THE EFFECTS OF COMPLEMENT AND ENDOTOXIN ON THE RELEASE OF LYSOSOMAL ENZYMES FROM MOUSE LEUKOCYTES *IN VITRO*

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CONTENTS

Page

ABSTRACT 1

INTRODUCTION 3

PART 1

REVIEW OF LITERATURE

Dental Plaque and Periodontal Disease . . 6

Classification of Allergic Tissue Damage 9

Immunological Features of Periodontal Disease 12

Biological Reactions of Complement . . . 16

Endotoxins as Pathogens in Periodontal Disease 19

Lysosomes in Cell and Tissue Damage . . . 23

Polymorphonuclear Neutrophils in Periodontal Disease 30

Summary 33

OBJECTS OF THE INVESTIGATION 35

PART II

MATERIALS & METHODS

INJECTION REGIMEN 37

Effect of Single Injection of Endotoxin . 37

Effect of Multiple Injections of Endotoxin 38

ASSESSMENT OF HISTOLOGICAL SLIDES 39

Page

RESULTS

HISTOLOGICAL ASSESSMENT OF SINGLE INJECTIONS OF ENDOTOXIN 42

HISTOLOGICAL ASSESSMENT OF MULTIPLE INJECTIONS OF ENDOTOXIN 49

Summary of Results 52

DISCUSSION 53

PART III

MATERIALS & METHODS

PREPARATION OF CELL SUSPENSIONS 56

Peritoneal Transudate Cells (P.T.C.) 57

Peritoneal Exudate Cells (P.M.N.'s) 58

Lymph Node Cells 59

PREPARTIONS OF CELLS WITH ENDOTOXIN AND ENZYME ASSAYS 60

Preparation of Sample Containing Enzyme Released During Incubation 61

Preparation of Samples Containing Total Enzyme Activity 62

The Effect of Ultrasonication of Peritoneal Transudate Cells 63

ENZYME ASSAYS 67

Beta-Glucuronidase 67

Acid Phosphatase 70

INCUBATION OF CELLS WITH ENDOTOXIN AND COMPLEMENT 75

Page

RESULTS

CALCULATIONS OF RESULTS 79

The Effect of the experimental Procedures on the release of acid phosphatase and beta glucuronidase from mouse leukocytes 83

ANALYSIS OF CELL MORTALITY 106

Correlations between the filtrable enzymes, cell concentration and mortality 107

SUMMARY OF RESULTS 113

PART IV

DISCUSSION 114

SUMMARY AND CONCLUSION 134

BIBLIOGRAPHY 137

TABLES

		Page
TABLE I	Effect of Ultrasonication on Percentage of Remaining Intact Cells and On Percentage Filtrable Beta-Glucuronidase	69
TABLE II	Contents of Assay Tubes for the Determination of Beta-Glucuronidase	72
TABLE III	Contents of Assay Tubes for the Determination of Acid Phosphatase	77
TABLE IV	Experimental Procedures: Contents of Each Incuabation Tube	
TABLE V	Results of Beta Glucuronidase Assay .... Replication 1	84
TABLE VI	Results of Beta Glucuronidase Assay Replication 2	85
TABLE VII	Results of Beta Glucuronidase Assay Replication 3	86
TABLE VIII	Results of Beta Glucuronidase Assay Replication 4	87
TABLE IX	Results of Beta Glucuronidase Assay Replication 5	88
TABLE X	Results of Beta Glucuronidase Assay Replication 6	89
TABLE XI	Results of Acid Phosphatase Assay Replication 1	90
TABLE XII	Results of Acid Phosphatase Assay Replication 2	91
TABLE XIII	Results of Acid Phosphatase Assay Replication 3	92
TABLE XIV	Results of Acid Phosphatase Assay Replication 4	93
TABLE XV	Results of Acid Phosphatase Assay Replication 5	94
TABLE XVI	Results of Acid Phosphatase Assay Replication 6	95

		Page
TABLE XVII	Mean Enzyme Release From Groups 1-6	97
TABLE XVIII	Mean Enzyme Release From Each Cell Type	98
TABLE IXX	Correlation Matrixes for Enzyme release and cell concentration	109
TABLE XX	Correlation coefficients between cell concentrations and enzyme released from procedures involving complement or heat inactivated complement	110
TABLE XXI	Correlation Matrixes for enzyme release and cell concentration and cell mortality	111

FIGURES

		Page
FIGURE 1	Average Cell Counts Expressed Against Subjective "+" Rating.	41
FIGURE 2	Single Injection 0.1 ug Endotoxin in Glucose Vehicle.	44
FIGURE 3	Single Injection 0.1 ug Endotoxin in Saline Vehicle.	45
FIGURE 4	Single Injection 1.0 ug Endotoxin in Saline Vehicle.	47
Figure 5	Single Injection 1.0 ug Endotoxin in Gluccse Vehicle.	48
FIGURE 6	Multiple Injections 0.1 ug Endotoxin in Saline Vehicle.	50
FIGURE 7	Multiple Injections 1.0 ug Endotoxin in Saline Vehicle.	51
FIGURE 8	Effect of Ultrasonication on Peritoneal Transudate Cells and Beta-Glucuronidase Release.	68
FIGURE 9	Experimental Design.	79
FIGURE 10	Group 1, Peritoneal Transudate Cells.. .	100
FIGURE 11	Group II, Lymph Node Cells.	101
FIGURE 12	Group III, Peritoneal Exudate Cells. . .	102
FIGURE 13	Group IV, Peritoneal Transudate Cells. .	103
FIGURE 14	Group V, Lymph Node Cells	104
FIGURE 15	Group VI, Peritoneal Exudate Cells . . .	105

ABSTRACT

The investigation was designed to identify the cells comprising the inflammatory infiltrate to multiple endotoxin injections in mice, and to investigate the roles of guinea-pig serum and endotoxin in the release of two lysosomal enzymes from each of the inflammatory cell types in vitro.

The buccal mucosa of mouse was used as the site for injection of single and multiple injections of endotoxin. The cellular response to multiple endotoxin injections was similar to periodontal disease with the exception of a more marked infiltrate of polymorphonuclear neutrophils. The most marked difference between single and multiple injections of endotoxin was a more rapid infiltration of P.M.N.'s and a more persistent infiltrate of chronic inflammatory cells. This study identified polymorphonuclear leukocytes, lymphocytes and plasma cells, and macrophages as the principal cellular inflammatory components following endotoxin injection. Based on this observation mouse peritoneal transudate cells, lymph node cells and peritoneal exudate cells (P.M.N.'s) were studied in vitro.

Guinea-pig serum released acid phosphatase and beta-glucuronidase from isolated peritoneal transudate cells, lymph node cells and polymorphonuclear leukocytes. This was probably due to a low-grade immune reaction at the cell surface. Polymorphonuclear neutrophils were totally dependent on the heat labile components of guinea-pig serum for the release of the enzymes, but the peritoneal transudate cells were only partially dependent on the heat-labile components of guinea-pig serum.

Lymph node cells were totally independent of the heat labile serum components.

Endotoxin did not cause release of enzyme into the medium when it was incubated with the leukocytes in vitro, nor did it significantly affect the release of enzyme from cells which were incubated simultaneously with guinea pig serum. An exception to this finding was that P.M.N.'s incubated with endotoxin and guinea-pig serum released more acid phosphatase than cells incubated with the serum alone.

The levels of released acid phosphatase correlated positively with those of beta-glucuronidase throughout the study which confirmed their validity as markers for all the lysosomal contents.

INTRODUCTION

Despite decades of research during which some old myths have been dispelled and more than a few new ones created, chronic inflammatory periodontal disease remains an insidious process affecting over 97% of the population. The condition claims most of the teeth extracted over the age of thirty-five, although this end result is merely an indication of the inception of the disease many years before.

Epidemiological studies have related socio-economic status to periodontal disease and demonstrated the importance of dental plaque, calculus, and local areas which trap food, in the initiation and maintenance of gingival inflammation. It is known that the most effective means of controlling gingivitis or periodontal disease is the removal of dental plaque by adequate oral hygiene procedures, although what is not known is the mechanism whereby persistent gingival inflammation achieves a net loss of tissues which support the teeth.

Two prominent hypotheses purport to explain the pathogenesis of periodontal disease: (1) That many tissue-damaging substances are elaborated by the bacteria comprising the dental plaque; (2) that the defense reaction of the host may produce substances which are injurious to the tissues. These hypotheses are obviously not mutually exclusive nor do they preclude a possible role of bacterial products or host response in the pathogenesis of acute necrotising ulcerative gingivitis. In contrast to periodontitis this latter condition results in a rapid tissue

necrosis usually affecting the interdental papillae and marginal gingivae.

The gingival milieu has been described as existing in a state of "dynamic balance" between the parasites of the dental plaque and the host resistance, where "normal" is a resulting low-grade inflammation. This thesis does not attempt to explain factors predisposing a shift in the dynamic balance - although this aspect is certainly open to further investigation - but presumes a shift in favour of the parasite system with subsequent increased access to the host defence mechanism.

Many attempts to investigate the effects of bacterial products and the host inflammatory and immune responses have been documented, but two questions usually remain: Firstly, how closely does an animal model resemble human periodontal disease? Secondly, what mechanism produces the observed changes? No immediate answer to these questions is apparent but an obvious model is the use of in vitro systems which can more closely control the experimental environment.

Endotoxins of gram negative bacteria have been implicated in periodontal disease and the immune response of the host to these and other antigens has been considered as a possible mediator of inflammation. From the obvious inflammatory potential of endotoxins and the less obvious detrimental effects of the host immune response, the lysosome

presents itself as a possible "common denominator" which may effect cell and tissue alteration. With these considerations in mind, endotoxins, complement, an immune reaction and leukocytes have been combined in an in vitro system to test the hypothesis that interactions between these components may be manifest in the release of lysosomal enzymes.

PART I

REVIEW OF LITERATURE

Dental Plaque and Periodontal Disease:

Inflammatory periodontal disease occurs in at least two distinct forms in humans. The first, almost universal, condition is a slowly progressive chronic inflammation with consequent net loss of both soft and hard tooth-bearing tissues and eventual impairment of dental function. Acute non-specific gingivitis, chronic non-specific gingivitis and periodontitis are the stages of the disease as it progresses from its initial to its established forms.

The second, also common, type of periodontal disease is an acute necrotic process most commonly affecting the interdental papillae and marginal gingivae, although severe forms may be more extensive. This condition is designated acute necrotising ulcerative gingivitis (ANUG).

Although the course and severity of periodontal disease may be modified by local factors affecting the environment of the periodontal tissues and by systemic conditions affecting host resistance, most recent research has centered around the pathogenic potential of the bacterial plaque and the host response to this plaque, which may also result in tissue damage. The bacterial or dental plaque is an integument of the teeth comprising micro-organisms embedded in a mass of protein and carbohydrate which is derived in part endogenously from saliva, the bacteria and the products of tissue metabolism, and in part exogenously from the diet (Macphee & Cowley, 1969; Socransky, 1970).

Initially when the teeth are clean the flora comprises gram positive cocci and rods. In the first two days after withdrawal of oral hygiene the gram-positive cocci and rods proliferate and there is an addition of 30% gram negative cocci and rods. After four days fusobacteria and filaments appear, each representing about 7% of the flora. Subsequently, up to nine days the flora is supplemented with Spirilla and Spirochaetes and after seven days gram negative organisms comprise about half the total flora (Theilade, et al, 1966). In addition to the longitudinal bacteriological investigation these workers concluded that the development of gingivitis was closely correlated with the rate of plaque accumulation, and that gingivitis usually disappeared one day after plaque removal. Other studies relating the accumulation of dental plaque with periodontal disease were those of Schei, et al (1959) who showed that toothbrushing efficiency, as estimated by plaque accumulation, correlated directly with radiographic measurements of bone loss; the most severe bone loss occurring in individuals with the most plaque. In a group of twelve dental students who ceased oral hygiene procedures, plaque and clinically observable inflammation developed, both of which subsided when oral hygiene was reinstituted (Loe, et al, 1965). Waerhaug (1956A, B, and C, 1957 and 1960) showed that when bacterial plaque adhered to dental restorations such as overhanging zinc phosphate cement fillings, gold foils,

metal wires or roughened tooth surface the inflammation was much more severe than when these devices were present without a covering of plaque.

The pathogenic products of dental plaque have been categorized into three major groups: 1) The exo- and endotoxins, although exotoxins have yet to be isolated from indigenous oral bacteria; 2) microbial enzymes, and 3) the immunogenic products or constituents of the plaque flora (Bahn, 1970). Socransky (1970) has also included as potential pathogens the "cytotoxic" agents in the plaque such as mucopeptides from gram-positive cell walls, ammonia, hydrogen sulphide, toxic amines, indole and a variety of organic acids. From this plethora of potential aetiological agents endotoxins of gram negative bacteria have received considerable attention in the recent periodontal literature. The evidence implicating endotoxins in periodontal disease will be reviewed later.

Classification of Allergic Tissue Damage:

The host response to the presence of dental plaque is a chronic inflammation with eventual net loss of tissue. Speculation on the mechanisms whereby the inflammation is maintained and how it causes loss of tooth-supporting tissues has aroused considerable interest in the possible role of immune mechanisms in the pathogenesis of periodontal disease (Berglund, 1970). Immune mechanisms which lead to tissue damage, thus confounding the teleological view of an antibody system acting solely to protect the host, can take the form of immediate, antibody-mediated hypersensitivity or delayed, cell-mediated hypersensitivity (Humphrey and White, 1970). Both of these forms are often referred to as allergy. The allergic reactions culminating in cell or tissue damage have been classified into types I, II, III and IV (Coombs and Gell, 1968).

Type I reactions are initiated by the reaction of antibody adsorbed on to a cell surface (Cytophilic antibody) and its corresponding antigen. The affected cell types are usually basophils and mast cells but possibly other cells, e.g. macrophages (Nelson and Boyden, 1967) and eosinophils (Coombs and Gell, 1969). Anaphylactic shock is a typical example of this reaction.

Type II reactions are mediated by the reaction of an antibody with a cell surface antigen with subsequent cell damage and release of lysosomal enzymes. The presence of complement

in this system greatly increase the capacity for cell damage. Ascites tumour cells have been used to demonstrate this process experimentally (Bitensky, 1963; Dumonde, et al, 1965; Weiss and Dingle, 1964). These reactions may have pathological significance in auto-immune processes.

Type III reactions result from the fixation of complement by antigen antibody interactions in the tissues or tissue fluids. The reaction products arising from complement fixation include factors chemotactic for polymorphonuclear leukocytes which in turn phagocytose the immune complexes with subsequent release of lysosomal components (Movat, 1968). The Arthus reaction is a typical experimental example of this type of allergic reaction (Cochrane & Dixon, 1968).

Type IV reactions are also referred to as delayed type hypersensitivity (D.T.H.). The predominant cell types in D.T.H. lesions are lymphocytes and macrophages. The condition may be transferred from sensitized donor to recipient by lymphocytes, but not by serum (Humphrey & White, 1970). The reactions in D.T.H. are as yet poorly understood but are initiated by the antigen acting on sensitized lymphocytes, and possibly macrophages (Nelson, 1969). When sensitized lymphocytes are incubated in the presence of specific antigen in vitro they have a cytotoxic effect, mediated through release of a cytotoxic factor, on tissue culture monolayers of fibroblasts (Ruddle and Waksman, 1968). The lymphocytes also "transform" by increasing in size and acquiring a larger cytoplasm which contains lysosomes (Humphrey & White,

1970; Alder, et al, 1970). The classical example of D.T.H. is that of tuberculin sensitivity as described by Zinsser and cited by Humphrey and White (1970).

Another type of hypersensitivity that does not fit readily into any of the other classes of allergic reactions is the local Schwartzman reaction (Schwartzman, 1937). If endotoxins are injected intradermally in adult rabbits and a similar dose administered intravenously 24 hours later the injected areas will develop purple lesions within 4 hours. Histological examination of these lesions shows polymorph infiltration, leucocyte-platelet thrombi and necrosis of vessel walls.

