for a total sonication time of 15 minutes.

The sample was clarified by filtration through a glass fiber pad (WHATMAN) and then through a 0.45μ membrane (GELMAN). The sample was concentrated by pressure filtration through a Millipore Pellicon membrane (10,000 m.w. cut off, PSAC06L10) at 55# pressure with stirring at 2°C. Approximately 7 ml of concentrate remained after eleven hours of pressure filtration. The concentrated plaque sonicate was filter sterilized and designated Plaque-Son. The protein concentration of Plaque-Son was determined, and the sample was divided into 0.5 ml portions, placed into snap-cap vials and stored at 72°C until the day of use.

3. Other Stimulants

These materials were available for testing and a description according to their experimental designation is given below.

PHA - A mitogenic extract of kidney bean. Commercially obtained Purified Phytohemagglutinin (WELLCOME REAGENTS #HA16) was prepared to a concentration of 1000 μg (dry weight)/ml.

SKSD - Vacuum dried Streptokinase-Streptodornase Veridase in a 20,000 SK to 5,000 SD unit ratio, product #NDC 0005 2201 66 (LEDERLE). The product had been dialized with saline to remove the Thimersol preservative agent and a concentration of 10,000 units/ml was prepared.

T14V-Son - A soluble extract of washed and sonicated *Actinomyces viscosus* (isolated strain #T14Vii) prepared by Dr. Benjamin Hammond of the University of Pennsylvania. A concentration of 10,000 μg (dry weight)/ml was prepared.

Lepto-Son-D - A Soluble extract of washed and sonicated *Leptotricia buccalis* (American Type Culture Collection #14201) was also prepared by Dr. Benjamin Hammond. This extract had been dialized to reduce inhibitory properties and a concentration of 10,000 µg (dry weight)/ml was prepared.

All of these materials were stored in small volumes at -72°C until their use and then filter sterilized (0.45µ Millex, MILLIPORE) prior to placement into culture tubes.

Statistical Methods

The mean, standard deviation and variance was established for all variables recorded separately in Group 1 (n=20) and Group 2 (n=20) and also for the combined data (n=40). A student's *t* test was used to compare the means of the variables between Group 1 and Group 2 in order to determine significant differences in lymphocyte transformation response and/or clinical variables. Correlation coefficients were calculated for certain clinical variables in relation to all other data for both groups separately and for the pooled population. Other statistical evaluations included the determination of multiple correlation coefficients for selected variables and the use of discriminate analysis in an attempt to identify variables which could be considered "predictive" of a history of Formocresol pulpotomy.

RESULTS

The purpose of this investigation was to determine the relative difference in immune response between two populations of patients which were known to vary with respect to previous Formocresol pulpotomy experience and to evaluate the degree to which Formocresol-treated and untreated extracts of human pulp tissue acted as antigenic stimulants in these patients.

It was first necessary to identify any variable which might differentiate the two groups on the basis of the available clinical and hematological data. Once this was established, analysis of the lymphocyte transformation responses could be used to more effectively demonstrate any difference in immune response between the two groups.

Clinical Variables

Criteria for the selection of patients were chosen to provide homogeneity with respect to age and sex distribution between and within the two groups of patients. A t test comparison of the mean age and sex distribution verified that no difference existed between Group 1 and Group 2. Table 1 provides the mean age and standard deviation as well as the sex distribution for both groups and for the total sample population.

A significantly higher mean caries index (DMFS) was recorded in the Group 2 patients when compared with Group 1 in which there was no history of Formocresol pulpotomy. Table 2 shows the mean DMFS values for

both groups and the t value associated with the means. This difference was expected due to the positive clinical relationship between the extent of caries involvement and the potential for pulpal involvement.

The extent of gingival inflammation as measured by the PMA index was not significantly different between the two groups. The mean number of inflamed papillae and marginal and attached gingival segments for both groups along with the respective t values are reported in Table 3.

The average extent of the Formocresol pulpotomy experience for the 20 subjects in Group 2 is represented in Table 4. There was an average of approximately three pulpotomies performed on each patient and the time from the last pulpotomy to the testing procedures averaged one year. This sample thus represented a group of individuals with a relatively extensive Formocresol pulpotomy experience and provided an evaluation of long term immune memory by the lymphocyte transformation analysis.

Hematological variables

The mean hematologic parameters of the sample populations were evaluated by *student's* t test. Of the 42 variables tested, only two showed a statistically significant difference between the treatment and control groups. The data are thus represented as the mean values for the total sample population (Tables 5, 6, and 7; n=40). The mean values were also compared with the "normal range" of pediatric values as published by Kempe et al (1976).

The mean values of the complete blood count and differential are given in Table 5. The mean hematocrit percentages were the only values shown to be significantly different, by t test, between Group 1 and 2.

The mean hematocrit values for each group were, however, within the published normal range of pediatric values. A comparison of other values in this table with normal pediatric ranges demonstrated a low monocyte count (281/mm^3 versus average normal of 430/mm^3) and a relatively low hemoglobin value (12.6 gm/100 ml versus a normal range of 12.5-15 gm/100 ml) for the total sample population.

The mean protein electrophoresis values are given in Table 6. No difference between Groups 1 and 2 was seen in the amounts of individual protein components present or the relative distribution of the globulin fractions. All of the mean values were within published pediatric standards.

The mean serum component values for the total sample population are given in Table 7. The mean serum cholesterol level was the only variable which differed significantly by *t* test comparison between both groups. The mean cholesterol values for Group 1 and Group 2 were both within the wide range of values considered to be normal for this age group. Further comparison of all other mean serum component values with established normal ranges for children revealed a high chloride level (110.5 meq/L versus a normal range of 97-104 meq/L) and an extremely high LDH level (389 IU/L versus a normal range of 17-59 IU/L).

Group Comparisons of the Lymphocyte Transformation Responses

The results of the lymphocyte transformation analysis demonstrated no difference between the mean values for Group 1 and Group 2. This observation was made for all concentrations of all materials tested, whether the mean values were calculated from the dissociations per minute (DPM), the log of DPM, or the stimulation index (SI). The mean peak DPM values

of the total sample population (n=40) for all of the test materials is given in Table 8. Table 9 shows the same data in which the logs of the peak DPM levels were used to calculate the mean peak response values for each stimulant.

The stimulation index is the ratio of the test material DPM value to the control DPM value in which no stimulant is added to the culture medium. The SI value thus measures the relative ability of the test material to act as a stimulant for lymphocyte blastogenesis. Table 10 shows the separate mean peak SI values of Group 1 and Group 2 for each of the materials tested. The "2-tailed" probability for each calculated t value demonstrated that the mean SI values of the two groups were not significantly different. Figure 1 represents the relative mean peak SI values, with standard error for each of the materials tested in Groups 1 and 2. Note the relationship of the pulp extracts (SI < 10) to the plaque sonicate (SI > 700) and to the mitogen PHA (SI > 2000).

Relative Stimulation by Test Materials

To further evaluate the stimulation potential of the test materials within each group, the mean control DPM was compared to the mean peak DPM value of each test material. Table 11 shows the calculated t values and the critical t value for significance at the 95% level of confidence. All transformation values except for PS-A and PS-B in Group 2 were seen to differ significantly from transformation values of their respective control cultures. The mean PS-A and PS-B DPM values were not statistically different from the mean 6-day control DPM value in Group 2, but the relatively large mean and standard deviation for the 6-day control in Group 2 may account for this lack of significance.

As stated previously, the stimulation index is generally considered to represent significant lymphocyte blastogenesis when the experimental DPM is at least two times greater than that of the control. Table 12 lists individual peak SI values of PS-A and PS-B for all subjects. The table divides the population into non-responders (Table 12A) with SI values of less than 2.5, and responders in which an SI equal to or greater than 2.5 was seen for either PS-A or PS-B (Table 12B). Twenty-five out of the forty individuals were considered to be immunologically responsive to either the treated or untreated human pulp extracts. There were both responders and non-responders to the pulp extracts in both groups of patients and only three of the non-responders could possibly be explained by excessively high control counts. The treated and untreated human pulp extracts appeared to be acting as immunologically specific stimulants for *in vitro* lymphocyte blastogenesis and can therefore be considered as antigens since not all persons responded to them.

Antigenic Differentiation of Untreated and Formocresol Treated Human Pulp Extracts

An attempt was made to determine whether the human pulp extracts differed in their ability to stimulate lymphocyte transformation. Table 13 shows a comparison of the peak SI values between PS-A and PS-B for both groups and the total population. *t* test values demonstrated significantly lower levels of transformation induced by PS-A as compared to PS-B. This indicates that the treated and untreated pulp extracts are acting as antigenically different materials and that the Formocresol treated extract is a significantly stronger stimulant of the

lymphocyte transformation analysis.

A scatter distribution of the individual peak SI values for pulp extract A and B is given in Figure 2. The mean peak SI values and standard error for each group is also shown. This figure demonstrates no significant difference between groups but a significant difference can be seen between the transformation levels obtained with the treated and untreated extracts.

Bivariate correlation coefficients were calculated for the peak SI response to PS-A and to PS-B to determine if the responses were related. The existence of such a relationship would tend to weaken the previous conclusion, based on a comparison of means, which suggested that the two materials were acting as independent variables. Figure 3 shows the scatter diagram with a least squares regression line calculated for the peak SI values of Group 1, Group 2, and of all responders (SI $\geq$ 2.5). The slope of the regression line (b), y axis intercept (a), Pearson correlation coefficient (r) and standard error of estimate (S_{xy}) are given for each diagram. A relatively high r value in Group 2 indicates a strong positive relationship between the response to PS-A and the response to PS-B. However, this strong correlation appears to be the result of a single bivariate point on the scatter diagram. When the r value is recalculated with the elimination of that bivariate point, a correlation coefficient of r = 0.405 results. This suggests that little real correlation exists between the peak responses induced by the pulp extracts. This supports the concept of the antigenic individuality of the two materials.

Correlations among Variables

The results presented thus far have primarily demonstrated a lack of difference between the mean values of Group 1 and Group 2 for most of the clinical, hematologic and lymphocyte transformation variables based on the student's t test. In an attempt to evaluate other possible relationships in the data, bivariate correlation coefficients were obtained between selected clinical variables and all other recorded variables. Although many statistically significant relationships were found, it was decided to limit interest to those that attained an r value of at least 0.450 ($r^2 = 0.20$).

Table 14 lists the clinical variables of interest and the variables with which they were correlated at a level of 0.45 or greater. The strongest bivariate correlation (DMFS and the extent of marginal inflammation established by the PMA index in Group 2) had an r value of 0.6434. An insight into the meaning of this relationship can be gained by using r^2 which is the coefficient of determination. An r^2 value of 0.4140 indicates that approximately 41% of the variation of one variable is statistically explained by the variation of the other variable. Figure 4 shows the scattergram of DMFS versus marginal gingival inflammation.

In addition to the bivariate correlations with selected clinical variables, an attempt was made to determine the possible relationship between certain salient variables and sets of predictor variables by means of a multiple regression analysis.

The method involved a stepwise determination of a series of independent variables which together showed the strongest correlation to the dependent variable of choice. This supplied a group of variables and a multiple correlation coefficient which represented the degree to which

these variables were capable of collectively predicting the value of the independent variable. A small set of two or three variables with a multiple r value of 0.700 was considered to be of possible biological significance on the basis that these few variables could predict approximately 50% of the observed values of the selected variable.

The variables of interest in this study were those associated with the peak lymphocyte transformation responses to the treated and untreated human pulp extracts. A multiple regression analysis was performed using the peak log and DPM responses to PS-A and PS-B as dependent variables. Independent variables were obtained from clinical and hematologic data and the analysis was separately run for Group 1, Group 2, and the total sample population.

Table 15 lists the multiple correlations in which an r value of at least 0.700 was attained for three variables. The results demonstrated only one strong multiple correlation for the total population and no obvious similarity between the variables selected when Group 1 and Group 2 were tested separately.

Discriminant Analysis

A final attempt was made to statistically distinguish between the two sample groups by using a discriminate analysis. The mathematical objective of discriminate analysis is to weight and linearly combine a set of discriminating variables so that the groups are forced to be as statistically distinct as possible. The results indicated that 77% of the sampled patients could be correctly classified as Group 1 or Group 2 individuals on the basis of three variables. The ability to correctly classify 67% of the known cases was provided by the serum cholesterol

value alone and a 10% increase in discriminating ability was added when the log response to SKSD at the 1000 U/ml concentration and the lymphocyte count were included as discriminating variables.

DISCUSSION

The lymphocyte transformation analysis was used to evaluate immune memory to Formocresol treated and untreated extracts of pooled human dental pulp tissue in two groups of patients, who differed on the basis of a past experience with Formocresol pulpotomies.

The discussion of the results of this analysis will cover three main areas of interest. First, the relationship between the Formocresol pulpotomy and the lymphocyte transformation and hematologic values will be discussed. Second, the ability of the homologous human pulp extracts to act as antigenic stimulants for the lymphocyte transformation analysis will be related to the presence of *in vivo* human pulp tissue antigens. Third, the implications of these research findings will be related to the current understanding of the role of the immune response in endodontics.

The lymphocyte transformation analysis resulted in positive responses to the untreated (PS-A) and Formocresol treated (PS-B) pulp extracts and to all other stimulants in both groups of subjects. There were no differences recorded in any of the mean lymphocyte transformation responses between the group of individuals with no history of Formocresol pulpotomy (Group 1) and the group of individuals with a history of multiple Formocresol pulpotomies (Group 2). This finding leads to the conclusion that the clinical Formocresol pulpotomy does not induce a significant systemic immune response as evaluated in this study. The basis for this support

relies upon the sensitivity of *in vitro* lymphocyte transformation as a correlate of immunologic memory and upon the extent of the clinical exposure to Formocresol experienced by the Group 2 patients.

The possibility that Formocresol pulpotomy was related to other systemic parameters was examined by a comparison of mean values for the hematologic variables recorded for Group 1 and Group 2. The mean hematocrit percentage (Table 5) and the mean serum cholesterol level (Table 7) were the only values which differed significantly between the groups. Both values, however, were within the normal pediatric limits and no biological relationship between these variables and the Formocresol experience could be postulated. These observed differences may be related to dietary differences between the groups, although no socioeconomic differences between the groups were evident. It was concluded that a history of Formocresol pulpotomy has no significant influence on or relationship with the hematological status of the subjects tested.

Evaluation of the mean hematologic values for the total population in relation to the published pediatric normal values revealed two points of interest. First, the relatively low hemoglobin level (mean 12.56 gm/ml) reported for the sampled individuals may reveal an iron deficiency in some members of the population from which this sample was taken. Hemoglobin values obtained by this study ranged from 11.2 to 14.3 gm/100 ml and it is possible that some of these individuals, although not seriously anemic, could benefit from dietary iron supplementation. Second, the mean serum LDH values demonstrated an extreme variation from the normal pediatric values. The values observed in this study ranged from 223 to 500 IU/L as compared to the normal pediatric range of 17 to 59 IU/L (Kempe et al 1976). This probably demonstrates a technical variability associated

with the automated quantitation of LDH used in this study.

The evaluation of possible relationships between variables was accomplished by calculating bivariate and multiple correlations for a number of clinical and lymphocyte transformation response values. The purpose of the analysis was to attempt to detect any biologically significant relationship between clinical, hematologic and immune response variables which were recorded for two groups of patients who differed on the basis of past Formocresol pulpotomy experience.

The strongest bivariate correlation (DMFS with marginal gingival inflammation in Group 2, r=0.6434) appears to be a statistical relationship that was explainable on the basis of a common etiology for both caries and periodontal disease (Table 14). The remaining bivariate selections demonstrated weaker statistical correlations and did not present obvious biological relationships. In addition, very few of the statistically significant correlations were observed when r was calculated for the total population (n=40). This indicates that many of the statistically significant correlations between variables in Group 1 or in Group 2 were a result of the relatively small number of values tested (n=20). The bivariate correlations which maintained a significant r value when n=40 were: the positive relationships between age and serum creatinine levels (p=.001), the positive relationship between age and serum gamma globulin levels (p=.001) and the positive relationship between gingival inflammation (PMA) and the peak response to the untreated pulp extract (p=.002). The levels of serum creatinine and serum gamma globulin could be expected to increase with age in as much as the normal adult levels of these components are higher than those of children.

The relationship between current oral inflammatory status, as measured by PMA, and peak responses to PS-A could be explained on the basis of a dependency of each upon a third variable such as oral hygiene which was not measured in this study. The lymphocyte transformation responses to pooled human dental pulp may thus be related to factors which influence oral health. Such factors could be associated with the general responsiveness to inflammatory by-products, possibly represented by the untreated extract.

The multiple correlations also demonstrated a number of statistically significant values and the same general observations can be made with respect to the biological significance of this data (Table 15). Some of the correlations between dependent variables and single entering variables in Group 1 and Group 2 can be explained by possible biologic relationships but the multiple groupings generally indicate no biologic relationship between the entering variables or to the dependent variable for which they were selected. A comparison of the same dependent variables in Groups 1 and 2 shows that no similarities were obtained in the correlated sets of variables selected.

One multiple correlation did meet the criterion of having three dependent variables with an r value of at least 0.700 when n=40. The multiple correlation coefficient of r=0.768 was obtained when the independent variable, PMA Index, was correlated with the following three variables: peak lymphocyte transformation response to PS-A, lymphocyte transformation response to Lepto-Son-D at 12 µg/ml, and number of lymphocytes. A measurement of current oral inflammatory status (PMA) was thus found to be positively related to measurements of immunologic memory for substances obtained from the oral environment (untreated extracts of dental

pulp and extracts of *Leptotricia buccalis*). This again supports the concept that PS-A is possibly associated with oral inflammatory processes.

The discriminate analysis was another attempt to determine if certain unexpected variables existed which could categorize individuals on the basis of past Formocresol experience. The strongest discriminating variable was found to be the level of serum cholesterol. Although this variable may indicate dietary differences between the two groups of patients, the mean values of both groups were within the published normal pediatric range. The discriminating power of serum cholesterol is interesting, but there is no apparent biological explanation for the relationship that is seen.

The second primary area of concern is the ability of the human dental pulp extracts to act as stimulants for the lymphocyte transformation analysis. The conclusion that the pulp extracts functioned as antigenic stimulants for the lymphocyte transformation analysis is based on the observation that 25 out of the 40 individuals were shown to have significantly elevated responses when compared to their own control cultures (Table 12). This selective response indicates that the materials were acting as antigenic stimulants rather than as mitogens which would have induced a generalized positive lymphocyte transformation response. A comparison with the other known antigens tested indicates that both extracts acted as minimal but real antigenic stimulants in both groups of patients (Table 11 and Figure 1).

The mean level of transformation as measured by the stimulation index was greater following stimulation with the Formocresol treated extract (PS-B) than with the untreated extract ($\overline{X}$ SI PS-A = 3.1, $\overline{X}$ SI PS-B = 8.5, $p < .005$, Table 13). This is evidence that the pulp extract

was antigenically altered by the Formocresol treatment. The response to PS-B was unrelated to past history of Formocresol pulpotomy as was the untreated extract.