Immunological Features of Periodontal Disease:

Raised levels of circulating antibodies specific for organisms normally found in the gingival sulcus have been demonstrated in patients with periodontal disease (Mergenhagen, et al, 1965; Courant and Gibbons, 1961; Evans, et al, 1966; and Steinberg, 1970), and raised serum immunoglobulin levels have also been demonstrated electrophoretically in patients with periodontal disease (Saito, et al, 1969). The gingival crevice fluid contains immunoglobulins (Brandtzaeg & Kraus, 1965) and complement fragments (Brandtzaeg, 1966), and Holmberg & Killander (1970) have demonstrated IgG, IgA and IgM in the crevice fluid in concentrations similar to serum.

Antibody forming cells which synthesize IgA, IgG and IgM antibodies are present in diseased periodontal tissue (Brandtzaeg & Kraus, 1965; Thonard, et al, 1966) while Platt, et al (1970) demonstrated plasma cells which were associated with IgM and IgG but they could not provide unequivocal evidence of the presence of IgA. Schneider, et al (1966) and Berglund (1971) have shown that the immunologlobulins in periodontally diseased tissue contain specific antibacterial antibody. Organ cultures of diseased gingivae have been shown to produce specific antibody when incubated with Fusobacterium antigens and this antibody production was inhibited when puromycin was added to the system (Berglund, 1971).

Evidence that bacterial antigens may enter the gingival tissues in periodontal disease was provided by positive fluorescent labelled antibody staining in the tissues which

was specific for Bacteroides melaninogenicus antigenic determinants. B. melaninogenicus is indigenous to the gingival crevice (Courant and Bader, 1966). Freedman, etaal (1968) and Gavin (1970) failed to demonstrate the presence of whole bacteria in chronically inflamed human gingivae, but Heylings (1967) and Listgarten and Socransky (1965) demonstrated bacteria in ANUG lesions by electron microscopy thus providing what may be a major clue in the different pathogenesis of ANUG and chronic periodontitis. Another notable feature of ANUG is that a sample of patients with the condition did not display different antibodies titres to F. fusiform, V. alcalescens and B. melaninogenicus from those of patients without a history of ANUG (Wilton, et al, 1971).

It can be seen from the foregoing that the components necessary for antigen-antibody interactions are present in both the gingival crevice and the adjacent diseased periodontal tissue. The intensity, site and significance of immune reactions in the gingival milieu has not yet been established, despite much speculation. It is highly likely, however, that the components of an immune reaction do combine with, perhaps, both beneficial and detrimental consequences to the host tissue. Some of the detrimental effects would be expected to be a result of one of the immediate-type hypersensitivity reactions, as already defined. Circumstantial evidence supporting this view exists in the form of a

positive correlation between patients with periodontal disease and those with a positive immediate hypersensitivity skin reaction to an actinomyces derivative at $P < 0.0001$ level (Nisengard, et al, 1968). Actinomyces naeslundii has plaque forming potential and is indiginous to the oral cavity (Socransky, 1970).

Recent evidence that delayed-type hypersensitivity may also be effective in both periodontal disease and ANUG has been presented. Peripheral lymphocytes from patients with chronic gingivitis or periodontitis were capable of transformation, as measured by increased tritiated thymidine uptake, by antigens from gram negative organisms associated with periodontal disease but not by Proteus mirabilis which is not indigenous to the mouth. Lymphocytes of patients with advanced periodontitis were not much more susceptible to transformation than the cells from the healthy control group. It is not clear whether this cell mediated response would exert a protective or destructive role in the pathogenesis of periodontal disease (Ivanyi & Lehner, 1970). Similar results were obtained by the action of the same antigens on lymphocytes from ANUG patients (Wilton, et al, 1971). The reaction of the lymphocytes to F. fusiform was greater in ANUG patients than chronic gingivitis patients which may suggest that this organism is associated with the development of the acute form of periodontal disease. Fusiform bacilli are a significant part of the flora associated with ANUG (Macphee & Cowley, 1969).

It can be seen that although investigations into cell-mediated hypersensitivity in periodontal disease have so far been less extensive than those involving antibody mediated reactions, there is a possibility that the former process may also play a significant role in the host response to the presence of dental plaque.

Immune damage to the periodontal tissues of rabbits (Rizzo and Mitchell, 1966) and squirrel monkeys (Ranney and Zander, 1970) has been achieved by application of ovalbumin to sensitized animals. Non-sensitized animals did not sustain signs of periodontal disease. It must be remembered, however that these models are not necessarily duplicating the human form of the disease.

Biological Reactions of Complement:

Complement is a system of serum proteins which is activated by IgG and IgM antibodies when they combine with their homologous antigens. The components of complement are designated C'1, C'4, C'2, C'3, C'5, C'6, C'7, C'8, and C'9 in order of their activation (WHO Bulletin, 1968). The details of the chain of reactions in the activation of the complement system has been reviewed by Muller-Eberhard (1968). As a result of complement fixation several important biological reactions occur which are attributable to products of the reaction. These include immune adherence, virus neutralization, phagocytosis, anaphylatoxin production, histamine release from mast cells, leukocyte chemotaxis, and immune cytolysis (Mergenhagen, 1970). Immune adherence is the ability of antigen-antibody-complement complexes to adhere to the surface of non-sensitized particles such as red cells, leukocytes and platelets. Furthermore, bacteria or viruses which attach to red cells due to immune adherence are more susceptible to phagocytosis (Nelson, 1953; 1956). Immune adherence is dependent upon bound C'3 (Muller-Eberhard, 1968) and it has been postulated that immune adherence is represented by a complement receptor system on macrophages or neutrophils (Nussenzweig, 1969). Anaphylatoxin activity has been generated from both the C'3 and C'5 components of human serum by earlier acting C' components or with proteolytic enzymes (Dias da Silva and Lepow 1965, 1967; Dias da Silva, et al (1967); Jensen, 1967; Cochrane & Mutter Eberhard, 1968).

Anaphylatoxins are substances of molecular weight 10,000 - 15,000 which have the ability to release histamine from mast cells, cause contractions of guinea-pig ileum and increase vascular permeability (Mergenhagen, 1970).

When activated, the complement system will release reaction products which are chemotactic for P.M.N.'s (Ward, et al, 1964; Ward, 1967; Ward & Becker, 1967; Snyderman, et al, 1968). There are two factors chemotactic for P.M.N.'s known to be derived from human complement. One is a high molecular weight compound comprising a complex of C'5, 6, and 7 which has been activated by preceeding C' components (Ward, et al, 1966). The other chemotactic factor is a low molecular weight fragment which can be split from C'3 by plasmin (Ward, 1967).

Complement has long been known for its ability to lyse red cell membranes when they are acted upon by complement-fixing antibody to antigenic determinants on the cell membrane. This property has been used in complement fixation techniques (Osler, et al, 1952), in the Jerne plaque technique for the detection of antibody forming cells (Jerne, et al, 1963) and in lymphocyte cytotoxicity assays (Batchelor, 1968). Lysis of the cell membrane is achieved by attachment of the C'8 component to the cell membrane with subsequent activation of C'9 and cell lysis (Muller-Eberhard, 1968). Recently the C'5 component has also been shown to possess some haemolytic activity (Polley, et al, 1971).

The nature of the sites of activation of the C'8 and C'9 components are circular lesions measuring 88 Å to 110 Å depending on the complement source, and appear as "holes" in electron micrographs, (Borsos, et al, 1964; Humphrey, et al, 1967). Similar lesions have also been produced in the endotoxins of gram negative bacteria (Mergenhagen, et al, 1968; Bladen, et al, 1967). These findings support the hypothesis that lipopolysaccharide is a substrate for the action of some complement components. The action of complement has also been implicated in the immune cytolysis of ascites tumour cells (Dumonde, et al, 1965). Complement activation not mediated by antibody antigen interactions seems to be implicated in paroxysmal nocturnal haemoglobinuria (Muller-Eberhard, 1968).

None of these biological functions of complement has, as yet, been demonstrated to be active in the process of periodontal disease although it is tempting to suspect that the complement system and its associated reactions form an integral part of the host response to the dental plaque.

The Relationship of Endotoxins to Periodontal Disease

Endotoxins are lipopolysaccharide constituents of the outer layer of the cell body of gram negative bacteria. They form physical aggregates which complex easily with other natural products thus explaining their high molecular weight which varies from one to twenty million (Nowotny, 1969). A recent comprehensive review of characteristic endotoxic reactions (Nowotny, 1969) categorizes their effects as follows: Pyrogenicity, release of endogenous pyrogen, immunogenicity, adjuvant effect and inhibition of antibody production, effect on "properdin" or natural antibodies, leukopenia and leukocytosis, protection against irradiation, effect on reticulo-endothelial system, development of endotoxin tolerance, enhancement of non-specific resistance, mobilization of interferon, changes in blood clotting, metabolic changes, endocrinological changes, release of and sensitization to histamine, vascular effects, Sanarelli-Schwartzman reaction, cytotoxicity, abortion, tumour-necrotizing effect, interaction with complement, shock and lethality. As with antigen-antibody reactions it is difficult to estimate which of the above reactions constitute part of the host response to the endotoxins of the dental plaque.

There is little doubt that there is abundant endotoxin in dental plaque. Socransky, et al (1963) have estimated that over 100 mg of bacterial debris can be collected from the gingival crevices of periodontally involved individuals

and 50 ug endotoxin could be derived from 1-2 mg of oral bacteria (Mergenhagen, et al, 1961).

The quantity of soluble endotoxin in dental plaque has been shown to correlate positively with the degree of clinical gingival inflammation ($P < 1\%$) (Simon, et al, 1970) although this association is not proof of a causal role of endotoxins in periodontal disease. Filtrates of seven day plaque, when a gram negative flora is established (Theilade, et al, 1966), inhibited the incorporation of ^{14}C-glucosamine into the ground substance (Powell, 1969). Twenty-four hour plaque does not contain a significant proportion of gram negative bacteria. This experiment demonstrates the potential of dental plaque, and probably its endotoxin component, to inhibit synthesis of intercellular ground substance of the gingival epithelium which would in turn result in loss of substances responsible for the integrity of the gingival seal. It must be suspected that epithelium compromised in this way is more easily traversed by the antigenic and toxic substances contained in dental plaque. Baboolal, et al (1970) demonstrated an increase in hydrolytic enzymes in dental plaque three days after withdrawal of oral hygiene. This coincided in an increase in gram negative bacteria and evidence of degenerating P.M.N.'s and epithelial cells. Veillonella endotoxin from oral bacteria when applied to the abraded epithelium "skin window" of the human forearm excited an immediate infiltrate of P.M.N.'s and from 4 - 10 hours mononuclear cells were also present. Phagocytosis of

carbon particles was enhanced in lesions also treated with endotoxin (Jensen, et al, 1966). *E. coli* endotoxin and lyophilized ground oral flora both inhibited bone growth in vitro and histamine acted synergistically with endotoxin to potentiate bone growth inhibition (Norton, et al, 1970). *Veillonella* endotoxins have produced abscesses in rabbit palatal mucosa with cellular proliferation in associated lymph nodes after a single injection of 50.0 ug while 1.0 ug endotoxin injected intramucosally was sufficient to produce a rise in body temperature and prepare the site for a Schwartzman reaction (Rizzo & Mergenhagen, 1964). *E. coli* endotoxin injected intra-mucosally in rabbits stimulated antibody forming cells at the site of injection and the local lymph nodes, and with larger endotoxin doses, antibody forming cells can be detected in the systemic antibody forming organs (Berglund, et al, 1969). Rizzo has studied the ability of endotoxin to cross the intact gingival epithelium of rabbits, but since he relied on the Schwartzman reaction as a positive indication of endotoxin penetration and since there were limited histological variations between experimental and control animals (Rizzo, 1968) his conclusion that endotoxin did not cross intact epithelium must be considered equivocal. Animal experiments must be assessed with caution since the results may indicate the potential pathogenic effects of endotoxin, although the experimental conditions may not often pertain to human periodontal disease.

Endotoxins can activate the terminal six complement components (Gewurz, et al, 1968) with the subsequent generation of a factor chemotactic for P.M.N.'s (Snyderman, et al, 1968), an anaphylatoxin (Lichtenstein, et al, 1969) and the production of lesions of 90 Å diameter on the lipopolysaccharide membrane resembling those produced on red cell membranes by activated complement (Bladen, et al, 1968).

An anomaly in the action of endotoxin exists in its different effects on lysosomes *in vivo* and *in vitro*. Martini (1959) first noted the increase in lysosomal enzyme activity in the livers of rats treated with endotoxin. This was confirmed in rabbit liver *in vivo* but could not be repeated on isolated lysosomes (Weissman & Thomas, 1964). Hirsch and Cohn (1960) could not demonstrate any alteration in isolated P.M.N. lysosomes when incubated with 200 ug/ml endotoxin in much the same way as it was by phagocytosis of bacteria. Similarly mouse peritoneal leukocytes released acid phosphatase from their lysosomes after endotoxin injection *in vivo* but only minimal changes could be detected *in vitro* (Weiner, et al, 1965). This raises the question of whether endotoxin mediates its *in vivo* effects through the complement system.

These properties of endotoxin are only a few of those which may play a role in the pathogenesis of periodontal disease. Unfortunately the lack of a satisfactory animal model which is known to duplicate the human periodontal lesion renders extremely difficult the evaluation of the role of any of the proposed aetiological agents.

Lysosomes in Cell and Tissue Damage:

Lysosomes are a heterogeneous group of cytoplasmic organelles containing hydrolytic enzymes which mediate the digestive and lytic processes of the cell. They are present in most cell types, with the exception of mammalian erythrocytes (Allison, 1966). Reviews of lysosomes and their roles in physiology (Duve & Wattiaux, 1966) and inflammation and disease (Weissman, 1967) and their possible role in periodontal disease (Hamp & Folke, 1968) have provided organization of the literature pertaining to this recently described organelle (Duve, et al, 1955).

The lysosomal enzymes may be categorized according to their substrates. Those degrading proteins and/or peptides are Cathepsins A, B, C, D, and E, collagenase, arylamidase and peptidase. The nucleic acids are degraded by acid ribonuclease and acid deoxyribonuclease. The orthophosphoric monoesters, phosphoproteins, FAD, ATP, phosphodiesters, phosphatidic acids are degraded by acid phosphatase, phosphoprotein phosphatase, acid pyrophosphatase, phosphodiesterase, and phosphatidic acid phosphatase respectively. The carbohydrate chains of glycoproteins and glycolipids are hydrolyzed by beta-N-acetyl hexosaminidase, alpha-N-acetylhexosaminidase, beta-galactosidase, beta-glucosidase, alpha-glucosidase, alpha-mannosidase, beta-xylosidase, alpha-fucosidase, sialidase, aspartylglucosylamime amino hydrolase and ortho-seryl-N-acetyl galactosaminide

glycosidase. Enzymes which degrade glycosaminoglycans (acid mucopolysaccharides) are lysozyme, hyaluronidase, beta-glucuronidase and aryl sulphatases A and B. This group is of importance because of its potential effects on the intercellular ground substance. Lastly, there is a group of lipases normally found in macrophages (Tappel, 1969).

Although these enzymes are present in different proportions and combinations in the lysosomes of each cell type, their overall potential to produce degradative tissue changes is apparent. The maximal activity of most of these enzymes occurs in the acid pH range (Tappel, 1969). An explanation of a mechanism whereby the acid hydrolases can act on their substrates has been forwarded by Weiss (1963A). This implicates the hyaluronate closely associated with each cell membrane which, because it possesses COO^- groups, may act as a polyanionic exchange resin and thus retain cations in the cell environment. Among these cations would be H+ the presence of which would effectively lower the local pH. In addition, the hyaluronate would impede the passage of sequestered lysosomal enzymes to the extracellular environment and retain them in the micro environment of the cell at a pH which may be 2 units below the bulk phase value (Weiss, 1963B). Lysosomal enzymes may be active under these conditions with the net result of loss of pericellular ground substance. Enzymes may also be active at neutral pH, e.g. P.M.N. collagenase (Lazarus, et al, 1968) and proteases (Folds, et al, 1971; Janff & Teligs, 1968).