Since lymphocyte transformation responses to Formocresol treated and untreated homologous pulp tissue extracts were found to be independent of past experience with Formocresol pulpotomy, it is necessary to provide possible explanations of the responses that were seen in each group. One possible explanation is that the prepared pulp extracts are cross-reactive with some antigen which is unrelated to pulp tissue. The lack of biologically significant correlations between peak responses to PS-A and peak responses to PS-B (Figure 3), as well as the significantly greater response induced by PS-B (Table 13), indicate that the two materials were acting as independent antigens. It seems unlikely that two materials with different antigenic determinants are both cross-reactive with some non-pulpal antigen. In addition, it is difficult to explain what type of non-pulpal antigen might be associated with cross-reactivity to the pulp extracts. If such an antigen were associated with an ubiquitous microorganism, all individuals should have responded and at relatively higher levels than those produced by the pulp extracts. Finally, cross-reactivity is generally not associated with cellular phenomenon in which carrier specificity requires that sensitized lymphocytes not only recognize the antigenic determinant but also recognize the molecular structure on which that determinant is presented.

It could be argued that the antigenicity of the homologous pulpal extracts might be due to histocompatibility antigens present in the pooled sample of pulp tissue that was used to prepare the extracts. The large number of individuals from which the nearly 300 unerrupted molar

teeth were obtained would represent a vast array of histocompatibility phenotypes. If such a representation were present in the prepared extracts, all of the tested individuals would be expected to respond. The lack of response in 15 of the 40 individuals tested indicates that histocompatibility antigens were not represented in the pooled sample. In addition, the sonication process which was employed in the preparation of the pulp extracts is not an efficient method for extracting histocompatibility antigens (Stabholz and McArthur 1978).

The preparation of autologous pulp tissue extracts would eliminate the possibility that the lymphocyte transformation responses that were seen were due to the presence of foreign histocompatibility antigens. The obvious problem with this approach would be the difficulty associated with obtaining enough tissue from each subject to insure an adequate concentration of autologous extract for the lymphocyte transformation technique. This point is illustrated in a study reported by Eleazer et al (1975) in which autologous pulp tissue or tissue from a periapical lesion were tested for their ability to induce lymphocyte transformation. They reported no significant transformation responses for any of the 23 cases evaluated. The authors did not report the concentration of prepared material that was added to the *in vitro* cultures. In 12 of the cases, however, pulp specimens from only a single tooth were used for the analysis. It is clear that this small amount of tissue would provide very low concentrations of the potential antigen for use in the lymphocyte transformation analysis and that these concentrations may have been suboptimal and responsible for the negative results which were reported.

The most reasonable explanation for the responses to the pulp extracts is that they contain some antigenic product of a common pulp-

related denaturation process and that the Formocresol treated extract has the closest antigenic similarity to the proposed antigen. An example of such an alteration process could be the pulpal inflammation associated with caries penetration into the dentin. This possibility would explain the presence of responses in both groups of subjects. If the pulp extracts represent an antigenic product related to the denaturation processes, the lack of response of some individuals can be explained on the basis of individual host variation, since the extent of these processes would be expected to vary among individuals. In addition, individual genetic variability might require higher levels of denaturation for immune sensitization to occur in some individuals.

It is thus concluded that the antigenicity of the prepared pulpal extracts is most probably related to the potential antigenicity of human pulp tissue, and that transformation responses induced by the extracts are based on sensitization to a product of pulp denaturation caused by inflammation.

A direct test of the ability of caries activity to induce antigenic pulpal alterations which leads to immunization of the host would involve a comparison of lymphocyte transformation responses in groups of age- and sex-matched individuals with varying degrees of caries experience. For example, a caries free group could be compared with groups having moderate and extensive caries experience. If the premise is supported, one would expect minimal lymphocyte transformation responses to pulpal extracts in the caries free group, increased responses in the moderate caries group, and the highest responses in the extensive caries group.

The final area of consideration will be a discussion of the results of this study as they relate to the current understanding of the immune

response in endodontics. The conclusion that the clinical Formocresol pulpotomy does not lead to immunization of the subjects is supportive of the study by Rolling and Thulin (1976). Negative patch tests were reported to the components of Formocresol for children who had received Formocresol pulpotomies. The present study was considered to be a more critical test of the potential for immune sensitization by pulpotomy because a broader and more sensitive immune assay was used and Formocresol altered pulp extracts were evaluated as the potential antigens.

The results reported by Block et al (1977), however, demonstrated the ability of Formocresol altered pulp tissue to stimulate the immune system as evaluated by lymphocyte transformation responses in dogs. Extracts of Formocresol treated pulp tissue were used to sensitize the animals by intramuscular injection with Freund's adjuvant as well as by repeated endodontic administration. The contrasting results that were found in the present study are most probably explained by the limited presentation of altered tissue to the host following clinical Formocresol pulpotomy as compared with the intense sensitization methods employed in their animal model. The study by Block et al did show that Formocresol treated autologous pulp could induce transformation responses, and that the untreated pulp tissue extracts induced weaker transformation responses than the treated extracts. They did not, however, report on possible lymphocyte transformation response of control dogs to the Formocresol treated pulp tissue. Therefore, the possibility exists that the Formocresol treated pulp tissue may have been acting independently of the sensitization process that was employed in their study.

A recent study by Stabholz and McArthur (1978) reported that patients with periapical pathosis had significant cell-mediated immune responses

to homologous necrotic pulp tissue as measured by the production of leukocyte migration inhibition factor (LIF) by peripheral lymphocytes. Lymphocytes of the control patients, in which there was no evidence of existing periapical pathosis, did not demonstrate leukocyte migration inhibition when tested with the pooled pulp antigen. This study supports the concept that human pulp tissue may become antigenic due to degenerative processes and that an immunopathologic mechanism may be involved in diseases of endodontic origin.

If the antigenic potential of *in vivo* denatured human pulp tissue is accepted, inferences can also be made about the cause of certain clinical problems. In the practice of endodontics, for example, repeated root canal therapy sometimes fails to alleviate obvious periapical pathology associated with necrotic pulp tissue. The patient may have had a history of endodontic treatment in several other teeth with a similar lack of success. This problem could theoretically be related to small remnants of denatured tissue within the apical portion of the root canal. In such individuals a type of hypersensitivity to their own altered pulp tissue might be preventing the clinical success of the treatment.

Additional research is needed in order to further evaluate the role of immunologic mechanisms in pulp related diseases and treatment. Characterization and identification of the postulated immunogenic pulp-tissue component and evaluation of mechanisms involved in its production would be worthy contributions to the science of endodontics.

SUMMARY AND CONCLUSIONS

The purpose of this study was to evaluate the potential for immune stimulation as a result of the clinical Formocresol pulpotomy experience in humans. Two groups of patients were selected on the basis of past Formocresol pulpotomy experience. Group 1 consisted of twenty individuals with no history of previous Formocresol exposure, while Group 2 consisted of twenty individuals with a history of two or more Formocresol pulpotomies which had been performed 5 to 18 months prior to the study.

The lymphocyte transformation analysis was employed as the assay most capable of evaluating immune memory. The extent of *in vitro* lymphocyte blastogenesis induced by a preparation of untreated and Formocresol treated homologous dental pulp extracts as well as a number of other potential stimulants was measured by radioactive thymidine incorporation. Clinical and hematologic variables were measured in the two groups to evaluate the extent of biological variation and to relate this potential variation to the results of the lymphocyte transformation analysis.

The following conclusions are based on the results of this study.

1. Extracts of homologous human pulp tissue contain a pulp-related antigenic component which can induce a positive lymphocyte transformation response in certain individuals.
2. Treatment of the pulp extract with Formocresol yielded an extract which led to the induction of significantly greater lymphocyte transformation responses than did the untreated

extract.

3. A history of clinical Formocresol pulpotomy was unrelated to a significant systemic immune response as measured by the lymphocyte transformation analysis.
4. A history of Formocresol pulpotomy is considered to be unrelated to the hematological parameters which were measured in this study.
5. The antigenicity of human pulp tissue extracts appears to be related to a pulpal antigen which is not uniquely associated with Formocresol pulpotomy, but which is possibly related to alterations of pulp tissue that has been brought about by inflammation.
6. There was no evidence to suggest that the clinical Formocresol pulpotomy should be discontinued because of concern that it will lead to significant immune sensitization of the patient.

BIBLIOGRAPHY

Al-Nashi, Y., and Al-Rubayi, A.: A case of sensitivity to tricresol formalin, Br. Dent. J. 142:52, 1977.

Bach, F., Meuwissen, H., Albertine R., and Good, R.: Cellular studies in Agammaglobulinemia, Birth Defects, Original Article Series, 4:245, 1966.

Barnes, G. and Langeland, K.: Antibody formation in primates following introduction of antigens into the root canal, J. Dent. Res., 45:1111, 1966.

Benacerraf, B.: The antigenicity of altered autologous proteins, a mechanism of autoimmune reactions, Ann. N. Y. Acad. Sci., 124:126, 1965.

Berger, J.: Pulp tissue reaction to Formocresol and zinc oxide-eugenol, J. Dent. Child., 32:13, 1965.

Block, R., Lewis, R., Sheats, J. and Fawley, J.: Cell-mediated immune response to dog tissue altered by Formocresol within the root canal, J. Endod., 3:424, 1977.

Buckley, J.: The chemistry of pulp decomposition, with a rational treatment for this condition and its sequelae, American Dental Journal, 3:1904.

Campbell, D., Garvey, J., Cremer, N. and Sussdorf, :Methods in Immunology, 2nd. ed., p. 76, W. A. Benjamin Inc., New York, 1970.