Lysosomes also contain a number of other basic proteins capable of antibacterial activity or of mediating and maintaining some aspects of the inflammatory response. These include phagocytin, endogenous pyrogen, leukocyte chemotactic factor, cationic inflammatory protein, permeability producing protein, unidentified mucopolysaccharides and glycoproteins. All of these substances are present in P.M.N. lysosomes (Weissman, 1967) along with kinin forming enzymes and a cationic protein in rabbit P.M.N. granules which is mastocytolytic (Janoff, 1967). The mast cell disrupting factor may be of significance in periodontal disease since mast cell populations are increased by chronic gingival irritation but sudden upsets of their environment cause their degranulation (Zachrisson, 1968). Heparin, which is a component of mast cell granules, has the ability to potentiate the action of parathyroid hormone on bone resorption and may thus influence the rate of resorption of alveolar bone (Goldhaber, 1965).

Experimental evidence of cell death following the release of lysosomal enzymes into the cytoplasm has been provided by Quie and Hirsch (1964). Leukocyte degranulation followed treatment of the cells with antilysosome antibody and complement with subsequent discharge of the granules into the cell cytoplasm and cell death. Allison (1965, 1966) achieved selective disruption of lysosomes by allowing them to concentrate such dyes as acridine orange and neutral red.

Subsequent exposure to light of an appropriate wavelength in the presence of oxygen resulted in photo-oxidation of the lysosomal membrane. The release of the lysosomal enzymes was followed by cell death. There is also evidence that lysosomal enzymes contribute to tissue damage. Lysosomes are involved in type II and III allergic reactions and the closely related granules in basophils and mast cells are involved in type I reactions (Coombs & Fell, 1969).

The effect of antibody and complement combining with antigen on the cell membrane surface of ascitos tumour cells causes immune cytolysis with release of lysosomal enzymes (Dumonde, et al, 1965). Since this is essentially a type II reaction, Fell, et al (1966) extended this model to investigate tissue changes which resulted from immune activation of the lysosomes. Bone rudiments from chick embryos incubated with complement sufficient fowl erythrocyte membrane antisera resulted in disintegration of the bone matrix within eight days.

Although only the cells in immediate contact with antisera were dead, the other cells lost their differentiated morphology. It is unlikely that there are antibodies to constituents of the cell membrane in periodontal disease (Brandtzaeg & Kraus, 1965). It is possible that antibody producing cells, cells with antigen receptors and cells which absorb antibodies on to their surfaces will react with antigens to release lysosomal enzymes in quantities depending on the intensity of the reaction at the cell surface. In lymphocytes it is immaterial whether the cell carries the

antigen or the antibody, the vigour of the reaction and the presence of complement determine whether stimulation or autolysis is the net result (Burnet, 1969). Platt, et al, (1970) have suggested that in human periodontitis P.M.N.'s will undergo morphological change when a cytophilic antibody complexes with specific antigens. This would support the concept of "modified" type I reaction occurring in periodontal disease.

It is well known that P.M.N.'s release their lysosomal enzymes in a wide variety of _in vivo_ situations such as the Arthus reaction (Humphrey, 1955 A & B), following phagocytosis (Hirsch, 1962; Zucker-Franklin & Hirsch, 1964) in serum sickness (Cochrane & Kniker, 1965), in experimental acute nephrotoxic nephritis (Cochrane, et al, 1965) and in the Schwartzman reaction (Lee & Stetson, 1965). With the exception of the Schwartzman reaction the release of lysosomal enzymes in these conditions causes the tissue damage of the type III allergic reaction. The basis of the reaction is the combination of complement-fixing antibody and specific antigen with release from C'3 and C'5, 6, 7 complex of the factors chemotactic for P.M.N.'s (Ward, 1967; Ward, et al, 1964; Ward & Becker, 1967; Snyderman, et al, 1968). There is strong evidence that the presence of P.M.N.'s is essential for tissue damage in type III reactions. The Arthus phenomenon is inhibited by administration of nitrogen mustard which depletes the P.M.N. population (Humphrey, 1955 A & B; Cochrane, et al (1959).

Similarly P.M.N. removal inhibited development of serum sickness in rabbits, which is a generalized accumulation of immune complexes in vessel walls (Kniker & Cochrane, 1965). In experimental acute nephrotoxic nephritis, which is the interaction between injected antibody and the host glomerular basement membrane antigen, P.M.N.'s were also a prerequisite for tissue damage. Furthermore, the concentration of P.M.N.'s in the infiltrate correlated with the degree of proteinuria (Cochrane, et al, 1965). In human diseases with a suspected immune aetiology immune complexes have been identified with an associated P.M.N. filtrate. These include lupus erythematosis (Lachman, et al, 1962), in post-streptococcal glomerulonephritis (Seegal, et al, 1965) and in the subcutaneous arteritis of early rheumatoid arthritis (Kunkel, et al, 1961; Solokoff, 1963). A chronic lesion has been shown to develop in rabbit kidney following prolonged administration of antigen in doses which form soluble complexes. Complexes eventually do form between the glomerular basement membrane and the epithelial cells in which position they are protected from the action of P.M.N.'s or macrophages (Cochrane & Dixon, 1968). Taichman (1970) has briefly reviewed the part played by soluble immune complexes in tissue injury. He also demonstrated that soluble immune complexes were able to release some acid protease and lysozyme from rabbit P.M.N.'s but no beta-glucuronidase or acid phosphatase (Taichman, et al, 1970).

As with many other factors implicated in periodontal disease the pathogenic potential of lysosomal enzymes has been demonstrated in other situations, but direct evidence that they play a causative role in tissue loss of periodontitis is lacking.

The fact that mink with Chediak-Higashi syndrome, an inborn lysosomal disease, had periodontitis involving more extensive bone loss and a more intense inflammatory reaction than standard mink (Gustafson, 1969) would justify a closer look at the role of lysosomes in periodontitis.

Polymorphonuclear Neutrophils in Periodontal Disease:

In view of the evidence indicating that P.M.N. infiltration is a sequel of immune reactions involving C' fixing antibody it is justified to consider the presence of P.M.N.'s in periodontal disease. Acute necrotising ulcerative gingivitis exhibits a heavy P.M.N. infiltrate (Glickman, 1964; Macphee & Cowley, 1969) and Heylings (1967) and Listgarten & Socransky (1965) have demonstrated bacteria in the superficial parts of the affected gingiva. Lehner (1969) has shown raised IgM concentrations in the first four days of onset of ANUG. Thus there is a strong possibility that a type III reaction is occurring in the tissues during an episode of ANUG and maybe a type I or II reaction if bacterial antigens react with antibody in the region of cell membranes.

In contradistinction the dominant cell type in periodontitis is of lymphoid origin, even clinically diagnosed acute gingivitis has a dominant plasma cell-type infiltrate (Platt, et al, 1970; Macphee & Cowley, 1969). Plasma cells are present in greater numbers than lymphocytes in most cases (Zachrisson & Schultz-Haudt, 1968; Oliver, et al, 1969; Wittwer, et al, 1969). Despite a lack of P.M.N.'s in the gingival connective tissue, they are present in considerable numbers in the gingival crevice and the crevice epithelium in both gingival health and disease (Egelberg, 1963; Freedman, et al, 1968; Gavin, 1970). Attstrom (1970) showed that

95 - 97% of cells in the gingival fluid were P.M.N.'s in both health and disease, although the number of cells was increased in chronically inflamed gingivae (Attstrom, 1970; Klinhamer & Zimmerman, 1969). There is little doubt that P.M.N.'s migrate from the tissues to the gingival crevice and thence to the saliva (Klinkhamer & Zimmerman, 1969; Rindom-Schiott and Loe, 1970) and that greater numbers of leukocytes migrate to the saliva when the gingival tissues are inflamed (Theilade, et al, 1966; Skougaard, et al, 1970). Therefore if the chemotactic factors derived from complement are participating in the cellular kinetics of periodontitis it would seem that most of the complement is being activated in the crevicular epithelium or crevice itself. Evidence that complement is activated in the gingival area was produced by Brandtzaeg (1966) who detected beta 1_A globulin, which is a breakdown product of activated C'3, in gingiva and crevice fluid. This does not preclude the possibility of many other factors influencing the migration of leukocytes in inflamed gingiva, such as bacterial chemotactic factor described by Keller & Sorkin (1967) and Ward, et al (1968) or chemotactic factors in saliva (Tempel, et al, 1970). In cases of neutropenia where the level of P.M.N.'s drops severe ulcerative gingivitis develops, thus implying that they play a protective role against oral bacteria (Platt, et al, 1970). However leukocyte enzymes have been shown to widen the intercellular spaces of the gingiva (Thilander, 1963), so a combined beneficial and detrimental effect of leukocyte enzymes could be expected,

the balance reflecting the state of gingival health.

A speculative conclusion from the above evidence is that much of the host defense against periodontitis takes place in the gingival crevice in the form of P.M.N.'s and contents of the gingival crevice fluid, and that tissue damage will result when the products of host inflammatory cells and bacterial metabolites gain access to the gingival tissue. When, for as yet unclear reasons, the host defense or "dynamic balance" (Macphee & Cowley, 1969) is upset, comparatively greater quantities of toxic substances may gain access to the gingival tissue and a more severe reaction develops in the gingiva instead of the crevice, which may lead to ANUG.

Summary:

This survey has described the two most common forms of human inflammatory periodontitis; acute necrotising ulcerative gingivitis and chronic periodontal disease. The latter ranges from acute non-specific gingivitis in its early form to chronic periodontitis in its established form. Considerations of the aetiology and pathogenesis of these conditions have introduced the following factors which may, under the proper conditions, play a role in alteration of tissue homeostasis in the periodontal lesion:

1. Dental plaque seems to be causally related to the development of non-specific gingivitis.
2. Immunological mechanisms can, under certain circumstances, act to the detriment of the host by evoking tissue damage.
3. All the components necessary for immune reactions, i.e. antigens, antibodies and complement, are present in periodontal disease.
4. The complement system can, upon activation, release factors with biological activities which may contribute to the overall host response to antigenic stimulation. Complement acting at a cell membrane may also induce release of the cell's lysosomal contents.
5. There is strong evidence implicating endotoxins in the pathogenesis of periodontal disease. They can also activate the terminal components of the complement system.
6. Lysosomes which are normal constituents of nucleated mammalian cells may, under appropriate conditions be induced to release their enzymes and other active substances with subsequent cell and tissue damage.

7. P.M.N.'s which are a prolific source of lysosomes are present in ANUG and periodontitis.

Objects of the Investigation:

The purpose of the study is to investigate the hypothesis that endotoxins, if present at a cell-associated immune reaction which fixes complement, will interact with complement to quantitatively affect the release of lysosomal enzymes from the involved cells. This investigation seeks to establish the following points in the development of the thesis:

A

(1) To establish an injection regimen for endotoxins which induces tissue responses which are distinct from control injections but which does not result in the supervention of haemorrhagic necrosis or abscess formation.

(2) To assess qualitatively and quantitatively the cellular response to single and multiple injections of endotoxin with a view to using these cell types for *in vitro* study.

(3) To remove the inflammatory cells from the injection sites enzymatically for subsequent use in *in vitro* studies.

B

(1) To establish whether guinea pig serum will release lysosomal enzymes from isolated polymorphonuclear neutrophils (P.M.N.'s), lymph node cells (L.N.C.'s), or peritoneal transudate cells (P.T.C.'s).

(2) To measure whether endotoxin alters the relative quantities of lysosomal enzymes released from P.M.N.'s, L.N.C.'s, P.T.C.'s by guinea-pig serum.

(3) To establish whether endotoxin could affect the availability of complement for action of the latter on the lysosomal enzymes.

(4) To compare the extent to which each cell type was reliant on the heat-labile components of guinea-pig serum to achieve release of its lysosomal enzymes.

(5) To measure the mortality rate sustained by experimental manipulation of each cell type and whether the experimental procedures induced increased mortality.

(6) To determine the correlations between the relative quantities of acid phosphatase and beta-glucuronidase on the basis that a strong positive correlation between these two parameters was required to support the view that they represented the release of lysosomal contents.

PART II

THE INFLAMMATORY RESPONSE OF MICE TO ENDOTOXIN INJECTIONS

MATERIALS & METHODS

A. INJECTION REGIMEN

The animals were an inbred stain of ICR strain Swiss White mice maintained in plastic cages with free access to chow and water. Six to ten week old male and female mice were used in the experiment with three mice representing a group from which evaluation of cellular infiltrate was made.

There were two main parts to the study, the first being the quantitative and qualitative assessment of the histological response to a single injection in one of two vehicles and the second being the assessment of the result of multiple injections of endotoxin.

(1) Effects of Single Injection of Endotoxin:

For the single injection groups there were six injection solutions comprising 0.1 ug per 0.1 ml, 1.0 ug per 0.1 ml and 10.0 ug per 0.1 ml of endotoxin each in 0.85% saline and 0.1 ug per 0.1 ml and 1.0 ug per 0.1 ml and 10.0 ug per 0.1 ml endotoxin in 5% glucose. The endotoxin was the Boivin extracted lipopolysaccharide from E. Coli 055:B5 (Difco Labs., Detroit, Michigan). The saline was made from pyrogen-free distilled water (Baxter Labs., Ontario). Glucose solution was also used as a vehicle because it has been reported that a 5% glucose solution dissolves endotoxin (Braude, 1964) and it was of interest to see if different vehicles elicited different reactions. Deionized water was used to make a 5% solution of glucose (Dextrose, Fisher Scientific).

Fifteen animals per group were anaesthetized by ether inhalation and both left and right buccal mucosae swabbed with 70% alcohol. They were injected with 0.1 ml of the experimental endotoxin solutions in the left side and with the same volume of the corresponding vehicle without endotoxin on the right side. Three of the mice were sacrificed immediately by dislocation of the cervical vertebrae and the others, also in groups of three, at 6, 24, 48 and 144 hours after injection. This procedure was repeated for each of the six injection solutions. The tissue was removed with razor blades and the fur trimmed from the outer aspect of the tissue after fixation in 10% formalin. Tissue sections were cut at 6 u thickness and five sections from different depths throughout each block stained with haematoxylin and eosin for histological assessment.

(2) Effect of Multiple Injections of Endotoxin:

The animals were injected in the left buccal mucosa at weekly intervals with 0.1 ug and 1.0 ug endotoxin in 0.85% saline for a period of eight weeks. The control injections of vehicle without endotoxin were performed simultaneously in the right side. The mice were sacrificed at 0, 6, 24, 48 and 144 hours after the final injection and the tissue removed and processed as before.

All the microscope slides were coded by a technician and histological assessment performed without knowledge of the experimental routine to which the animal had been subjected.

B. ASSESSMENT OF HISTOLOGICAL SLIDES:

A survey of the slides showed that the cellular infiltrate comprised polymorphonuclear neutrophils (PMN's) and a variety of cells appearing later. These included lymphocytes, plasma cells, monocytes and macrophages. Also present were large numbers of cells with chromatin distribution in the nuclei similar to that of plasma cells, but whose narrow crescentic cytoplasm seemed much more related to the lymphocyte group of cells. Rather than attempting to differentiate between these cells this latter type along with lymphocytes and plasma cells were described as "round cells" and assessed as one group. Since PMN's and round cells were the most dominant and consistently present cell type they were used as indicators of severity of the cellular response to endotoxin injections.

Probably because the injection solution was evenly distributed throughout the tissue, there was no observed difference in the severity of inflammation seen in the slides taken from various depths of the same tissue block. Thus only one result per tissue was required to express the response for the entire block.

The nature of the inflammatory infiltrate was usually a series of pockets between tissue components, e.g. between muscle bundles or in fat spaces and the cell density in these pockets could be taken as an indication of the intensity of the infiltrate.

FIGURE 1

A semi-quantitative assessment of cellular infiltrate expressed in terms of the subjective assessment showed that an observed difference between "+" ratings represented a logarithmic increase in cell numbers in a given area.

The PMN's and round cells were each assigned on a + to ++++ basis ranging from a detectable light infiltrate to a heavy infiltrate. Where no infiltrate of a cell type was seen a negative reading was given, and those sections with frank abscess formation or tissue necrosis were noted.

All slides were re-read to test the consistency of the subjective assessment. About 70% of the second assessments corresponded with the first and none of those which differed did so by more than one + unit. These slides were again re-read and a final + rating assigned to each one.