Creamer, H. and Harold, W.: (In preparation)

Cunningham-Rundles, S., Hansen, J., and Dupont, B.: Clinical Immunobiology, (Bach, F., Good, R., ed.), Vol. 3:151-194, Academic Press, New York, 1976.

Curtis, J., Hersh, E., Harris, J., McBride, C. and Freireich, E.: The human primary immune response to keyhole limpet haemocyanin interrelationships of delayed hypersensitivity, antibody response and *in vitro* blast transformation, Clin. Exp. Immunol., 6:473-491, 1970.

Davies, A., Leuchars, E., Wallis, V., and Doenhoff, M.: A system for lymphocytes in the mouse. Proc. R. Soc. Lond., B 176:369, 1971.

Dennert, G., and Tucker, D.: Selective priming of T cells by chemically altered cell antigens, J. Exp. Med., 136:656, 1972.

Dutton, R. and Eady, J.: An *in vitro* system for the study of the mechanism of antigenic stimulation in the secondary response, Immunology, 7:40, 1964.

Eisen, S., Lyle, L. and Parker, C.: Antigen stimulation of ^{3}H thymidine incorporation by subpopulations of human peripheral blood cells, J. Immunol. 3:962, 1973.

Eleazer, P., Faber, P. and Seltzer, S.: Lack of lymphocyte stimulation by root canal products, J. Endod., 1:388, 1975.

Epstein, E. and Maibach, H.: Formaldehyde allergy, Arch. Dermatol., 94:196, 1966.

Fisher, A.: Formaldehyde: Some recent experiences, Current Contact News, 17:665, 1976.

Fraenkel-Conrat and Olcott: The reaction of Formaldehyde with proteins. V. Cross-linking between amino and primary amide or guanidyl group. J. Am. Chem. Soc., 70:2673, 1974.

French, D. and Edsall, J.: The reaction of Formaldehyde with amino acids and proteins, Advances in Protein Chemistry (Anson and Edsal, eds.), p. 278-336, 1945.

Gowans, J. and McGregor, D.: The immunologic activities of lymphocytes, Prog. Allergy, 9:1, 1965.

Greaves, M., Janossy, G. and Doenhoff, M.: Selective triggering of human T and B lymphocytes *in vitro* by polyclonal mitogens, J. Exp. Med., 140:1, 1974.

Hadden, J., Hadden, E., Sadlik, S. and Coffey, V.: Effects of concanovalin A and a succinylated derivative on lymphocyte proliferation and cyclin nucleotide levels, Proc. Natl. Acad. Sci. U.S.A., 73:1717, 1975.

Horsfall, F.: Formaldehyde and serum proteins. Their immunological characteristics, J. Immunol., 27:553, 1934.

Kempe, C., Silver, H., O'Brien, D.: Current Pediatric Diagnosis and Treatment, 4th. Ed., p. 1015-1023, Lange Medical Publications, Los Altos, California, 1976.

Klein, H., Palmer, C. and Knutson, J.: Studies on dental caries. I. Dental status and dental needs of elementary school children, Public Health Rep., 53:751, 1938.

Kobayashi, Y. and Maudsley, D.: Biological Applications of Liquid Scintillation Counting, p. 25-27, Academic Press, New York, New York, 1974.

Langeland, L., Dowden, W., and Langeland, K.: Formocresol, "mummification" tissue disintegration, microbes, inflammation, resorption, and apposition, I.A.D.R. Abstracts #268, J. Dent. Res., 55, B, 1976.

Lawrence, H. and Landy, M. (eds): Mediators of Cellular Immunity, Academic Press, New York, New York, 1969.

Lazar, P.: Reactions to nail hardeners, Arch. Dermatol., 99:446, 1966.

Lischner, H., Punnett, H. and DiGeorge, A.: Lymphocytes in congenital absence of the thymus. Nature (London), 214:580, 1967.

Loos, P., Straffon, L., and Han, S.: Biological effects of Formocresol, J. Dent. Child., 40:193, 1973.

Massler, M., Schour, I. and Chopra, B.: Occurrence of gingivitis in suburban Chicago school children, J. Periodontol., 21:146, 1950

Massler, M. and Mansukhani, N.: Effects of Formol-Cresol on the dental pulp, J. Dent. Child., 4th quarter, 277, 1959.

Merck Index, 8th ed., P.G. Stecher, ed., Merck and Co., Rahway, New Jersey, 1968.

Milgrom, F.: Antibodies to denatured autologous antigens, Ann. N.Y. Acad. Sci., 124:118, 1965.

Moorhead, J., Connolly, J., McFarland, W.: Factors affecting the reactivity of human lymphocytes *in vitro*. I. Cell number, duration of culture and surface area. J. Immunol., 99:413, 1967.

Myers, D., Shoof, H., Dirksen, T., Pashley, D., Whitford, G. and Reynolds, K.: Distribution of ^{14}C-formaldehyde after pulpotomy with Formocresol, J.A.D.A., 96:805, 1978.

Nishida, O., Okada, H., Kawagoe, K., Tokunaga, A., Tanihata, H., Aono, M., and Yokomizo, I.: Investigation of homologous antibodies to an extract of rabbit dental pulp, Arch. Oral Biol., 15:733, 1971.

Nowell, P.: Phytohemagglutinin: an initiator of mitosis in cultures of normal human leukocytes, Cancer Res., 20:462, 1960.

Okada, H., Aono, M., Yoshida, M., Munemoto, K., Nishida, O. and Yokomizo, I.: Experimental study on focal infection in rabbits by prolonged sensitization through dental pulp canals, Arch. Oral Biol., 12:1017, 1967.

Oppenheim, J., Blaise, R. and Waldmann, T.: Defective lymphocyte transformation and delayed hypersensitivity in Wiskott-Aldrich Syndrome. J. Immunol., 104:835, 1970.

Oppenheim, J., and Rosenstreich, D.: Lymphocyte transformation: Utilization of automatic harvesters, in *In Vitro* methods in cell-mediated and tumor immunity, Bloom, B. and David, J. (ed.) p. 584, Academic Press, San Francisco, California, 1976.

Oppenheim, J. and Schecter, B.: Lymphocyte transformation in Manual of Clinical Immunology, Rose, N. and Friedman (ed.) p. 81, Amer. Society for Microbiology, Washington, D.C., 1976.

O'Quinn, S., and Kennedy, C.: Contact dermatitis due to formaldehyde in clothing textiles, J.A.M.A., 194:593, 1965.

O'Reilly, R., and Lopez, C.: Cyclic deficiency of lymphokine production in patients with recurrent herpes labialis or progenitalis, Fed. Proc., 34:947, #4124, 1975.

Owen, J.: The origins and development of lymphocyte populations, in Ontogeny of Acquired Immunity, Ciba Found. Symp. (North-Holland, Amsterdam, London), 1972.

Pearmain, G., Lycette, R., and Fitzgerald, P.: Tuberculin-induced mitosis in peripheral blood leukocytes, Lancet I, 637-638, 1963.

Peck, S. and Politz, L.: Sensitization to facial tissues with urea-formaldehyde resin, J.A.M.A., 162:1226, 1956.

Rolling, I., Hasselgren, G., and Tronstad, L.: Morphologic and enzyme histochemical observations on the pulp of human primary molars 3 to 5 years after Formocresol treatment, Oral Surg., 42:518, 1976.

Rolling, I. and Thulin, H.: Allergy tests against formaldehyde, cresol, and eugenol in children with Formocresol pulpotomized primary teeth, Scand. J. Dent. Res., 84:349, 1976.

Rosengren, L.: The antibody response to experimental streptococcal infection (S84) of the dental pulp of the cat. Scand. J. Dent. Res., 70:265, 1962.

s'-Gravenmade, E.: Some biochemical considerations of fixation in endodontics. J. Endod. 1:233, 1975.

Stabholz, A. and McArthur, W.: Cellular immune response of patients with periapical pathosis to necrotic dental pulp antigens determined by release of LIF, 4:282, J. Endod., 1978.

Steffen, C., Keresztesi, K., Endler, A., Smolen, J. and Menzel, J.: Experimental pulpal immunization I. Route of application and demonstration of immune response, Immunitaets Forsch, Immunobiol. 152:42, 1976.

Sweet, C.: Root canal treatment in deciduous teeth including pulp exposure, Pacific Dental Gaz., 31:718, 1923.

Sweet, C.: Treatment of vital primary teeth with pulp involvement, J. Colorado D.A., 33:10, 1955.

Thoden vanVelzen, S. and Feltkamp-Vroom, T.: Immunologic consequences of formaldehyde fixation of autologous tissue implants, J. Endod., 3:179, 1977.

Wadsworth, A., and Pangborn, M.: The reaction of formaldehyde with amino acids, J. Biol. Chem., 115:423, 1936.

TABLE 1: AGE AND SEX OF POPULATION

	Group 1	Group 2	All
n	20	20	40
Male	10	9	19
Female	10	11	21
Mean age (years)	7.3	6.9	7.1
S.D. age	1.1	1.5	1.3

TABLE 2: MEAN CARIES INDEX OF GROUPS (DMFS)

	Mean DMFS	S.D.	t*
Group 1	18	14	-4.28
Group 2	38	16	

*"1-tailed" t value for significance at 95% level (38 d.f.) =-1.68

TABLE 3: MEAN GINGIVAL HEALTH INDEX OF GROUPS (PMA)

	Group 1 mean (S.D.)	Group 2 mean (S.D.)	t*
P - No. of inflamed papillae	6.7 (3.5)	6.9 (3.2)	-0.24
M - No. of inflamed marginal gingivae	4.1 (3.3)	3.7 (3.4)	0.37
A - No. of inflamed attached gingivae	0.2 (0.5)	0.1 (0.2)	0.83

* "2-tailed" t value for significance at 95% level (38 d.f.) = $\pm$ 2.02

TABLE 4: HISTORY OF FORMOCRESOL PULPOTOMY (GROUP 2)

	Mean	Range	S.D.
No. of pulpotomies per patient	3.0	2-6	1.2
No. of pulpotomy appointments/patient	2.5	1-4	0.7
Time from first pulpotomy to lymphocyte transformation analysis (weeks)	53	22-77	18
Time from last pulpotomy to lymphocyte transformation analysis (weeks)	49	20-75	18

TABLE 5: BLOOD COUNT AND DIFFERENTIAL VALUES OF POPULATION (n=40)

	Mean number of cells per mm^3	S.D.
Leucocytes	6140	1375
Lymphocytes	2968	779
Monocytes	281	146
Polymorphonuclears	2602	1049
Eosinophils	181	208
Basophils	35	50
Band cells	67	118
Erythrocytes	4.61×10^6	0.41×10^6
	Mean	**S.D.**
Hemaglobin (gm/100 ml)	12.56	0.74
Hematocrit (%)	38.1*	1.85

* A significant difference in mean hematocrit values was obtained by *t*-test. Group 1 mean hematocrit = 37.5%, Group 2 mean hematocrit = 38.7%, "2-tailed" probability = 0.047.

TABLE 8: PEAK LYMPHOCYTE TRANSFORMATION VALUES (DPM) FOR TOTAL POPULATION (n=40)

	Mean	S.D.
PHA	37217	41846
3-Day Control	22	23
PS-A	71	176
PS-B	112	159
Plaque-Son	5688	6267
SKSD	7567	7380
Lepto-Son-D	1149	1032
T14V-Son	13065	7032
6-Day Control	54	159

TABLE 9: PEAK LYMPHOCYTE TRANSFORMATION VALUES (log) FOR TOTAL POPULATION (n=40)

	Mean	S.D.
PHA	4.459	0.259
PS-A	1.418	0.485
PS-B	1.715	0.538
Plaque-Son	3.458	0.560
SKSD	3.572	0.725
Lepto-Son-D	2.868	0.457
T14V-Son	4.052	0.252

TABLE 10: PEAK LYMPHOCYTE TRANSFORMATION VALUES (STIMULATION INDEX) FOR GROUP 1 AND GROUP 2

	n	Mean	S.D.	S.E.	t*
PS-A					
Group 1	20	3.4	4.1	0.9	0.59
Group 2	20	2.7	2.7	0.6	
PS-B					
Group 1	20	7.5	7.6	1.7	-0.50
Group 2	20	9.5	15.3	3.4	
Lepto-Son-D					
Group 1	20	102.1	106.6	23.8	-0.62
Group 2	19	138.2	232.3	53.3	
SKSD					
Group 1	20	814.9	746.5	166.9	1.05
Group 2	19	560.9	768.9	176.4	
Plaque-Son					
Group 1	20	734.0	1494.8	334.2	0.04
Group 2	19	716.1	1244.9	285.6	
T14V-Son					
Group 1	20	1331.3	973.8	217.8	-0.57
Group 2	19	1618.3	1969.2	451.8	
PHA					
Group 1	19	2411.8	1989.2	456.4	-1.04
Group 2	19	3696.8	4995.3	1146.0	

*"2-tailed" t value for significance at 95% level (38 d.f.) = $\pm$ 2.02

TABLE 11: COMPARISON OF CONTROL VALUES (DPM) WITH PEAK STIMULATION VALUES (DPM) IN GROUP 1 AND GROUP 2

	Mean	S.D.	t*
	Group 1		
3-day Control	20	18	--
PHA	33169	22822	-6.50
6-Day Control	14	13	--
PS-A	32	28	-2.54
PS-B	88	116	-2.82
SKSD	8044	7959	-4.5
Plaque-Son	5604	7168	-3.5
T14V-Son	11840	6354	-8.3
Lepto-Son-D	1130	1225	-4.1
	Group 2		
3-Day Control	24	28	--
PHA	41265	55172	-3.3
6-Day Control	93	221	--
PS-A	110	245	-0.2**
PS-B	136	193	-0.6**
SKSD	7066	6899	-4.5
Plaque-Son	5766	5352	-8.4
T14V-Son	14356	7638	-8.4
Lepto-Son-D	1169	816	-5.7

*"1-tailed" t value for significance at 95% level (38 d.f.) = -1.68
**Mean peak DPM of test material not significantly greater than control DPM

TABLE 12: INDIVIDUAL RESPONSE TO PULP EXTRACTS BASED ON STIMULATION INDEX

A. NON-RESPONDERS (SI < 2.5 FOR PULP EXTRACTS)

	Subject Number	6-Day Control (DPM)	Extract A (Peak SI)	Extract B (Peak SI)
Group 1	5	7	0.9	2.3
	15	11	2.1	1.4
	23	18	2.1	1.2
	25	17	1.7	1.5
	27	48	0.3	0.5
	32	20	1.3	1.4
	39	14	1.7	1.9
Group 2	9	796	0.5	0.5
	18	159	0.1	0.1
	19	11	0.6	0.9
	21	656	1.5	1.2
	22	14	0.7	0.9
	30	7	1.6	1.4
	35	15	0.9	2.2
	40	39	0.9	1.7

TABLE 12: INDIVIDUAL RESPONSE TO PULP EXTRACTS BASED ON STIMULATION INDEX

B. RESPONDERS (SI $\geq$ 2.5 FOR PULP EXTRACTS)

	Subject Number	6 Day Control (DPM)	Extract A (Peak S.I.)	Extract B (Peak S.I.)
	1	3	19.0	24.7
	4	48	2.7	8.3
	6	13	2.4	14.8
	7	4	3.5	13.3
	12	7	3.0	3.9
	13	16	1.9	17.7
Group 1	16	8	1.6	3.6
	17	14	1.2	23.0
	24	3	5.3	5.1
	28	16	3.0	3.3
	29	5	1.1	4.7
	33	7	8.2	14.0
	38	8	4.8	4.0
	2	3	4.3	24.6
	3	4	2.5	3.3
	8	22	0.5	4.8
	10	10	1.2	15.2
	11	14	4.2	14.6
	14	10	1.4	12.1
Group 2	20	5	11.4	66.3
	26	3	4.8	3.2
	31	6	3.5	7.9
	34	7	4.0	23.2
	36	4	4.8	3.4
	37	79	5.5	1.6

TABLE 13: COMPARISON OF PEAK STIMULATION INDEX FOR PULP EXTRACTS

		Mean	Variance	t*
Group 1	Peak SI PS-A	3.39	16.777	-2.143
	Peak SI PS-B	7.53	57.851	
Group 2	Peak SI PS-A	2.745	7.231	-1.926
	Peak SI PS-B	9.455	235.561	
All	Peak SI PS-A	3.067	11.805	-2.750
	Peak SI PS-B	8.492	143.888	

*"1-tailed" t value for significance at 95% level (38 d.f.) = -1.68

TABLE 14: Pearson Correlation Coefficients for Selected Clinical Variables and all other Variables in which $r \geq 0.450$ or $r \leq -0.450$

Selected Clinical Variable	Independent Variable	r Total	r Group 1	r Group 2
Age	PLPKD[1]			-0.500
	PLPKS[2]		-0.497	
	Number of lymphocytes			-0.623
	Number of eosinophils		-0.492	
	Serum glucose		0.563	
	Serum CO_2		0.636	
	Serum inorganic phosphorus		-0.555	
	Serum creatinine	0.550	0.563	0.558
	Serum LDH		-0.530	
	Serum protein			0.458
	Serum gamma globulin	0.464	0.298	0.585
	Gamma as % of total protein			0.532
	Gamma as % of total globulin			0.540
	Alpha 2 as % of total globulin			-0.510
PMA	DMFS			0.489
	APKD[3]			0.478
	APKL[4]	0.452	0.306	0.584
	APKS[5]	0.455	0.427	0.517
	PLPKD[1]		-0.482	
	PLPKL[6]		-0.455	
	Serum SGOT			0.556
DMFS	Marginal gingival inflammation			0.643
	Attached gingival inflammation			0.508
	Serum alpha 2 globulin		0.483	-0.454
	Serum triglyceride		0.438	
	Serum BUN			-0.461
	Alpha 2 as % of total protein			-0.460
	Alpha 2 as % of total globulin			-0.540
Number of pulpotomies	LPPKD[7]	*	*	0.573
	LPPKL[8]			0.461
	Beta as % of total globulin			-0.487
Time from first pulpotomy	Serum Na^+	*	*	-0.585
	Serum Cl^-			-0.560
Time from last pulpotomy	PHAPKS[9]	*	*	-0.432
	Number of eosinophils			-0.496
Number of pulpotomy appointments	Serum CO_2	*	*	0.575

1. PLPKD - DPM of peak Plaque-Son response
2. PLPKS - S.I. of peak Plaque-Son response
3. APKD - DPM of peak PS-A response (untreated pulp extract)
4. APKL - log of peak PS-A response (untreated pulp extract)
5. APKS - S.I. of peak PS-A response (untreated pulp extract)
6. PLPKL - log of peak Plaque-Son response
7. LPPKD - DPM of peak Lepto-Son response
8. LPPKL - log of peak Lepto-Son response
9. PHAPKS - S.I. of peak PHA response

*The remaining clinical variables apply only to Group 2 patients.