To accord each + rating with a numerical value for statistical analysis five slides which had been assigned each + rating were selected at random. The area of densest infiltration on each was selected and a cell count performed for each cell type at X400 magnification. Each field was counted three times and the average cell count calculated. These values were in turn averaged for the five slides selected from each + rating and this average used for the particular + rating in the statistical analysis. A graph plotted on semi logarithmic axis (Fig. 1) showed that the cellular infiltrate increased logarithmically for each subjective + unit. The values selected were + = 25 cells per field; ++ = 55 cells per field; +++ = 150 cells per field, and ++++ = 300 cells per field.

All the slides were decoded and subjected to an analysis of variance.

AVERAGE CELL COUNTS EXPRESSED AGAINST
SUBJECTIVE "+" RATING

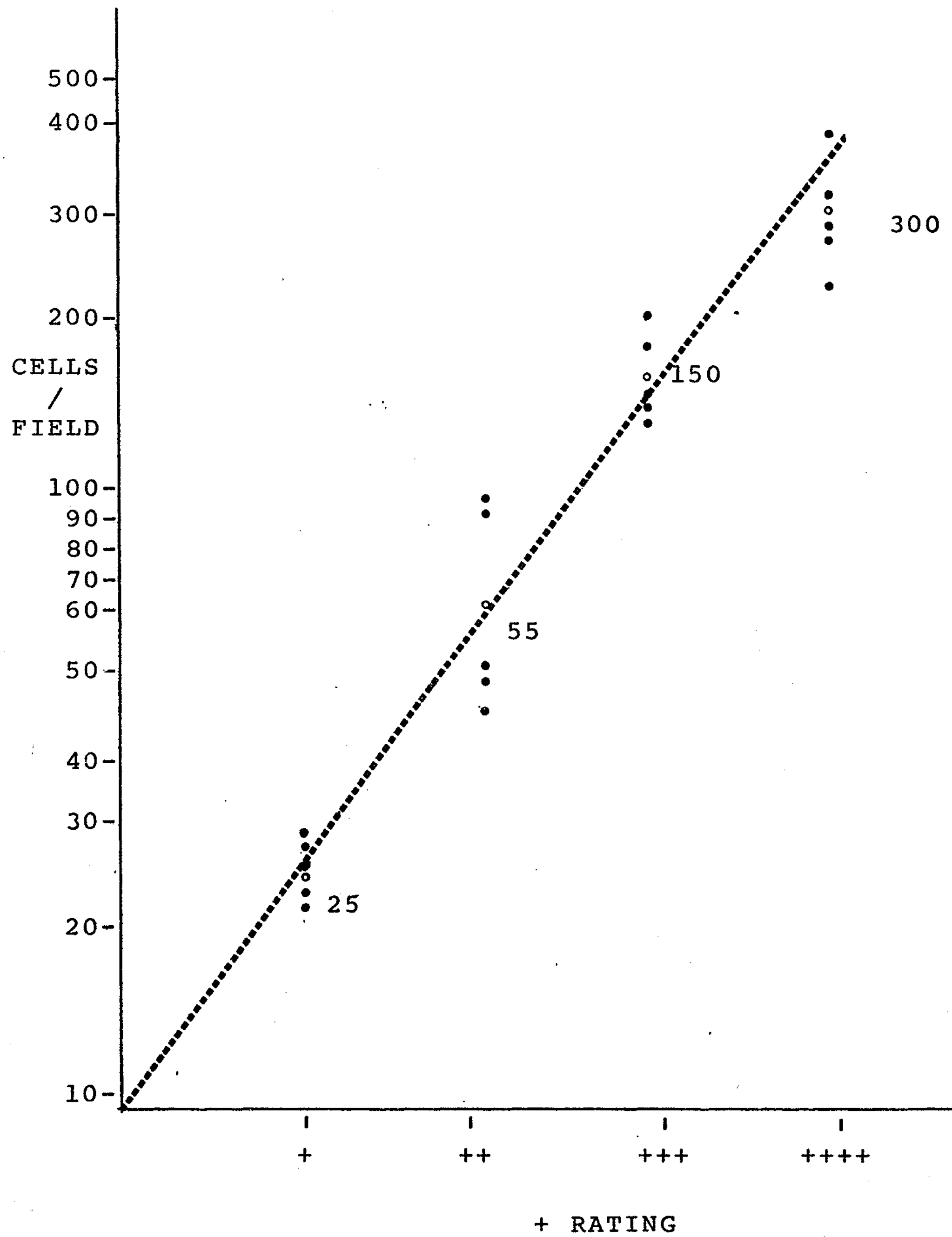


FIGURE I

RESULTS

A. HISTOLOGICAL ASSESSMENT OF SINGLE INJECTIONS OF ENDOTOXIN

(1) Injections Containing 10.0 ug Endotoxin:

The animals receiving 10.0 ug of endotoxin quite often developed small abscesses and necrosis of muscle bundles. In addition this dose almost invariably produced gross dilatation of the blood vessels often containing thrombi. These changes were considered to be too profound to represent an acceptable model of periodontal disease and the study of this group was discontinued.

(2) Injections Containing 0.1 ug Endotoxin:

Following an injection of 0.1 ug endotoxin in glucose the highest average value for polymorphs was between 180 and 190 with a standard error of 25 (fig. 2). At no time was a PMN reading consistently high within a group to maintain an average infiltrate of ++++. Although individual responses within a group were different each was paralleled by its own control side thereby maintaining a significant experimental control difference.

For the round cell response at this dose there is never a marked infiltrate. The level of over forty falls short of an average ++ count, which is a light, patchy infiltrate and not convincingly different from the control.

The group receiving 0.1 ug endotoxin in saline produced a similar profile to the glucose vehicle (fig. 3).

(3) Injections Containing 1.0 ug Endotoxin:

The saline and glucose vehicles produce similar profiles of inflammatory infiltration (figs. 4 and 5). A notable

KEY FOR FIGURES 2 - 7

Round Cell Control *

Round Cell
Experimental *

* Standard Error Single Injection = ± 16.765
" " Multiple Injections = ± 13.681

Polymorphonuclear
Neutrophils Control **

Polymorphonuclear
Neutrophils
Experimental **

** Standard Error Single Injection = ± 25.499
" " Multiple Injections = ± 23.731

SINGLE INJECTION 0.1 ug ENDOTOXIN
IN GLUCOSE VEHICLE

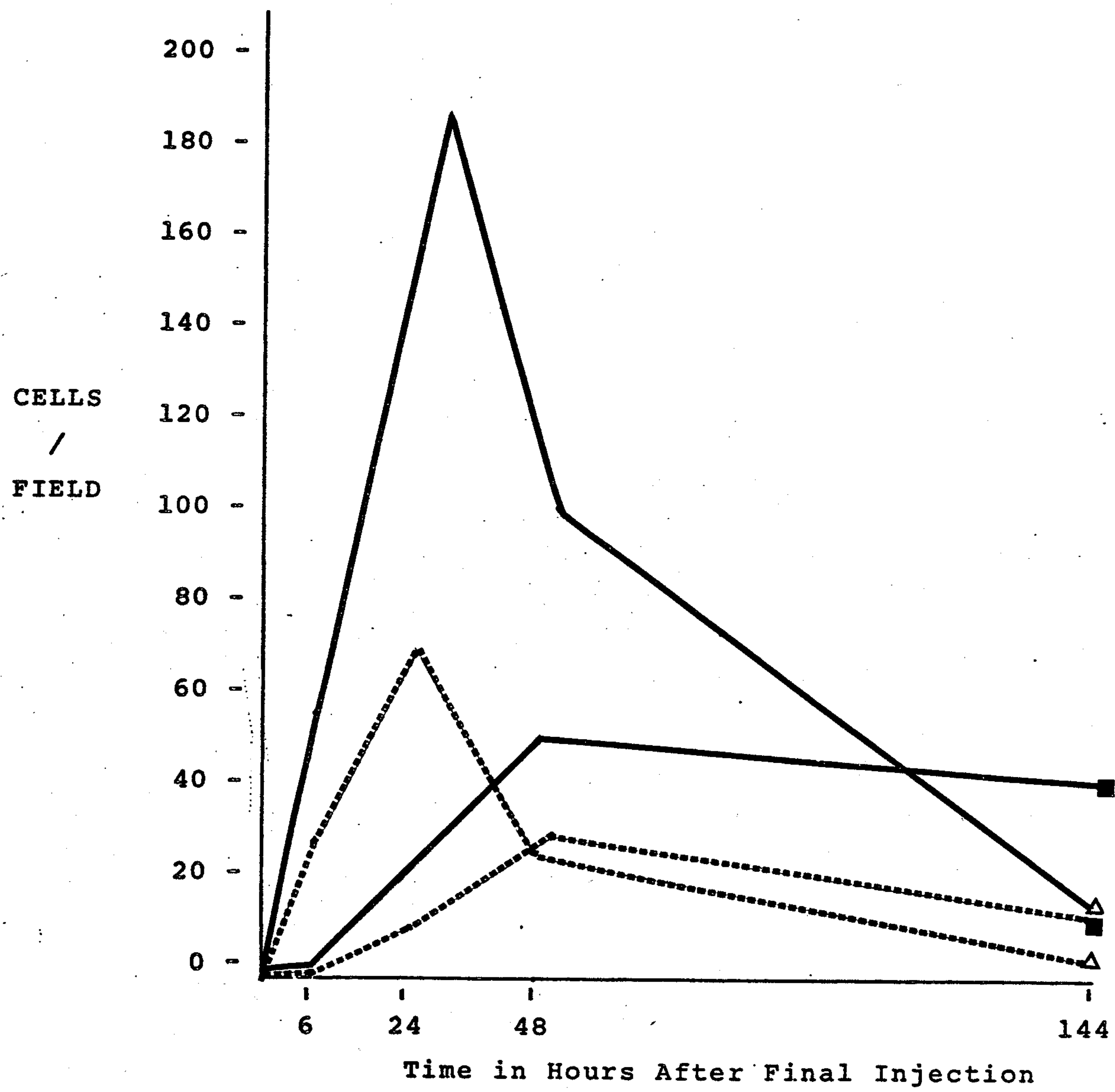


FIGURE 2

SINGLE INJECTION 0.1 ug ENDOTOXIN
IN SALINE VEHICLE

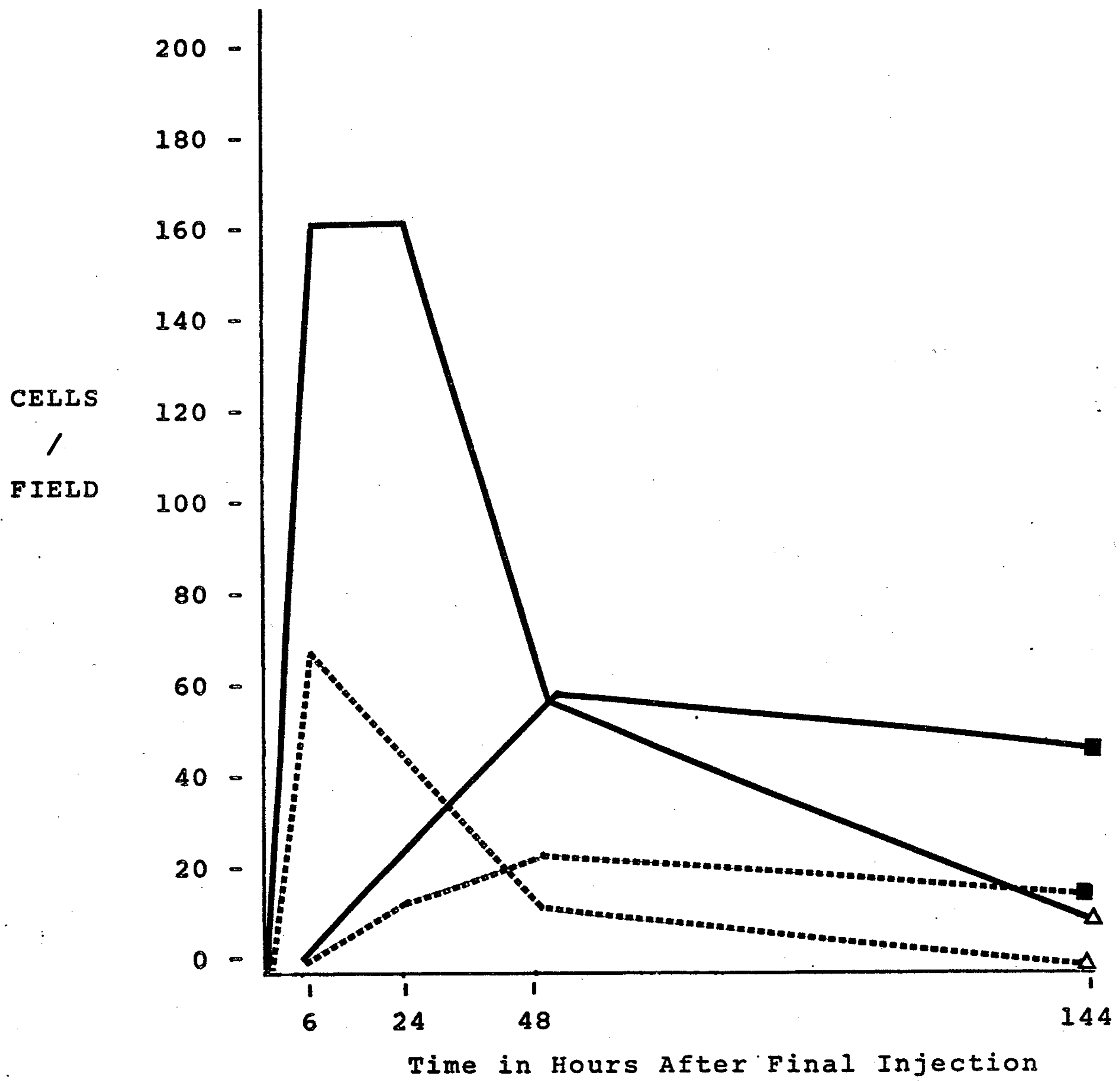


FIGURE 3

finding is that although the dose of endotoxin is ten times that in the other two groups the intensity of PMN infiltration does not reach a higher level. In contrast to the animals receiving 0.1 ug endotoxin there is an increase in the round cell infiltration which is significantly greater than in the control sides. The infiltrate declined quickly after the injections.