TABLE 15: Multiple Correlation for Selected Variables in which Multiple $r \geq 0.700$ for Three Variables

B. Group 2 (n=20)

Dependent Variable	Entering Variable	r
Peak DPM response to PS-A	Serum SGOT	0.596
	Serum glucose	0.714
	Blood type*	0.785
Peak log response to PS-A	Serum SGOT	0.649
	Number of monocytes	0.811
	Alpha 1 as % of total protein	0.880
Peak DPM response to PS-B	Serum SGOT	0.561
	Serum d. bilirubin	0.708
	Number of monocytes	0.833
Peak log response to PS-B	Serum Cl^-	0.600
	Serum inorganic phosphorus	0.703
	Number of monocytes	0.792
PMA	Serum SGOT	0.534
	DMFS	0.689
	Hemoglobin	0.791
DMFS	Marginal gingival inflammation	0.640
	Hemoglobin	0.734
	Age	0.805

* Non-parametric values were used to code this data; therefore no statistical interpretation can be made for this variable

C. Total (n=40)

Dependent Variable	Entering Variable	r
PMA	Log of peak PS-A response	0.509
	Log of Lepto-Son-D at 12 μg/ml	0.736
	Number of Lymphocytes	0.768

FIGURE 1: MEAN PEAK STIMULATION INDEX FOR ALL STIMULANTS

The mean SI value (experimental DPM/control DPM) and standard error for each of the stimulants used in the lymphocyte transformation analysis is plotted for both groups of subjects. SI values are plotted on a log scale.

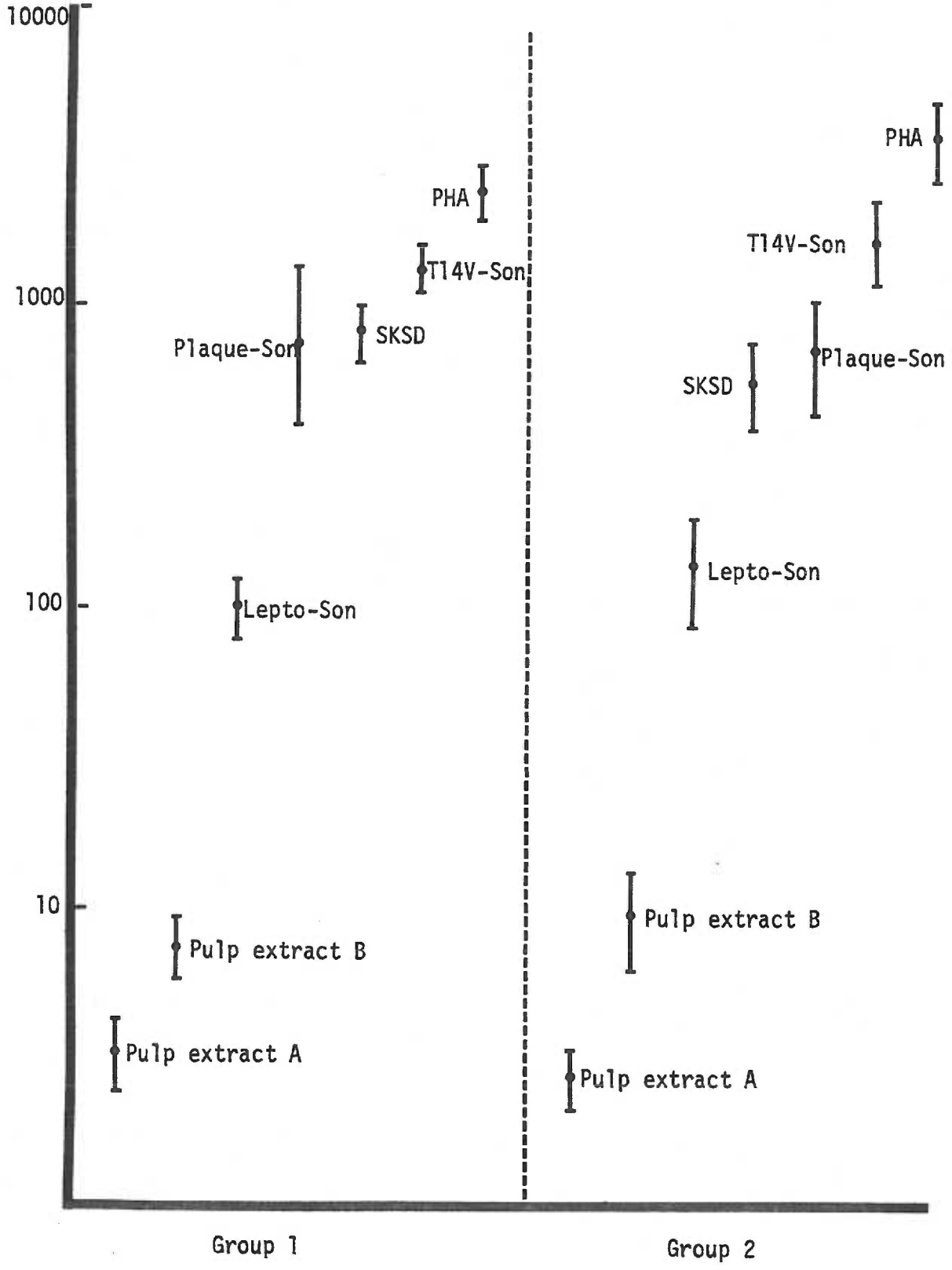

10000
1000
100
10
PHA
T14V-Son
Plaque-Son
SKSD
Lepto-Son
Pulp extract B
Pulp extract A
PHA
T14V-Son
SKSD
Plaque-Son
Lepto-Son
Pulp extract B
Pulp extract A
Group 1
Group 2

FIGURE 2: DISTRIBUTION OF PEAK SI VALUES FOR PULP EXTRACTS

The individual peak SI values (experimental DPM/control DPM) for both groups are plotted for pulp extract A (untreated) and pulp extract B (Formocresol treated). The mean group SI values and standard error are given for both extracts.

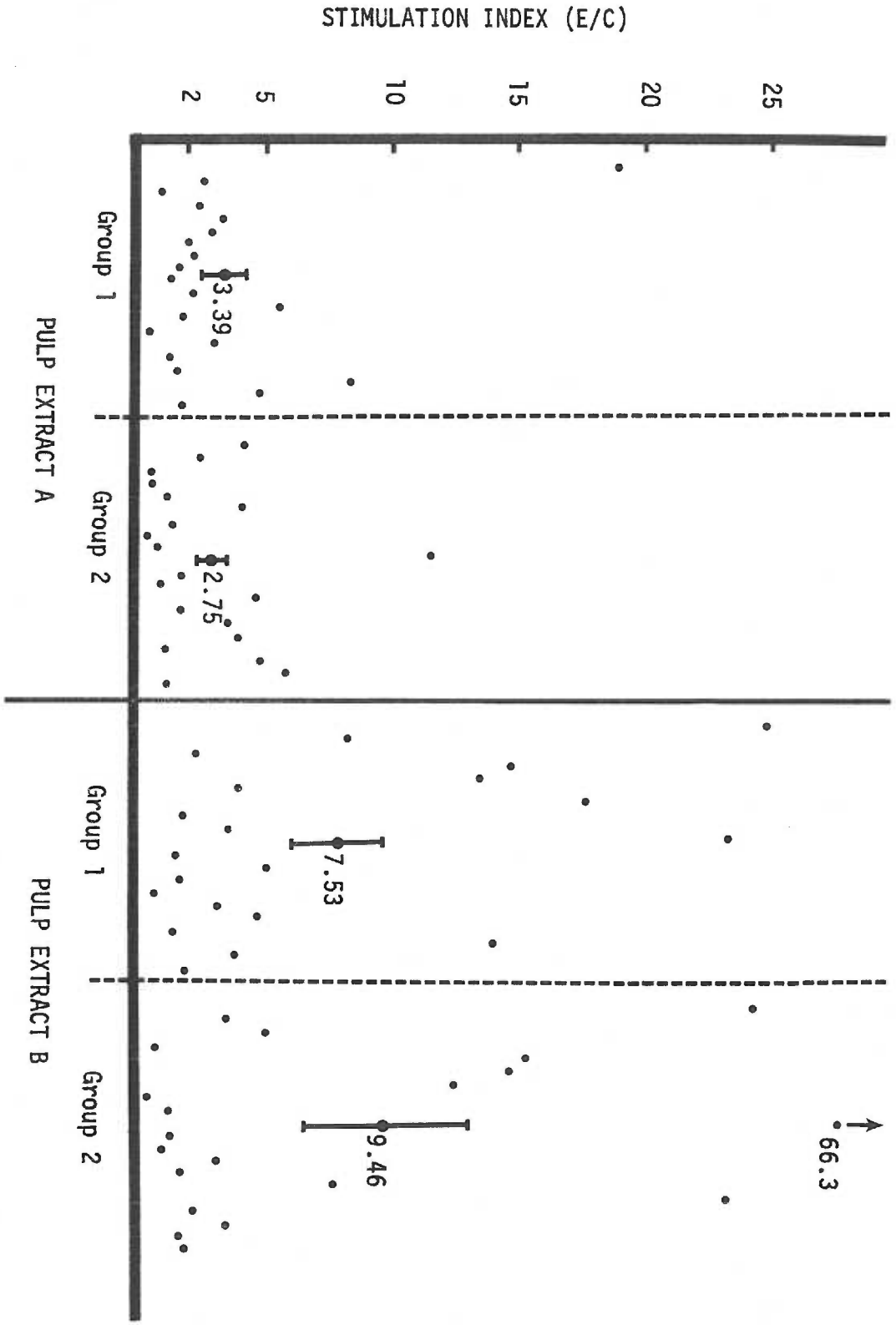
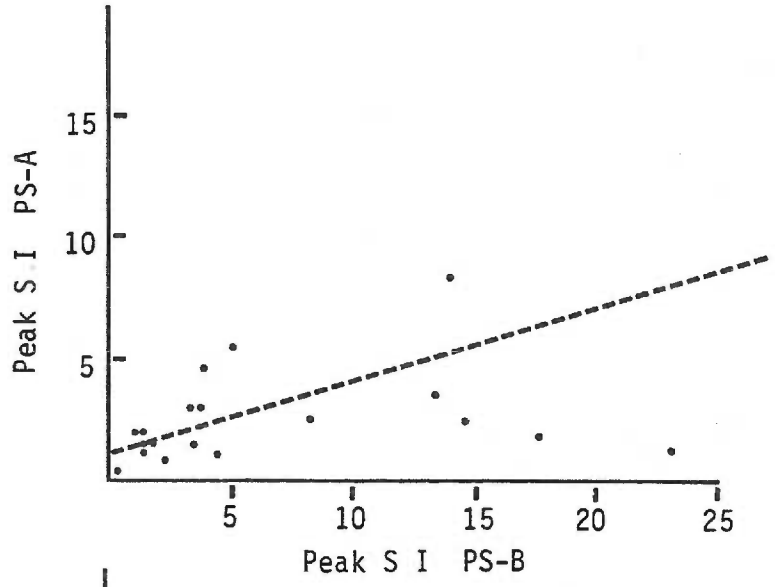