SINGLE INJECTION 1.0 ug ENDOTOXIN
IN SALINE VEHICLE

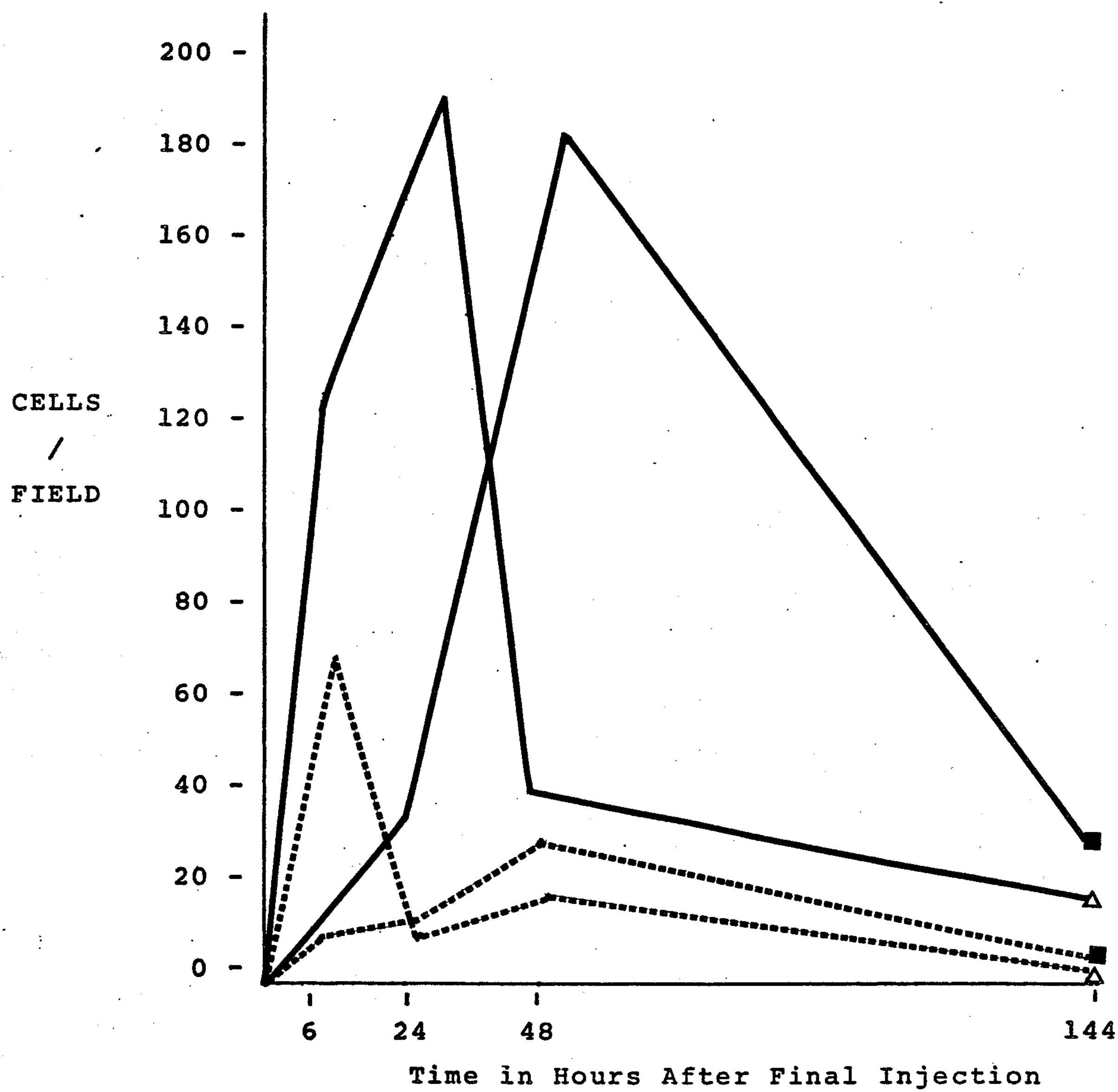


FIGURE 4

SINGLE INJECTION 1.0 ug ENDOTOXIN
IN GLUCOSE VEHICLE

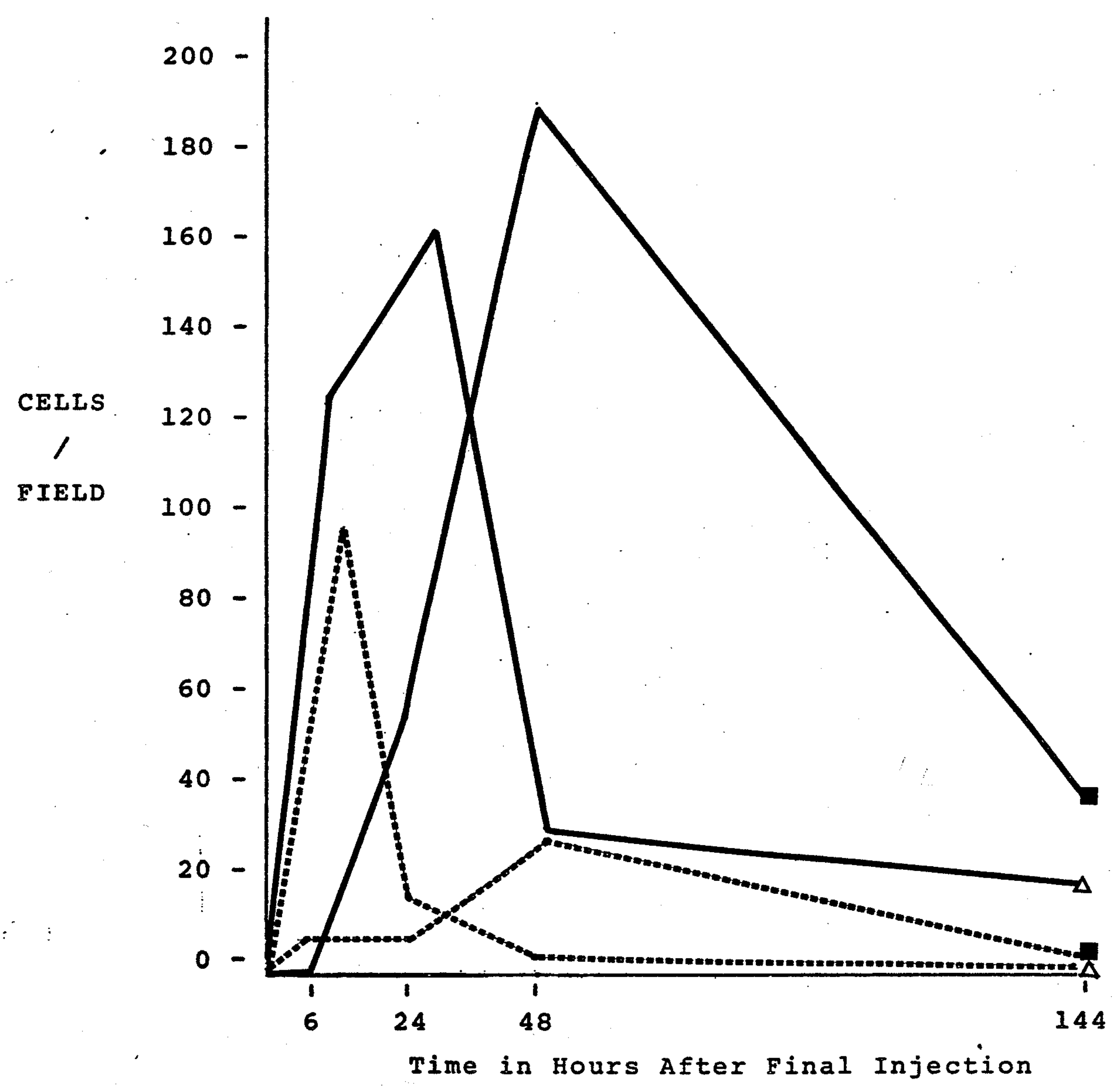


FIGURE 5

B. HISTOLOGICAL ASSESSMENT OF MULTIPLE INJECTIONS OF ENDOTOXIN

1. Multiple Injections of 0.1 ug Endotoxin in Saline:

Rather than any cumulative effect of endotoxin it can be seen that there is a marked dampening of all the cellular responses to an extent where the peaks in the profiles at certain time intervals are not satistically different from each other or their corresponding controls (Fig. 6).

2. Multiple Injections of 1.0 ug Endotoxin in Saline:

This regimen produced a histological appearance which was distinct from its controls and which, from 48 hours after the final injection, resembled the appearance of periodontal disease. There was no evidence of abscess formation or tissue necrosis. A marked difference observed in this group (Fig. 7) was that the initial P.M.N. infiltrate had subsided by 24 hours whereas after single injection procedures the P.M.N.'s attained their peak density at 24 hours. As expected there was some round cell infiltrate at the time of injection which was probably a residual effect of the previous injections. The round cell infiltrate seemed slower to decline than after single injections.

It was concluded that this injection regime produced a cellular infiltrate which was due to the action of endotoxin and which resembled the histological appearance of periodontal disease.

MULTIPLE INJECTIONS 0.1 ug ENDOTOXIN
IN SALINE VEHICLE

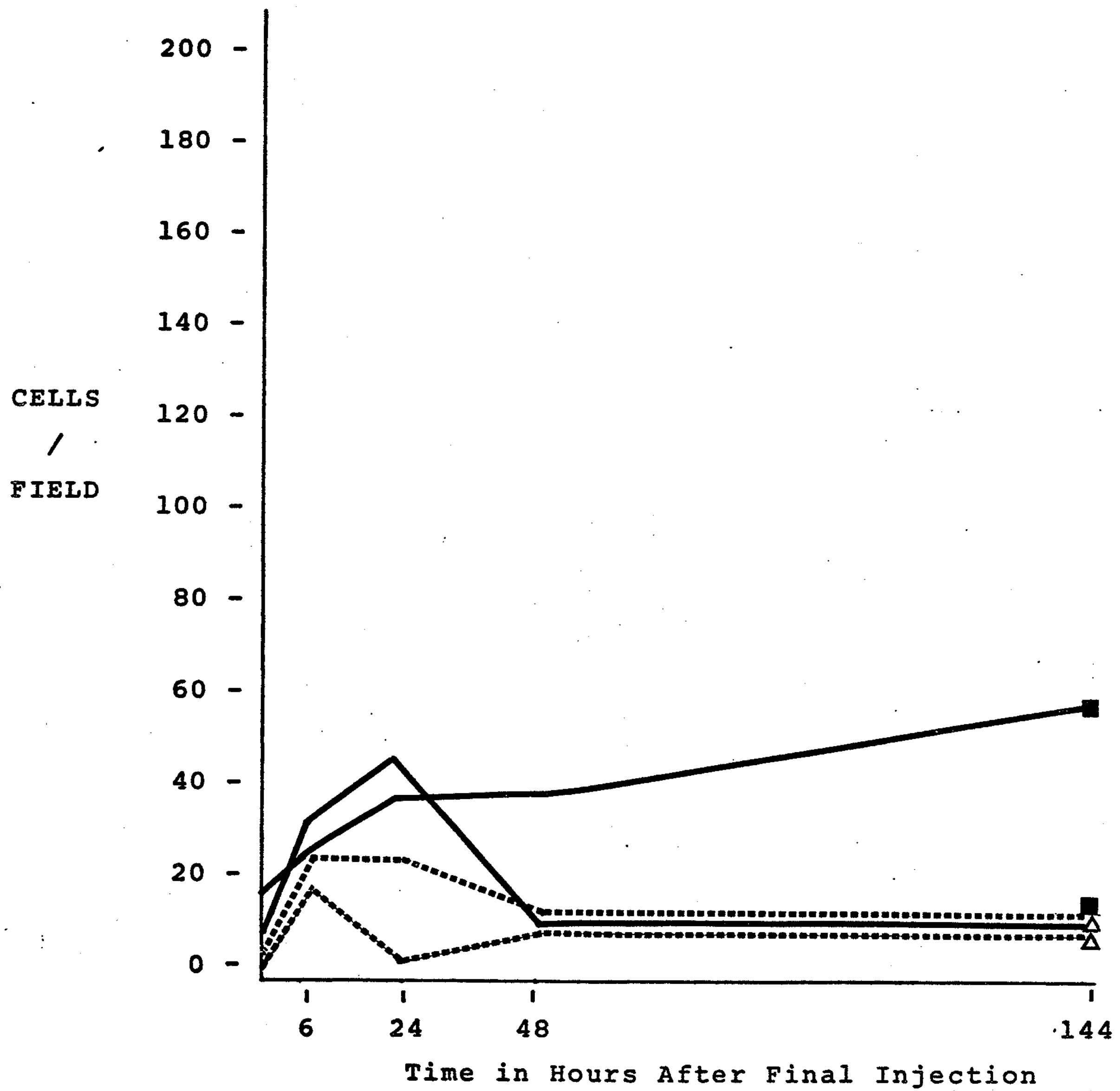


FIGURE 6

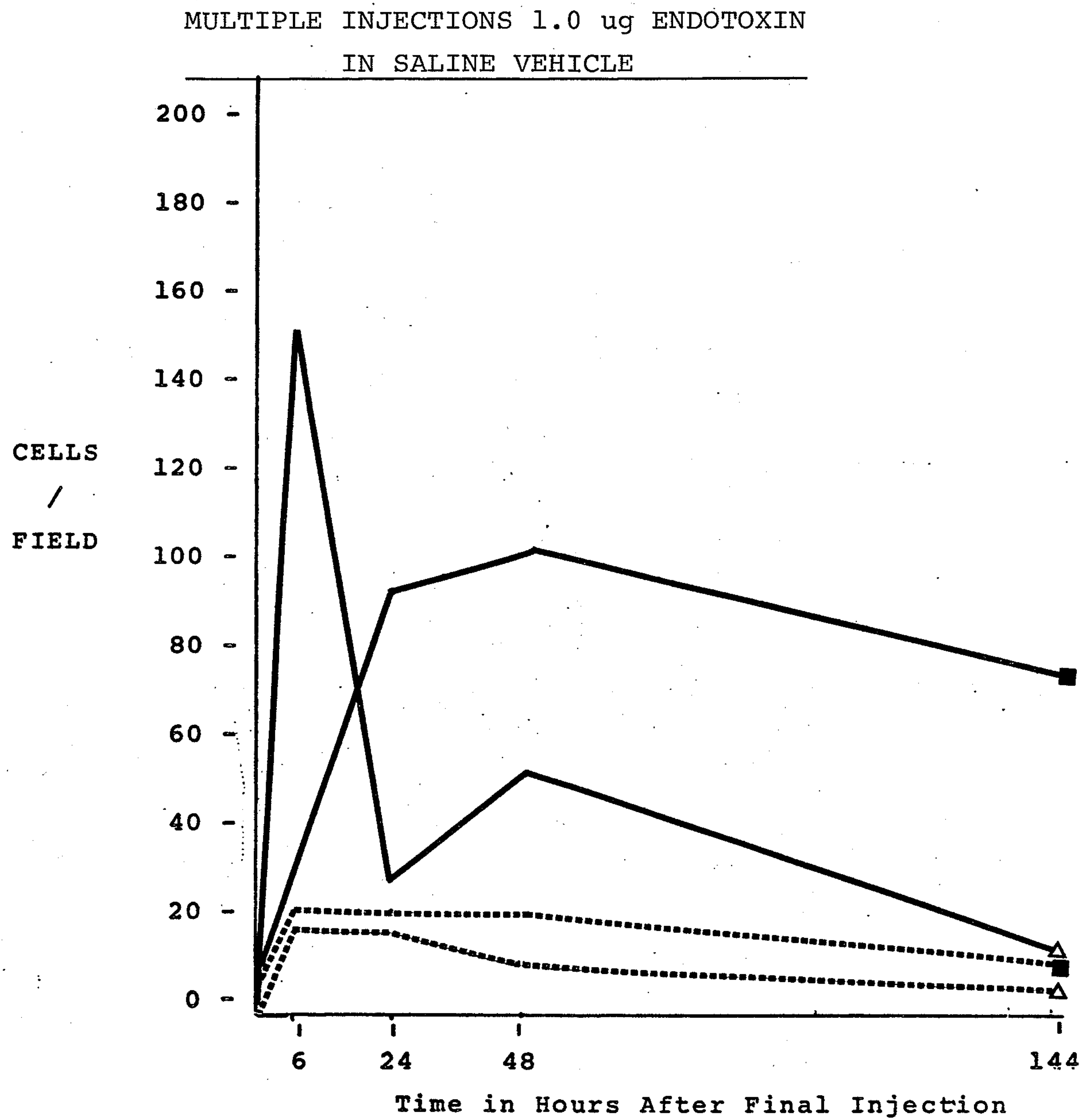


FIGURE 7

Summary of Results:

(i) There was no significant qualitative or quantitative difference between the cellular infiltrate produced by endotoxin in a saline vehicle and a glucose vehicle.

(ii) Single injections of 1.0 ug endotoxin produce a greater round cell infiltrate than 0.1 ug endotoxin although they both evoke a similar PMN response.

(iii) Multiple injections of 0.1 ug of endotoxin fail to produce a histological response which is significantly different from its control.

(iv) Multiple injections of 1.0 ug endotoxin induce an accelerated PMN response and a more persistent round cell response when compared with the same dose after a single injection.

(v) With these doses of endotoxin there was no subsequent abscess formation or haemorrhagic necrosis in the tissues.

DISCUSSION

The use of buccal mucosa in this experiment provided more tissue than did gingivae, which were not essential to study the histopathological effects of endotoxin injection. The palates of rabbits have also been used for this purpose to prime the site for the Schwartzman reaction and to induce abscess formation (Rizzo & Mergenhagen, 1964).

The effect of 5% glucose on the solubility of endotoxin was not as Braude had reported. The mixture appeared the same as the saline/endotoxin mixture which was a cloudy suspension. If some of the endotoxin was dissolved in the glucose solution it was impossible to estimate how much. Taking these findings into consideration there is no reason to suppose that the two vehicles should have produced different histological results, and this was borne out by the experimental results. The conclusion to be drawn from the study was that this endotoxin preparation did not dissolve in glucose solution, but no conclusions could be made asserting that there is no difference between the histological responses to endotoxin suspensions and solutions. It was therefore considered unnecessary to perform the multiple endotoxin injections with more than one vehicle.