STIMULATION INDEX (E/C)
2
5
10
15
20
25
PULP EXTRACT A
PULP EXTRACT B
Group 1
Group 2
Group 1
Group 2
3.39
2.75
7.53
9.46
66.3

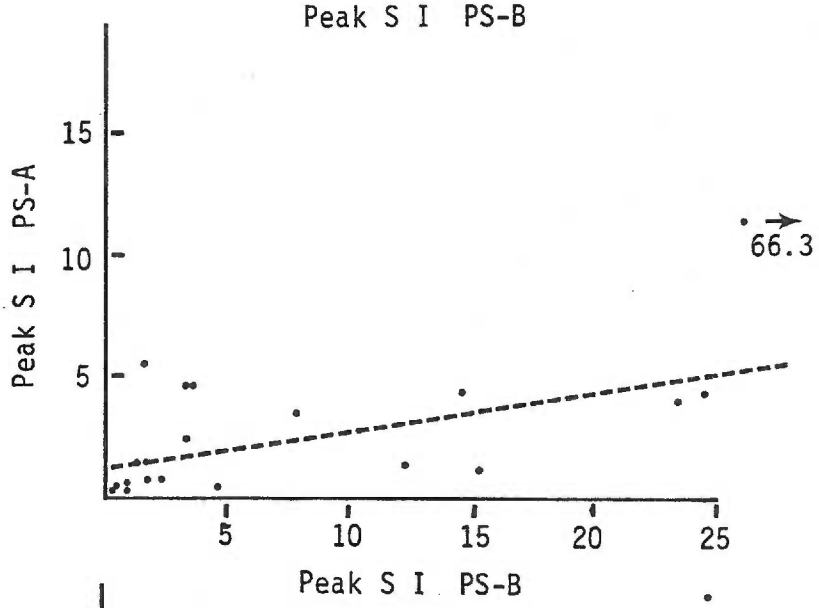
FIGURE 3: CORRELATIONS FOR PEAK SI VALUES OF PULP EXTRACTS

The individual peak SI values (experimental DPM/control DPM) for pulp extracts A and B are plotted for Group 1, Group 2, and all responders (SI $\geq$ 2.5 for either A or B extract). The slope of the regression line (b), y axis intercept (a), Pearson correlation coefficient (r) and standard error of estimate (S_{xy}) are given for each diagram.



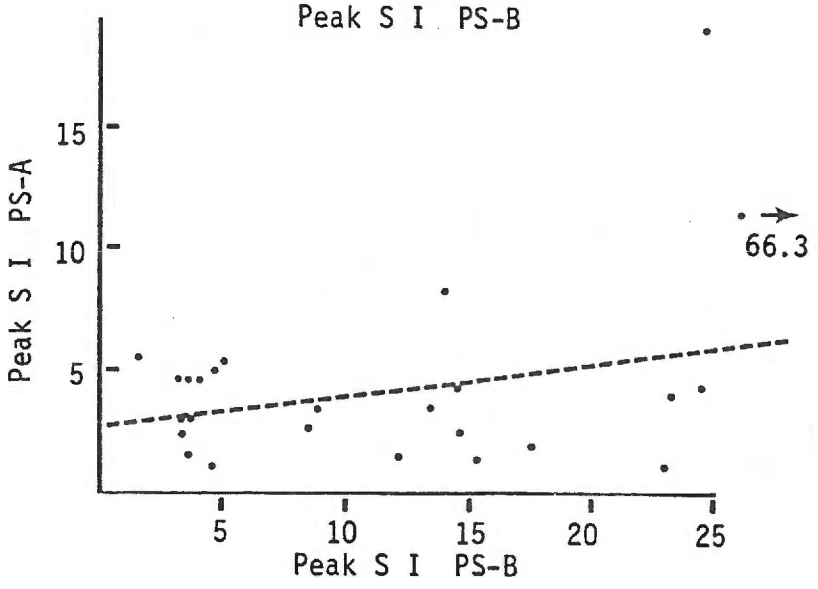
A. Group 1

b= 0.30
a= 1.11
r= 0.56
S_{xy} = 3.30



B. Group 2

b= 0.14
a= 1.44
r= 0.79
S_{xy}= 1.61

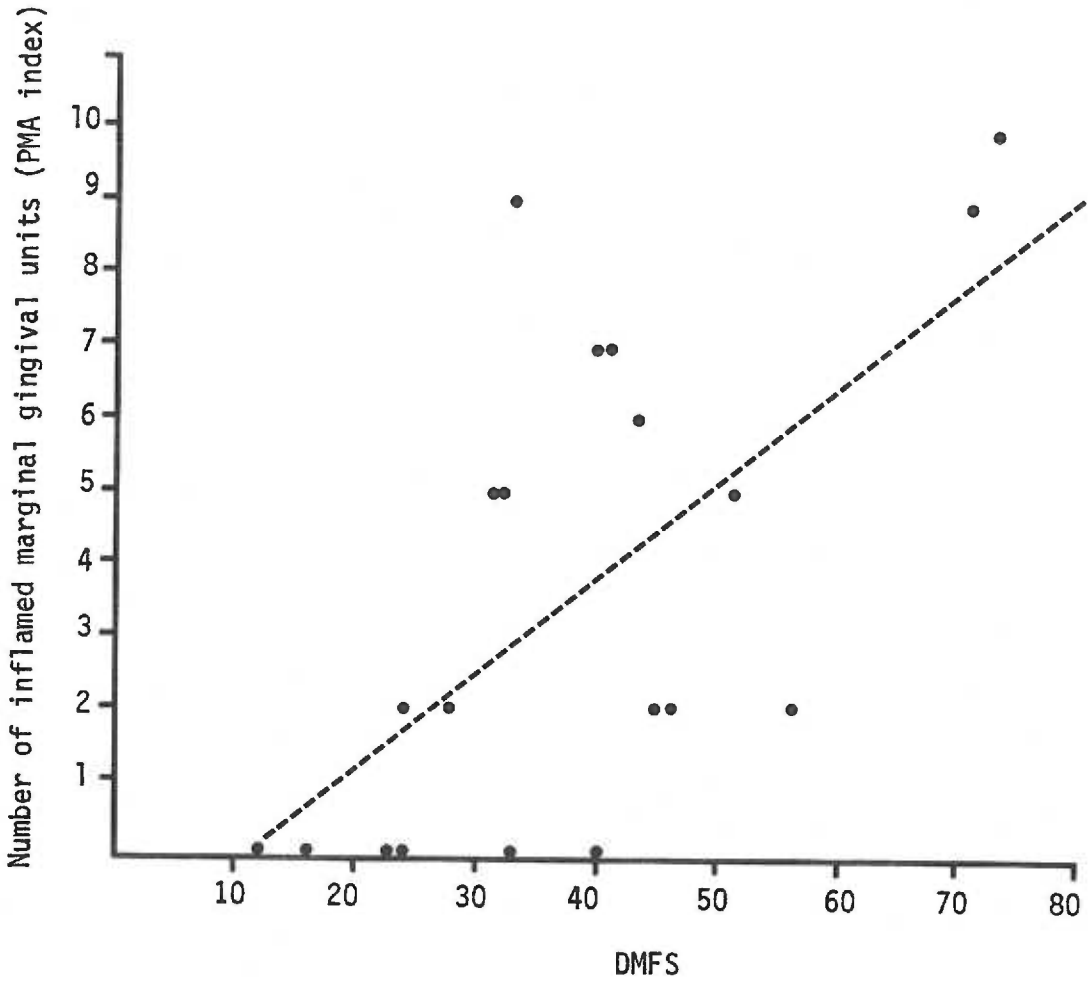


C. All Responders
(SI $\geq$ 2.5)

b= 0.12
a= 2.87
r= 0.44
S_{xy}= 3.32

FIGURE 4: CORRELATION FOR MARGINAL GINGIVAL INFLAMMATION AND CARIES IN GROUP 2

A scatter diagram with the least squares regression line is shown for the strongest bivariate correlation. The values given are the slope of the regression line (b), y axis intercept (a), Pearson correlation coefficient (r), the coefficient of determination (r^2), the standard error of estimate (S_{xy}), the mean number of inflamed marginal gingival units for Group 2 ($\overline{X}_M$), and the mean DMFS for Group 2 ($\overline{X}_{DMFS}$).



b = 0.1369

a = -1.5724

r = 0.6434

r^2 = 0.4140

S_{xy} = 2.5651

$\overline{X}_M$ = 3.650

$\overline{X}_{DMFS}$ = 38.150

APPENDIX I

A. Criteria for employing the Formocresol pulpotomy technique:

1. Radiographic appearance of caries approximating the coronal pulp chamber of a deciduous tooth.
2. Radiographic appearance of normal root resorption and a lack of interradicular or apical bone destruction (radiolucency).
3. Adequate restorability of the tooth.
4. No history of spontaneous pain.
5. Absence of mucosal swelling or fistula.
6. Appearance of clinically vital pulp tissue when the pulp chamber is exposed.