The histological responses of each animal often differed within the same groups, however the control for each individual consistently maintained a relationship with the experimental side which was constant for the whole group. This variable reaction of different individuals to endotoxin has also been noted in rabbits (Lee & Stetson, 1960).

The more rapid appearance and decline of the PMN infiltrates in the animals given multiple injections of 1.0 ug endotoxin may bear some relationship to the accelerated cutaneous reaction to endotoxins noted by Lee and Stetson (1960). In this phenomenon intradermal injections in rabbits result in a cutaneous reaction with maximum intensity at 24 hours. Animals which had received intravenous injection of endotoxin 1 day to 1 month previously and were subsequently given intradermal injections reacted with maximal intensity at six hours, and exhibited a more intense infiltrate than the group receiving an intradermal injection only.

This model obviously falls short of a slow continuous introduction of endotoxin into the gingival tissue. It does, however, establish a chronic lesion in which the effects of the injection trauma alone can be assessed. It permits prediction of the nature of the infiltration up to 144 hours after either a single injection or multiple injections of 0.1 ug and 1.0 ug of endotoxin. The cell types involved in the inflammatory response to endotoxin were PMN's, lymphocytes, plasma cells and macrophages. The object of the investigation was to determine the cellular response after endotoxin injections, with the view of removing these cells enzymatically from the tissue for subsequent investigation on the effects of complement and endotoxin on their lysosomal enzymes. Subsequent experimentation showed that the yield of lysosomal enzymes substantially

decreased following this method. About 70% of the lysosomal enzyme activity was lost in the supernatants following centrifugation, thereby leaving only a small and probably resistant, population of lysosomes with which to work. It was decided to use other sources of cells which morphologically resembled the cells observed in the tissues following administration of endotoxin. These were PMN's from a peritoneal exudate, macrophages from a peritoneal transudate and lymph node cells.

PART III

THE EFFECTS OF COMPLEMENT AND ENDOTOXIN ON THE RELEASE OF LYSOSOMAL ENZYMES FROM MOUSE LEUKOCYTES *IN VITRO*

MATERIALS & METHODS

A. PREPARATION OF CELL SUSPENSIONS

The animals used for cell harvesting were an inbred strain descendant from ICR strain Swiss white mice supplied by Canadian Breeding Laboratories. Six to ten week old males and females were used. The animals were maintained in plastic cages with free access to Purina Laboratory Chow (Ralston Purina Chow, st. Louis, Missouri) and water.

The medium in which the cells were suspended was reconstituted lyophilised TC 199 medium without phenol red (Difco Laboratories, Detroit, Michigan). Phenol red indicator which is normally in this medium would have interfered with the spectrophotometric estimations of free phenolphthalein, which is the basis of the beta glucuronidase assay. When reconstituted with distilled water the pH of the medium was adjusted to 7.4 with 2 molar Tris solution. The Tris buffering system is more stable at different ambient temperatures than the bicarbonate system which is usually incorporated in the TC 199, and therefore obviates the need for phenol red indicator. Tris buffer was found not to affect test assays of acid phosphatase and beta-glucuronidase. Penicillin G and Streptomycin sulphate (Difco Laboratories, Detroit, Michigan) were added to the medium in a concentration of 100,000 units per litre and 10,000 ug per litre respectively. The medium was sealed in autoclaved glassware and stored at -20°C till needed. The pH was checked immediately before use at 37°C.

Cell Harvesting:

All glassware used in the harvesting, incubation and sonication of cells was siliconised by immersion in a 1% solution of "Siliclad" (Clay Adams, Canadian Laboratories, Winnipeg) for 20 seconds and dried at room temperature for 24 hours.

(1) Peritoneal Transudate Cells (P.T.C.):

This procedure was based on the method described by Whitby and Rowley (1959). Six mice were used to provide cells for one experiment. The mice were killed by dislocation of the cervical vertebrae. Ether was not used at any time as it may have changed the cell membrane permeability. Immediately after death the animals were pinned to the dissection board and the abdominal skin reflected without the abdominal wall being punctured. Animals which suffered a ruptured spleen or other cause of haemorrhage were discarded to minimise contamination of the cell sample by peripheral blood cells. The abdominal wall was swabbed with 70% ethyl alcohol and 1.0 to 1.5 ml. of TC 199 injected into the abdominal cavity through a sterile gauge 20 needle on a disposable plastic syringe. The needle was pointed away from the liver or spleen. The fluid was washed around the peritoneal cavity for a few seconds by manipulating the abdominal wall with the fingers, but without removing the needle. The fluid was then withdrawn slowly by keeping the tip of the needle away from fat or mesentery to avoid clogging,

and the cloudy fluid expressed gently into a 10 ml. beaker keeping the point of the needle in contact with the glass. The yield was approximately 7-8 mls. of cell suspension. A fibrin clot formed after a few minutes at 37°C and was removed by filtering the suspension through a fine nylon screen. Heparin was not used to prevent fibrin formation because increased rat plasma levels of beta-glucuronidase, acid RNAase have been recorded after heparin injection (Levy, 1967). Heparin has also been demonstrated to increase phagocytic activity (Filkins and Luzio, 1966) and pinocytotic activity (Cohn and Parks, 1968).

The yield was 3.0×10^6 cells per ml. to 10.0×10^6 cells per ml., about 65-75% of which were macrophages as judged by their ability to phagocytose neutral red particles. The rest appeared to be monocytes.

(2) Peritoneal Exudate Cells (P.M.N.'s):

This procedure was based on the method described for rabbits by Cohn and Hirsch (1960). A peritoneal exudate was induced in six mice by injection of 0.1% glycogen (British Drug Houses) in normal saline prepared from sterile pyrogen-free water (Baxter Laboratories, Alliston, Ontario). The mice were killed four hours later. The peritoneal exudate washings were obtained as described for the transudate cells. Animals which had intra-peritoneal haemorrhage were rejected. The fibrin clot was removed as

before. Examination revealed 75-85% polymorphonuclear leukocytes with the remainder classed as macrophage or monocyte with occasional small lymphocytes.

(3) Lymph Node Cells (L.N.C.):

Three mice were killed by dislocation of the cervical vertebrae. The cervical, axillary and inguinal lymph nodes were dissected out, freed from fat, mesentery and blood as far as possible and transferred to a plastic petri dish over ice containing TC 199 for washing. They were subsequently placed in 6-7 ml. TC 199 in another petri dish and gently teased open with sterile guage 25 injection needles. When the suspension had become cloudy it was filtered through three layers of nylon screen into a 10 ml. siliconized beaker. Microscopic examination revealed little or no contamination by fibrous stroma of the lymph node. This procedure yielded 25×10^6 - 35×10^6 cells per ml. 90% of which had the morphology of small lymphocytes, the rest comprising plasma cells and macrophages. No fibrin clot formed after this procedure.

None of the cells were washed further after harvesting because centrifugation released up to 20% of the total lysosomal enzyme activity into the supernatant. This may have represented a distinct lysosome population which would not be subjected to experimental procedures.

The cells were then ready to dispense into their respective incubation tubes.

B. PREPARATION OF CELL SUSPENSIONS FOR ENZYME ASSAYS

Following incubation of the cell suspensions for one hour the total cell count and percentage mortality was calculated for each experimental procedure. Trypan blue exclusion was used as the criterion of cell viability. A 1% stock solution of trypan blue was diluted 1:20 in TC 199 and pre-tested on unincubated fresh cell suspensions of lymph node cells, peritoneal transudate cells and peritoneal exudate cells to ensure that the trypan blue solution had no primary cytotoxic effect. The total number of viable white cells and stained dead cells present in each experimental incubation tube were counted in a Levy counting chamber using a white blood cell pipette to dilute the cells with the trypan blue solution. A minimum of 100 cells was considered to give a representative sample. The mortality was expressed as a percentage of dead cells in the total white cell count.

The results of the experiment were expressed as the percentage of the total enzyme activity of each suspension which was released into the medium during incubation. Thus two samples had to be prepared from each experimental procedure, the first representing the enzyme which had been released into the medium and the second representing 100% enzyme activity.

(1) Preparation of Sample Containing Enzyme Released During Incubation:

After incubation the cell suspensions were filtered through a millipore filter and prefilter in a Swinnex filter holder (Millipore Corp., Montreal) which was attached to a 10 ml. disposable syringe. The filter pore size was 0.22 μ and the diameter 25 mm. The filtration had two purposes. Firstly it removed the cloudiness of cell debris which interefered with optical density. This was important with Triton treated (see below) samples which could not be cleared by centrifugation at conventional speeds. Secondly it prevented whole cells or lysosomes from being included in the enzyme assay mixture, which at pH 5.0 and 37°C would have released lysosomal enzymes which were not the result of experimental procedure (Gianetto & de Duve, 1956).

The efficacy of millipore filtration was tested to assess any inaccuracies that may have arisen from it.

A pool of 6 ml. of peritoneal transudate cells was sonicated over ice for 20 seconds. This procedure ruptures most of the cell walls but has only a limited effect on the release of beta glucuronidase (see preparation of samples containing total enzyme activity). The sonicated cells were divided into three 2 ml. samples. Two of these were filtered and then Triton X-100 (Sigma Chem. Co., St. Louis) added to one sample in a final concentration of 1%. Triton X-100 is a non-ionic detergent which solubilises

membrane-bound lysosomal enzymes (Wattiaux and deDuve, 1956). The other filtered sample was made up to the same volume with normal saline. Triton X-100 was added in the same concentration to the third sample which was then filtered after it had remained at room temperature for 20 minutes. Equal volumes (0.2 ml.) of the three filtrates were assayed for beta-glucuronidase content as described in "enzyme assays". The results showed that the tube treated with Triton before filtration had about four times the activity of the other two samples. This indicated that most of the beta-glucuronidase of the other two samples was membrane bound before filtration. The beta-glucuronidase activity of the Triton treated filtrate was the same as that treated with saline only, which indicated that Triton was not solubilising membrane-bound beta-glucuronidase in the filtered samples. These experiments were replicated six times.

(2) Preparation of Samples Containing Total Enzyme Activity:

The 1.0 ml. samples which were drawn from each incubation tube were dispensed in 5 ml. beakers and maintained on ice. Each was ultrasonicated for two minutes with a Bronson sonifier using a microtip adaptor at 60 watts power. The membrane-bound enzymes were solubilised by adding 0.5 ml. of 3% Triton to yield 1.5 ml. of sample total enzyme assay. This was then filtered as previously described and the filtrate used to represent total enzyme activity.

As the effect of Triton on isolated lysosomes has been studied, (Wattiaux and deDuve, 1956) ultrasonication was used in this experiment to rupture cell membrane and so expose the lysosomes to the action of Triton. The effect of ultrasonication on cell membrane integrity and lysosomal enzyme release was assessed beforehand on peritoneal cells.

The Effect of Ultrasonication of Peritoneal Transudate Cells:

A pool of peritoneal transudate cells in a volume of 12 mls. was collected from nine mice. A 1.0 ml. sample was taken before sonication over ice with a Bronson sonifier fitted with micro-adaptor. The sonicator was stopped every four seconds and a 1.0 ml. sample withdrawn each time until the remaining cells had been sonicated for 28 seconds. The tip of the micro adaptor was adjusted so that it was always immersed to the same depth in the cell suspension. Each sample withdrawn at a 4 second time interval was assayed for the percentage of total beta-glucuronidase present in the filtrate, and subjected to an intact white cell count which was expressed as a percentage of the total cell count in the sample taken before sonication. This procedure was repeated six times and the results subjected to an analysis of covariance and regression. The calculated regressions of both intact cell population and filtrable beta-glucuronidase with ultrasonication time are shown in Figure 8. The means of the readings of intact cells

FIGURE 8

Ultrasonication of peritoneal transudate cells ruptured almost all the cell membranes but only released a small proportion of beta-glucuronidase into the medium, thus confirming the need for Triton X-100 to solubilise the lysosomal enzymes for estimation of 100% enzyme activity.

Effect of Ultrasonication on Peritoneal Transudate Cells and Beta Glucuronidase Release

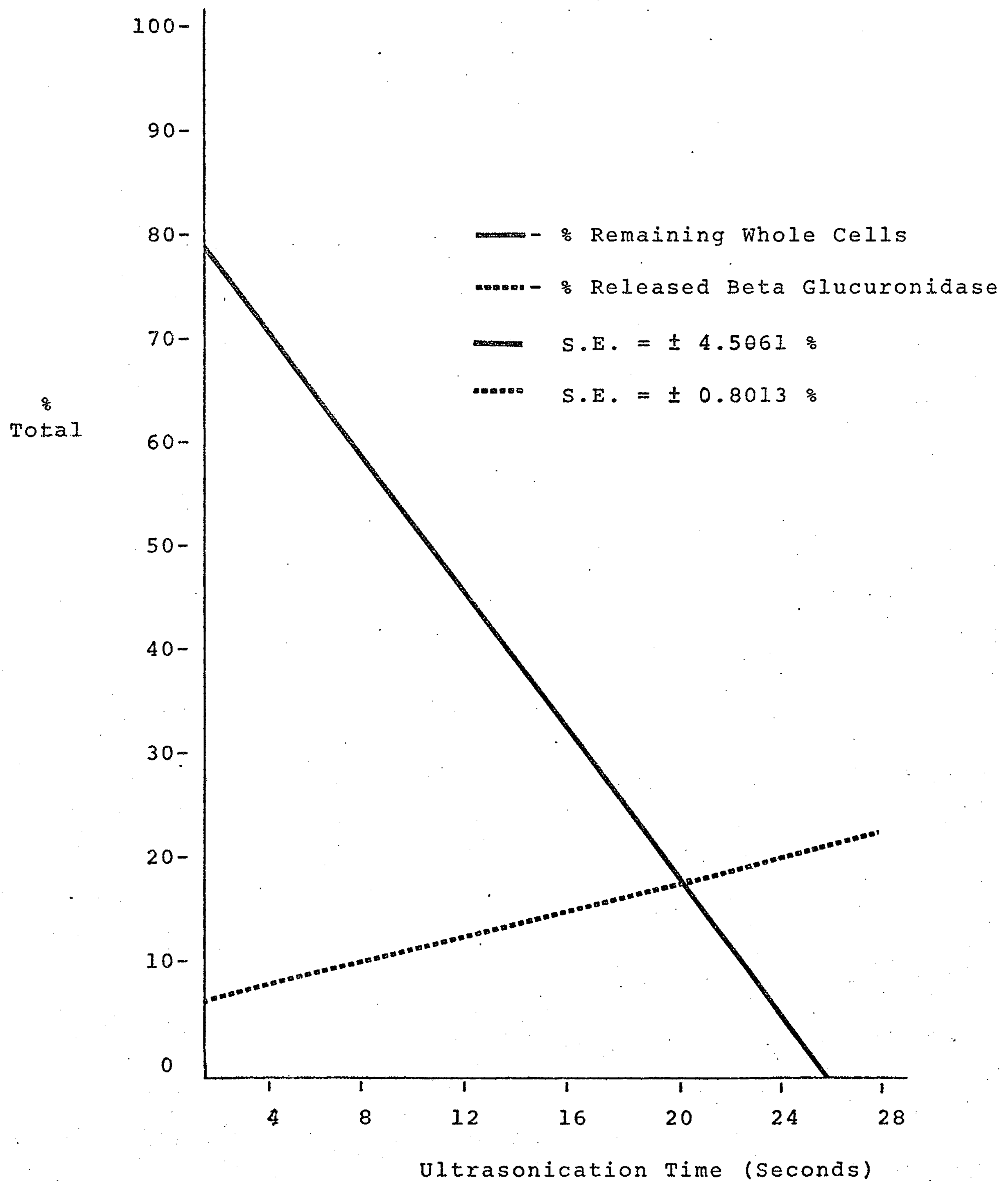


FIGURE 8

TABLE I

EFFECT OF ULTRASONICATION ON PERCENTAGE OF REMAINING INTACT CELLS AND ON PERCENTAGE FILTRABLE BETA-GLUCURONIDASE

Sonication Time In Seconds	Cell Count Mean % Total	Optical Density of Phenolpthalien Mean % Total Beta Glucuronidase
0	100	5.12
4	66.4	7.66
8	47.2	10.66
12	33.7	13.75
16	18.9	16.63
20	10.7	18.93
24	6.2	20.95
28	3.7	22.03
	S.E. ± 4.5061	S.E. ±0.8013

The mean values of six experiments estimating the effect of ultrasonication times on peritoneal transudate cells are shown above. The statistical analysis of these results is expressed graphically in Fig. 8.

and percentage of filtrable beta-glucuronidase at each time interval are shown in Table II . The percentage of remaining intact cells had a negative correlation with ultrasonication time which was significant at the 0.001 level. The filtrable beta-glucuronidase had a positive correlation with time, also significant at the 0.001 level.

From Figure 8 it can be seen that there would be a projected intact cell population of 0% between 24 and 28 seconds. The experimental cells were sonicated for two minutes to leave a margin of error which may have arisen with differences in cell type and variations in the intensity of power transmitted through the microtip of the sonifier.

C. ENZYME ASSAYS

(1) Beta-Glucuronidase:

The estimation of beta-glucuronidase was based on the method of Gianetto & de Duve (1955). The principle of the method relies on the action of beta-glucuronidase in the prepared sample acting on the synthetic substrate, phenolphthalein glucuronide, at pH 5.0 which is near the optimum activity of the enzyme (Bowers, et al, 1967). Free Phenolphthalein is released from the substrate in amounts dependent on the concentration of enzyme in the sample and its typical pink colour may be developed with a glycine buffer at pH 10.7. The colour intensity relates to the amount of enzyme present in the sample and it may be measured by a spectrophotometer.

Materials Used in beta-glucuronidase Assay:

The substrate solution was Phenolphthalein glucuronic acid-sodium salt (Sigma Chemicals Co., St. Louis) dissolved in TC 199 to make a 0.0075 M stock which was stored at -20°C to minimize spontaneous hydrolysis.

Glycine buffer was as follows:

2.5730 gms. of sodium carbonate monohydrate (0.083 M)
0.9789 gms. of sodium chloride (0.067 M)
2.4961 gms. of glycine (0.133 M)

all in 250 mls. of distilled water. The pH was adjusted to 10.7 with 2N sodium hydroxide.

TABLE II

CONTENTS OF ASSAY TUBES FOR THE DETERMINATION OF BETA GLUCURONIDASE

Tube No. and Total Volume		Distilled Water	Acetate Buffer	Enzyme Sample	Substrate	Source of Sample
1.	1.0 ml	0.4 ml	0.2 ml	0.2 ml	0.2 ml	E+C' Filtrable Enzyme
2.	1.0 ml	0.3 ml	0.2 ml	0.3 ml	0.2 ml	E+C' Total Enzyme
3.	1.0 ml	0.4 ml	0.2 ml	0.2 ml	0.2 ml	C' Filtrable Enzyme
4.	1.0 ml	0.3 ml	0.2 ml	0.3 ml	0.2 ml	C' Total Enzyme
5.	1.0 ml	0.4 ml	0.2 ml	0.2 ml	0.2 ml	E+IC' Filtrable Enzyme
6.	1.0 ml	0.3 ml	0.2 ml	0.3 ml	0.2 ml	E+IC' Total Enzyme
7.	1.0 ml	0.4 ml	0.2 ml	0.2 ml	0.2 ml	IC' Filtrable Enzyme
8.	1.0 ml	0.3 ml	0.2 ml	0.3 ml	0.2 ml	IC' Total Enzyme
9.	1.0 ml	0.4 ml	0.2 ml	0.2 ml	0.2 ml	E Filtrable Enzyme
10.	1.0 ml	0.3 ml	0.2 ml	0.3 ml	0.2 ml	E Total Enzyme
11.	1.0 ml	0.4 ml	0.2 ml	0.2 ml	0.2 ml	L Filtrable Enzyme
12.	1.0 ml	0.3 ml	0.2 ml	0.3 ml	0.2 ml	L Total Enzyme
13.	1.0 ml	0.4 ml	0.2 ml	0.2 ml*	0.2 ml	Substrate Hydrolysis Control
14.	1.0 ml	0.5 ml	0.2 ml	0.1 ml**	0.2 ml	BG Content of Complement Alone
15.	1.0 ml	0.5 ml	0.2 ml	0.1 ml***	0.2 ml	BG Content of Inactive Complement Alone

N.B. 0.3 ml of enzyme sample are required in "total enzyme Assays to compensate for previous dilution by Triton X-100.

* of TC 199
** Complement
*** Inactivated Complement

Acetate buffer at pH 5.0 used in the assay mixture to maintain an optimum pH for beta-glucuronidase activity was made up from two stock solutions A and B:

A. 2.15 ml. of acetic acid in a total volume of 100 ml. distilled water.

B. 5.103 gm. sodium acetate trihydrate in a total of 100 ml. distilled water.

A combination of 14.8 ml. A and 35.2 ml. B were made up to 100 ml. which had a molarity of 0.375. The pH was adjusted to 5.0. This buffer was used in both acid phosphatase and beta-glucuronidase assay mixtures.

Beta-glucuronidase Assay:

When the "filtrable" and "total" enzyme samples had been prepared from each of the procedures in an experimental group 0.2 ml. of "filtrate" and 0.3 ml. "total" enzyme samples were added to their respective assay tubes as listed in Table III. The "total" enzyme sample had been diluted by Triton X-100 and therefore required 0.3 ml. to compensate. In addition, one tube was prepared without enzyme sample so that the effect of storage and the assay procedure on substrate hydrolysis could be assessed. The complement and inactivated complement used in the cell incubation procedures were also assayed for beta-glucuronidase content. Another fifteen tubes numbered 1a, 2a, etc. were prepared in the same way as the assay tubes with the exception that the 0.2 ml. of phenolphthalein glucuronide substrate was replaced by

0.2 ml. TC 199. Thus the optical density of the reagents in each tube could be measured without the masking effect of phenolphthalein.

The assay tubes were then incubated simultaneously with the acid phosphatase assay mixtures (see below). The lymph node cell preparations were incubated at 37°C for 3-1/2 hours and the peritoneal cells for 16 hours, since trial studies showed that these times yielded end products in concentrations which could be measured sensitively by the spectrophotometer.

After incubation the thirty tubes were made up to five ml. with the glycine buffer. The positive samples turned pink instantaneously and were stable. The optical densities were recorded in a Unicam SP 600 spectrophotometer at 540 mu wavelength using the blue light filter against glycine buffer blanks.

(2) Acid Phosphatase:

The estimation of acid phosphatase was based on the method of Allen and Gockerman (1964). The activity of acid phosphatase was measured by the estimation of alpha-naphthol liberated enzymatically from sodium alpha-naphthyl acid phosphate (Mann Research Labs., Orangeburg, N.Y.) at pH 5.0. The free α-naphthol was then reacted with the diazonium compound Fast Red ITR (Matheson, Coleman & Bell, Canadian Laboratories, Winnipeg) at pH 8.0 in a post-coupling solution. The purple end-product was measured in a spectrophotometer.

Materials Used in Acid Phosphatase (AP) Assay:

The substrate solution was a 0.05 M solution of sodium α-naphthyl phosphate in TC 199 which was stored at -20 C. The TC 199 maintained a neutral pH during storage thus minimising spontaneous hydrolysis, which is accelerated in acidic conditions.

The acetate buffer at pH 5.0 was the same as that described in the beta-glucuronidase assay.

The post-coupling solution containing Fast Red ITR was compounded from stock Michaelis buffer and working Michaelis veronal buffer. Stock Michaelis buffer contained 14.7 gm. of diethyl barbiturate and 9.7 gm. of sodium acetate in a final volume of 250 ml. distilled water. Working Michaelis comprised 2 parts stock Michaelis, 3 parts water and one part 0.2N HCl added in that order to avoid precipitation of the salts in solution. The final pH was adjusted to 8.0. Sodium lauryl sulphate (Fisher Scientific) was added to the working Michaelis veronal buffer in a concentration of 4 gms. per 100 ml. This mixture was stored in a sealed bottle at room temperature. Immediately before coupling with the acid phosphatase assay system 100 mg. of Fast Red ITR is added to 100 ml. of the post-coupling solution. This preparation of Fast Red ITR was unstable in this solution, with a gradual increase in brown colouring after 40 minutes which affected optical density unless spectrophotometer readings were made within this time.

Acid Phosphatase Assay:

The contents of the enzyme assay tubes were made up as listed in Table IV. For any single experiment the "filtrable" and "total" enzyme samples were the same preparations used for the beta-glucuronidase assay. The substrate hydrolysis control and the assay of acid phosphatase in complement and inactive complement are also performed in the same way as in the beta-glucuronidase assay. Similarly another fifteen tubes labeled 1a, 2a, etc. were made up without substrate so that the contributions of the assay mixtures to optical density could be assessed. The tubes were all shaken, sealed and incubated along with the beta-glucuronidase assay tubes for the same lengths of time. After incubation the Fast Red ITR was added to complete the post-coupling solution and all the tubes, both enzyme assay and blanks made up to 5 ml. In the positive samples the colour turned light brown at first and to purple in a few seconds. These mixtures were read immediately at 545 mu while the colour remained stable at purple, against blanks of coupling solution.

The capacity of the substrate solutions depended on the total amount of phenolphthalein or α-naphthol which could be liberated from the substrates, and thus on the substrate itself. To ensure that the capacities of the incubation mixtures were not exceeded under these experimental conditions the acid phosphatase and beta-glucuronidase substrate mixtures were incubated with 0.3 ml. of a "total"

TABLE III

CONTENTS OF ASSAY TUBES FOR THE DETERMINATION OF ACID PHOSPHATASE

TUBE No. And Total Volume	Assay Substrate Mixture				Source of Sample
	Distilled Water	Acetate Buffer	Enzyme Sample	Substrate Solution	
1. 2.0 ml	1.3 ml	0.3 ml	0.2 ml	0.2 ml	E+C' Filtrable Enzyme
2. 2.0 ml	1.2 ml	0.3 ml	0.3 ml	0.2 ml	E+C' Total Enzyme
3. 2.0 ml	1.3 ml	0.3 ml	0.2 ml	0.2 ml	C' Filtrable Enzyme
4. 2.0 ml	1.2 ml	0.3 ml	0.3 ml	0.2 ml	C' Total Enzyme
5. 2.0 ml	1.3 ml	0.3 ml	0.2 ml	0.2 ml	E+IC' Filtrable Enzyme
6. 2.0 ml	1.2 ml	0.3 ml	0.3 ml	0.2 ml	E+IC' Total Enzyme
7. 2.0 ml	1.3 ml	0.3 ml	0.2 ml	0.2 ml	IC' Filtrable Enzyme
8. 2.0 ml	1.2 ml	0.3 ml	0.3 ml	0.2 ml	IC' Total Enzyme
9. 2.0 ml	1.3 ml	0.3 ml	0.2 ml	0.2 ml	E Filtrable Enzyme
10. 2.0 ml	1.2 ml	0.3 ml	0.3 ml	0.2 ml	E Total Enzyme
11. 2.0 ml	1.3 ml	0.3 ml	0.2 ml	0.2 ml	L Filtrable Enzyme
12. 2.0 ml	1.2 ml	0.3 ml	0.3 ml	0.2 ml	L Total Enzyme
13. 2.0 ml	1.3 ml	0.3 ml	0.2 ml*	0.2 ml	Substrate Hydrolysis Control
14. 2.0 ml	1.4 ml	0.3 ml	0.1 ml**	0.2 ml	AP Content of Complement
15. 2.0 ml	1.4 ml	0.3 ml	0.1 ml***	0.2 ml	AP Content of Inactivated Complement

N.B. 0.3 ml of enzyme sample are required in "total" enzyme assays to compensate for previous dilution by Triton X-100

* of TC 199
** Complement
*** Inactivated Complement

enzyme preparation from lymph node cells for 18 hours which is about five times the length of an experimental incubation time. When the solutions were colour developed their optical densities safely exceeded any of those obtained in experimental readings. It was thus assumed that sufficient substrate was present in the reaction.

D. INCUBATION OF CELLS WITH ENDOTOXIN AND COMPLEMENT

Boivin extracted endotoxin of Escherichia coli 055:B5 (Difco Laboratories, Detroit, Michigan) was used throughout the study. Two stock suspensions were prepared in concentrations of 5 mg per 50 ml. TC 199 and 50 mg per 50 ml. TC 199 for experiments requiring final concentrations of 1.0 ug per ml. and 500.0 ug per ml. respectively. These were dispensed in disposable culture tubes and stored at -20°C until required.

The source of complement was reconstituted lyophilized guinea-pig serum (Sylvana, Winley-Morris, Montreal) which is a satisfactory substitute for fresh serum (Batchelor, 1967). The serum was reconstituted as directed and dispensed in 1 ml. amounts into disposable culture tubes, sealed with parafilm and stored at -20°C until required. Reconstituted lyophilized complement retains its haemolytic activity at this temperature for at least 48-62 days (Osler, et al, 1952; Levine, 1968). Only a total of 10 ml. was made up at one time and only tubes within a batch were used in any single experiment. The complement was inactivated by heating to 56°C in a water bath for 40 minutes, the temperature being monitored with the thermometer bulb in the complement. Heat inactivation destroys its capacity for membrane lysis (Bladen, et al, 1967).

The effect of endotoxin on complement was tested using two concentrations of endotoxin and 10% complement

on each of the three cell types giving a total of six experimental groups designated as follows:

Group I	Peritoneal Transudate Cells incubated in 10% complement and 1.0 ug/ml. endotoxin.
Group II	Lymph Node Cells incubated in 10% complement and 1.0 ug/ml. endotoxin.
Group III	Peritoneal Exudate Cells incubated in 10% complement and 1.0 ug/ml. endotoxin.
Group IV	Peritoneal Transudate Cells incubated in 10% complement and 500.0 ug/ml. endotoxin.
Group V	Lymph Node Cells incubated in 10% complement and 500.0 ug/ml. endotoxin.
Group VI	Peritoneal Exudate Cells incubated in 10% complement and 500.0 ug/ml. endotoxin.

Each of the above experimental groups was divided into six procedures to permit estimation of separate and combined effects of the reactants involved. The series of procedures performed in a single experiment were abbreviated as follows, with the endotoxin concentration and cell type in each series depending on the experimental group.

E+C'	= Leukocytes + Endotoxin + 10% Complement.
C'	= Leukocytes + 10% Complement.
E+IC'	= Leukocytes + Endotoxin + 10% Inactivated Complement.
IC'	= Leukocytes + 10% Inactivated Complement.
E	= Leukocytes + Endotoxin.
L	= Leukocytes alone.

This series was repeated six times for each experimental group, the cells for one series always being derived from the same cell pool.

When a pool of cells was prepared for an experimental group 1 ml. of the cell suspension was decanted into each of six 10 x 75 mm. disposable culture tubes. The incubation mixtures were made up with complement, endotoxin and TC 199 to a volume of 3 ml. according to the experimental group being studied (Table I). After gentle mixing, 1 ml. of each cell suspension was taken for estimation of the total enzyme activity, details of which are described on Page 66 The tubes were covered with parafilm which was pricked twice with a pin to allow gaseous exchange. The tubes were then incubated for 1 hour at 37°C as this time and temperature will permit depletion of complement by endotoxin (Bladen, et al, 1967). The excess complement and heat inactivated complement which were to be tested separately for enzyme activity were also incubated with the experimental tubes.

The experimental design is summarized in Figure 9.

TABLE I

EXPERIMENTAL PROCEDURES

Contents of Each Incubation Tube

Procedure	E+C'	C'	E+IC'	IC'	E	L
Leukocytes	1 ml	1 ml	1 ml	1 ml	1 ml	1 ml
Complement	0.3 ml	0.3 ml	-	-	-	-
Inactive Complement	-	-	0.3 ml	0.3 ml	-	-
Endotoxin	0.03 ml	-	0.03 ml	-	0.03 ml	-
TC 199	1.67 ml	1.7 ml	1.67 ml	1.7 ml	1.97 ml	2 ml
Total Volume	3 ml	3 ml	3 ml	3 ml	3 ml	3 ml

This is an example of experiments using 1.0 ug/ml. endotoxin which is taken from the 5 mg/50 ml. stock suspension. For experiments requiring 500.0 ug/ml. endotoxin, the volume of endotoxin added would be 1.5 ml. of the 50 mg/50 ml. stock suspension, and the volume of TC 199 in the appropriate tubes adjusted to 0.2 ml. The tubes containing endotoxin and leukocytes only had their proportion of TC 199 adjusted accordingly.