B. Procedure for the Pulpotomy Technique:

1. Regional anesthesia of tooth and tissue.
2. Isolation of the tooth with a rubber dam.
3. Removal of caries without entering pulp chamber.
4. Removal of pulp chamber roof with a high-speed rotary bur.
5. Removal of the coronal pulp with a spoon excavator or round bur.
6. Achievement of hemostasis at the amputation sites.
7. Application of Formocresol* to the pulp on a moist cotton pellet for 5 minutes.
8. Placement of a zinc oxide and eugenol base directly on the amputation site.
9. Restoration of the tooth with a stainless steel crown.

---

*Roth International, Chicago, Illinois

APPENDIX II

DEPARTMENT OF ENDODONTOLOGY

SCHOOL OF DENTISTRY

UNIVERSITY OF OREGON HEALTH SCIENCES CENTER

CONSENT FORM

_____ _____
Subject's Name Age

I hereby consent for the above-named to serve as a subject in the project entitled, "Lymphocyte Transformation Responses to Pulp Antigens In Formocresol Pulpotomy Patients", by Dr. F. James Marshall and Dr. Dennis Longwill. The investigation is designed to gain knowledge about the ways in which the immune system may respond to a common type of dental treatment.

I agree to allow the above-named to have approximately 28 ml (less than one ounce) of blood drawn by a licensed laboratory technician. The amount of blood drawn is minimal and will not jeopardize the health of the subject. Injury to the vein is transient and only slightly painful.

I understand that participation in the project will provide the following benefits:

1. A complete blood count, chemical analysis and urinalysis will be performed as a health screening service for the subject, without charge. Results can be made available to a physician at my request.
2. A dental appointment consisting of diagnostic x-rays, oral prophylaxis, and fluoride treatment will be provided at no charge.
3. The satisfaction of participating in an endeavor designed to help expand knowledge in the health sciences.

An offer to answer any inquiries concerning these procedures was made by Dr. Dennis Longwill of the Department of Endodontology.

I understand that I may refuse to participate, or withdraw my child from this study at any time without affecting my relationship with, or treatment at, the University of Oregon Health Sciences Center.

I have read the foregoing statements.

_____
Signature of Parent or Guardian Relationship Date Time

_____
Signature of Parent or Guardian Relationship Date Time

_____
Signature of Witness Date Time

APPENDIX III

Concentration of Test Materials for Lymphocyte Transformation

Test Material	Culture Tube #	Concentration in 0.4 ml Supplemented MEM-S
PHA	1 - 3	12 µg dry wt/ml
	4 - 6	3 µg dry wt/ml
	7 - 9	0.75 µg dry wt/ml
	10 - 12	0.19 µg dry wt/ml
3-Day Control	13 - 15	0 µg protein/ml
PS-A	16 - 18	200 µg protein/ml
	19 - 21	40 µg protein/ml
	22 - 24	8 µg protein/ml
	25 - 27	1.6 µg protein/ml
6-Day Control	28 - 30	0 µg protein/ml
PS-B	31 - 33	200 µg protein/ml
	34 - 36	40 µg protein/ml
	37 - 39	0.8 µg protein/ml
	40 - 42	1.6 µg protein/ml
SKSD	43 - 45	1000 U/ml
	46 - 48	250 U/ml
	49 - 51	50 U/ml
Plaque-Son	52 - 54	30 µg protein/ml
	55 - 57	6 µg protein/ml
T14V-Son	58 - 60	600 µg protein/ml
	61 - 63	150 µg protein/ml
	64 - 65	30 µg protein/ml
Lepto-Son-D	67 - 69	150 µg protein/ml
	70 - 72	30 µg protein/ml
	73 - 75	6 µg protein/ml
	76 - 78	1.2 µg protein/ml

APPENDIX IV

Preparation of Pulp Extracts

Collection

A sample of 280 impacted maxillary and mandibular human molar teeth was obtained from several local oral surgeons and the Oral Surgery Department of the University of Oregon Health Sciences Center, School of Dentistry. Immediately following extraction, the teeth were placed in a dry container and frozen at -10 to -20°C until the time of pulp removal. The teeth were individually grooved with external longitudinal cuts prepared with a straight fissure bur in a high speed dental handpiece and split with end cutting pliers. The intact pulp specimens were removed and stored in a glass container at -72°C.

Extraction

The pooled tissue (7.8 grams) was allowed to thaw at room temperature for 30 minutes and placed in a petri dish. The tissue was diced with scissors into homogenous fragments and washed twice with 10ml of 0.2 M PBS (phosphate buffered saline, Sorenson's, pH 7.3) to remove blood components. The washed tissue was cooled over ice and minced with a scalpel blade for 15 minutes. The sample (7.8 ml) was transferred to a 125 ml Erlinmeyer flask and five volumes (39.0 ml) of cold 0.2 M PBS was added. The suspension was frozen at -72°C for 20 minutes and immediately thawed at 38°C for 7 minutes. The freeze-thaw cycle was performed three times and the contents were stored overnight in a refrigerator at 4°C.

The contents were transferred to a 100 ml beaker and sonicated at 80 watts (Sonifer Cell Disruptor, Model W 185, ULTRASONICS INC.) for ten one minute intervals, allowing two minutes for ice bath cooling between each interval. The sonicated mixture was equally divided into centrifugation tubes and centrifuged (Servall Refrigerated - Automatic Centrifuge, IVAN SORVAL INC.) at 2°C for 10 minutes. The relative centrifugal force (RCF) for 10,000 RMP was determined to be 12,100 X G. The supernatant was decanted into a 125 ml Erlenmeyer flask and stored at 4°C. The sediment which was left in the centrifuge tubes was washed into a 100 ml Erlinmeyer flask with 0.2M PBS and sonicated for 10 seconds. The centrifugation cycle was repeated and the supernatant was decanted into the sample flask which was stored overnight in a refrigerator at 4°C. The entire sample was then divided into centrifugation tubes and spun at 11,000 RPM (14,500 X G RCF) for 30 minutes at 2°C to separate particulate matter. The extract was clarified by filtration through a glass fiber pad (Type AE, GELMAN), followed by filtration through a 0.45μ membrane filter (Millex, MILLIPORE CORP.).

Formocresol Treatment

One half of the sample (42 ml), weighing 2.8 mg/ml, was placed in a 100 ml beaker with a magnetic stirrer. The addition of 0.86 ml of Formocresol (ROTH DRUG CO.) containing 50% formalin provided a 1% concentration of formalin to the pulp extract. The pulp extract-Formocresol mixture which had formed a particulate suspension was allowed to stir at room temperature for 5 minutes. The Formocresol treated pulp extract was poured into a 100 ml screw cap bottle and designated as PS-B. The remaining 42 ml of untreated extract was sterilized

through a 0.45μ membrane filter (Millex, MILLIPORE CORP.) directly into a sterile 100 ml screw cap bottle and designated as PS-A. Both bottles were placed into a refrigerator at 4°C and incubated for 7 days.

Following incubation the PS-B extract was dialized at 2°C for 68 hours against four 4.0 liter changes of 0.2 M PBS to remove any residual Formocresol. The PS-B extract was transferred to an ice bath and sonicated ten times at one minute intervals. After sonication the extract was poured into a sterile 100 ml screw cap bottle and refrigerated at 4°C.

Protein Determination

The protein concentration of PS-A and of PS-B was determined by the Lowry method as described by Campbell (1970). A bovine albumin standard (10g/100 ml, SIGMA), diluted to a concentration of 1 ug/ul, was used to establish an initial calibration curve. A series of 200 ul tube duplicates at 0, 10, 20, 40, 60, 80 and 100 ug protein in 0.2 M PBS were prepared in 4 ml Coleman cubettes. The prescribed reagents were added to the samples and read in a Coleman Junior Spectrophotometer at 500λ. The alteration in light transmission was converted to a value of optical density and the change in optical density (Δ0.D.) was plotted against the known protein concentrations.

Single tubes of 0, 10, 50, 100, and 200 ul PS-A were analyzed by the same method and the Δ0.D. values were compared to those of the albimin standard. On the basis of this estimation, a triplicate sample of 35 ul of PS-A was analyzed against a duplicate standard series containing 0-100 ug protein. The results indicated the protein concentration of the PS-A extract to be approximately 2.8 mg/ml.

A final calculation of the protein concentration was achieved by analyzing the albumin standard (0 - 80 ug protein) against triplicate 25 ul volumes of both PS-A and PS-B. The ΔO.D. of all samples was plotted on a graph and the value of the protein concentration was calculated using a simple linear regression:

$$C = m\ (x) - b$$

Where:

C = protein concentration per tube (ug)
m = constant established by the standard curve
x = mean ΔO.D. for PS-A and PS-B
b = calculated correction factor

The protein concentration for both extracts was similar and an average value of 2500 ug/ml was taken to be the test concentration used in the transformation analysis.

The protein concentration of the non-precipitated portion of the PS-B extract was also determined from a volume which had been cleared of particulate matter by filtration. A value of less than 50 ug/ml protein was estimated and it was therefore decided to use the particulate suspension of PS-B (2500ug/ml protein) as the test material for the transformation analysis.

Storage

The extracts were divided into 0.2 ml aliquots by pipetting into snap-cap vials. The vials were designated as PS-A or PS-B and were frozen at -72°C until the day of use.