EXPERIMENTAL DESIGN

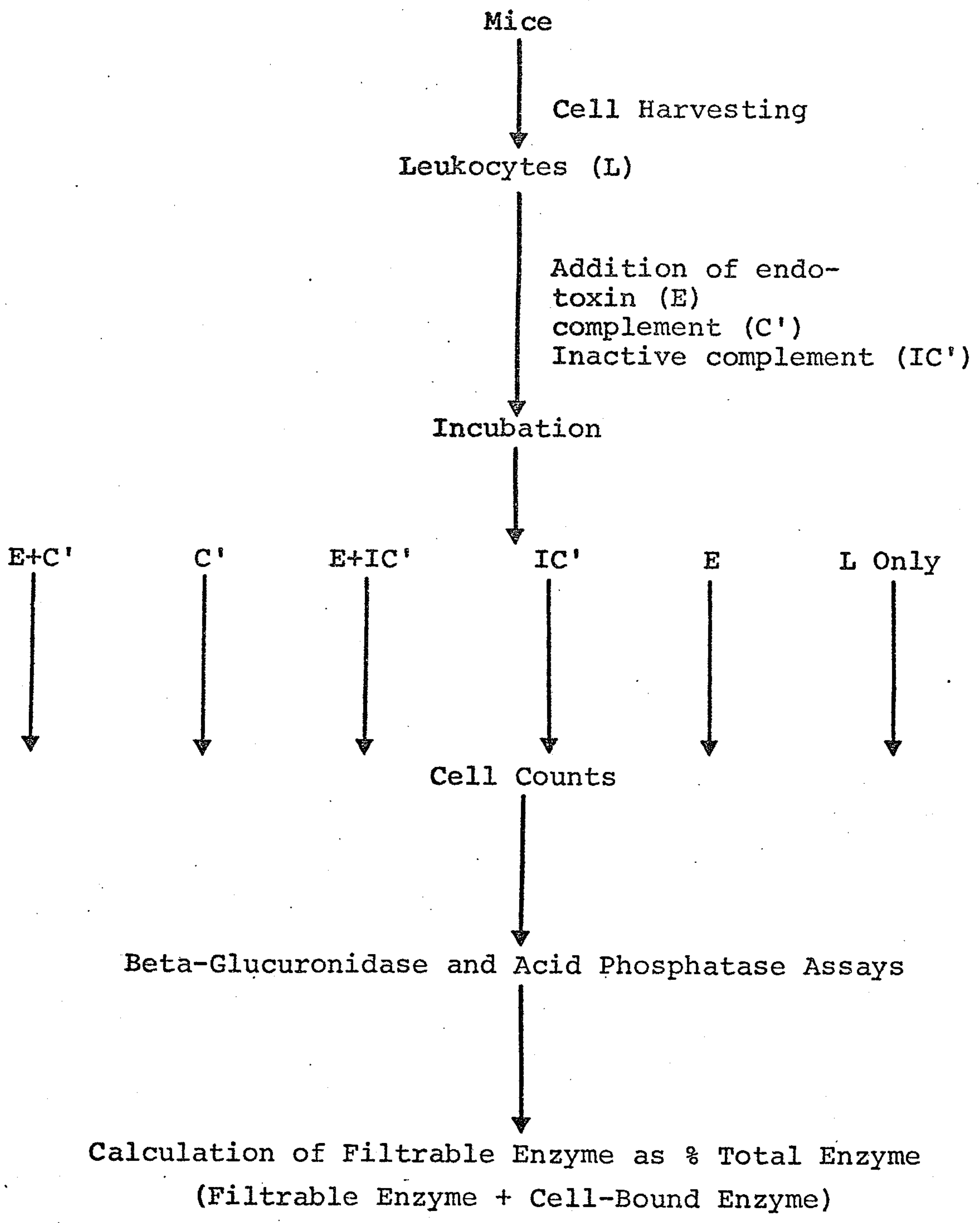


FIGURE 9

RESULTS

A. CALCULATION OF RESULTS

The corrected optical density for an incubation mixture was calculated by subtracting the contributions of reaction components which did not represent an experimental release of enzyme. The factors which contributed to optical density were:

(i) Cell filtrate + TC 199 solution + endotoxin (where applicable).

(ii) Cell filtrate + TC 199 Triton + endotoxin (where applicable).

(iii) Spontaneous hydrolysis of substrate.

(iv) Optical density of complement or inactive complement.

(v) Enzyme activity of complement. (Tube 14)

(vi) Enzyme activity of inactive complement. (Tube 15)

Numbers (i), (ii), (iii) could be measured directly from preparations made for that purpose. Enzyme activities of complement and inactive complement were estimated separately in amounts five times that in the individual assay tubes. Addition of 0.1 ml complement to the assay permitted the use of a delivery vehicle similar to that used in making up the enzyme substrate mixtures, thus minimizing the inaccuracy that would have resulted in changing to a pipette capable of delivering 0.02 ml. complement. In addition 0.2 ml. complement provided enough enzyme to permit a more sensitive spectrophotometer reading.

The corrected optical densities were calculated thus:

(1) In assays not involving C' or IC':

The spontaneous hydrolysis of substrate and the optical density (O.D.) of the mixture made up without substrate was subtracted from the O.D. of the corresponding assay, e.g. O.D. tube 9 - O.D. tube 9A

- Spontaneous hydrolysis of substrate

= Corrected optical density of tube 9. (The filtrable enzyme released by the action of endotoxin on leukocytes).

(2) In assays including C' or IC':

Enzyme activity of C' (or IC') - spontaneous hydrolysis of substrate - O.D. of C' without substrate

= Enzyme activity of 0.1 ml. C'

÷ 5 = Enzyme activity of 0.02 ml. C' (which is the volume in an assay mixture).

Enzyme activity of 0.02 ml. C' + O.D. of:

Cell filtrate

+

TC 199 (+ Triton and/or endotoxin where applicable)

+

Spontaneous hydrolysis of substrate

+

O.D. of C' without substrate

= Total O.D. of factors not representing experimental enzyme activity (error).

This error is calculated for all assays containing C' or IC' and subtracted from its corresponding original O.D.

to give the corrected optical density of the assay.

Thus, the percentage of filtrable enzyme released by an experimental procedure is:

$$= \frac{\text{Corrected O.D. filtrable enzyme}}{\text{Corrected O.D. total enzyme}} \times \frac{100}{1}$$

B. <u>The effect of the Experimental Procedures on the release of Acid Phosphabase and Beta Glucuronidase from Mouse Leukocytes</u>.

An example of the results obtained in the enzyme essays for one experimental group is shown in Tables V - XVI . These tables show the optical densities of the enzyme assay mixtures and the calculations which disclose the percentage of filtrable enzyme released in an incubation tube. The cell concentration and the cell mortality of each incubation tube are also contained in the beta-glucuronidase and acid phosphatase result tables respectively. The results for all the experimental groups were treated in an identical manner.

When the values of the filtrable enzyme expressed as a percentage of the total enzyme of each tube were obtained for each of the six replications of all the procedures in the six experimental groups they were subjected to statistical analysis. The mean values and the standard error of each procedure in each group are presented in table XVII. An alternative form of presentation is shown on table XVIII where each mean is taken from a procedure on a cell type irrespective of the concentration of endotoxin used. On its right-hand column Table XVII lists the mean percentages of enzymes released by any group of cells irrespective of the procedure. Also shown are the mean percentages of enzymes release resulting from any procedure in groups I, II and III, which employed 1.0 ug/ml endoboxin, or from groups VI, V or VI which employed 500.0 ug/ml endoboxin. With the exception of the "E + C" procedures releasing acid phosphabase it can be seen that there is no significantly different pattern of enzyme release obtained by varying the endobixin concentration. The exception which is due to

TABLES V - VXI

The Beta Glucuronidase assay results (Tables V - X) include the cell concentration in each incubation tube. The individual assay tube numbers correspond to those described in Table II . Since "E + C' (F) and "E + C' total" etc. are derived from the same incubation tube only one cell concentration value applies to each pair. Similarly the % cell mortality of these same incubation tubes is recorded in the acid phosphatase assay results (Tables XI - XVI). The assay tube numbers in Tables XI - XVI correspond to those described in Table III . The calculation of the correction factor was described on pp.

Table V

Tube No.	Procedure	Recorded O.D. Units	Correction Factor	Corrected O.D. Units	% Enzyme Release	Cell Concentration per cu. mm.
	Group V		Beta glucuronidase Assay		Replication	No. 1
1	E ± C' (F)	0.084	- 0.015	= 0.069	23.72	$1.45 \times 10^4/mm^3$.
2	E+C' total	0.311	- 0.020	= 0.291	100	
3	E+IC' F	0.096	- 0.014	= 0.082	35.0	1.49×10^4
4	E+IC' total	0.253	- 0.019	= 0.234	100	
5	C' (F)	0.092	- 0.015	= 0.077	25.2	1.38×10^4
6	C' total	0.326	- 0.020	= 0.306	100	
7	IC' F	0.091	- 0.014	= 0.077	25.4	1.22×10^4
8	IC' total	0.322	- 0.019	= 0.303	100	
9	E (F)	0.009	- 0.007	= 0.002	0.42	1.41×10^4
10	E total	0.486	- 0.011	= 0.475	100	
11	L (F)	0.006	- 0.007	—	0	1.60×10^4
12	L total	0.515	- 0.011	= 0.504	100	
13	Substrate only	0.005				
14	C' + substrate only	0.027				
15	IC' + substrate only	0.018				

Table VI

Group V Beta Glucoronidase Assay Replication No. 2

Tube No.	Procedure	Recorded O.D. Units	Correction Factor	Corrected O.D. Units	% Enzyme Release	Cell Concentration per cu. mm.
1	E + C' (F)	0.079	- 0.019	= 0.060	22.2	1.07×10^4
2	E+C' total	0.294	- 0.024	= 0.270	100	
3	E+IC' (F)	0.078	- 0.016	= 0.062	21.1	1.33×10^4
4	E+IC total	0.315	- 0.021	= 0.294	100	
5	C' (F)	0.105	- 0.019	= 0.086	32.3	1.26×10^4
6	C' total	0.290	- 0.024	= 0.266	100	
7	IC' (F)	0.084	- 0.016	= 0.068	22.6	1.34×10^4
8	IC' total	0.328	- 0.024	= 0.304	100	
9	E (F)	0.010	- 0.011	-	0	1.21×10^4
10	E total	0.347	- 0.016	= 0.331	100	
11	L (F)	0.011	- 0.011	= 0	0	1.07×10^4
12	L total	0.463	- 0.016	= 0.447	100	
13.	Substrate only	0.007				
14	C' + substrate	0.036				
15	IC' + substrate	0.020				

Table VII

Group V   Beta Glucoronidase Assay   Replication No. 3

Tube No.	Procedure	Recorded O.D. Units	Correction Factor	Corrected O.D. Units	% Enzyme Release	Cell Concentration per cu. mm.
1	E+C' (F)	0.110	- 0.025	= 0.085	23.6	1.4×10^4
2	E+C' total	0.388	- 0.030	= 0.358	100	
3	E+IC' (F)	0.104	- 0.017	= 0.087	24.9	1.32×10^4
4	E+IC' total	0.372	- 0.022	= 0.350	100	
5	C' (F)	0.110	-0.025	= 0.085	26.5	2.1×10^4
6	C' total	0.352	- 0.030	= 0.322	100	
7	IC' (F)	0.096	- 0.017	= 0.079	23.9	1.52×10^4
8	IC' total	0.352	- 0.022	= 0.330	100	
9	E (F)	0.016	- 0.013	= 0.003	0.79	1.6×10^4
10	E total	0.397	- 0.018	= 0.379	100	
11	L (F)	0.013	- 0.013	= 0	0	1.38×10^4
12	L total	0.464	- 0.018	= 0.446	100	
13	Substrate only	0.007				
14	C' + substrate	0.062				
15	IC' + substrate	0.024				

Table VIII

Group V Beta Glucoronidase Assay Replication No. 4

Tube No.	Procedure	Recorded O.D. Units	Correction Factor	Corrected O.D. Units	% Enzyme Release	Cell Concentration per cu. mm.
1	E+C' (F)	0.133	- 0.021	= 0.112	36.5	1.17×10^4
2	E+C' total	0.333	- 0.026	= 0.307	100	
3	E+IC' (F)	0.119	- 0.018	= 0.101	33.8	1.05×10^4
4	E+IC'total	0.322	- 0.023	= 0.299	100	
5	C' (F)	0.123	- 0.021	= 0.102	30	1.20×10^4
6	C' total	0.340	- 0.026	= 0.340	100	
7	IC' (F)	0.108	- 0.018	= 0.090	29.8	1.27×10^4
8	IC' total	0.325	- 0.023	= 0.302	100	
9	E (F)	0.013	- 0.014	= -	0	1.19×10^4
0	E total	0.350	- 0.021	= 0.337	100	
1	L (F)	0.012	- 0.014	= -	0	1.05×10^4
2	L total	0.486	- 0.021	= 0.465	100	
3	Substrate only	0.007				
4	C' + Substrate	0.040				
5	IC' + Substrate	0.026				

Table IX

Group V Beta Glucoronidase Assay Replication No. 5

Tube No.	Procedure	Recorded O.D. Units	Correction Factor	Corrected O.D. Units	% Enzyme Release	Cell Concentration per cu. mm.
1	E+C' (F)	0.097	- 0.020	= 0.077	31	1.97×10^4
2	E+C' total	0.274	- 0.025	= 0.249	100	
3	E+IC' (F)	0.089	- 0.020	= 0.069	27	2.17×10^4
4	E+IC' total	0.280	- 0.025	= 0.255	100	
5	C' (F)	0.096	- 0.020	= 0.076	31	2.19×10^4
6	C' total	0.270	- 0.025	= 0.245	100	
7	IC' (F)	0.099	- 0.020	= 0.079	22.8	2.03×10^4
8	IC' total	0.372	- 0.025	= 0.347	100	
9	E (F)	0.009	- 0.011	-	0	2.11×10^4
10	E total	0.333	- 0.015	= 0.318	100	
11	L (F)	0.005	- 0.011	-	0	2.27×10^4
12	L total	0.358	- 0.015	= 0.343	100	
13	Substrate only	0.007				
14	C' + Substrate	0.017				
15	IC' + Substrate	0.018				

Table $\overline{X}$

Group V Beta Glucoronidase Assay Replication No. 6

Tube No.	Procedure	Recorded O.D. Units	Correction Factor	Corrected O.D. Units	% Enzyme Release	Cell Concentration per cu. mm.
1	E+C' (F)	0.097	- 0.010	= 0.087	34.6	1.48×10^4
2	E+C'total	0.264	- 0.013	= 0.251	100	
3	E+IC' (F)	0.115	- 0.010	= 0.105	40.3	1.61×10^4
4	E+IC'total	0.273	- 0.013	= 1.260	100	
5	C' (F)	0.111	- 0.010	= 0.010	39.8	1.37×10^4
6	C' total	0.266	- 0.013	= 0.253	100	
7	IC' (F)	0.124	- 0.010	= 0.114	45.2	1.64×10^4
8	IC' total	0.265	- 0.013	= 0.252	100	
9	E (F)	0.005	- 0.005	= 0	0	1.72×10^4
10	E total	0.364	- 0.008	= 0.356	100	
11	L (F)	0.004	- 0.005	= -	0	1.55×10^4
12	L total	0.442	- 0.008	= 0.434	100	
13	Substate only	0.003				
14	C' + Substate	0.020				
15	IC' + Substate	0.017				

Table XI

Group V Acid Phosphatase Assay Replication No. 1

Tube No.	Procedure	Recorded O.D. Units	Correction Factor	Corrected O.D. Units	% Enzyme Released	Cell Mortality
1	E+C' (F)	0.163	- 0.097	= 0.066	9.5	34%
2	E+C' total	0.795	- 0.102	= 0.693	100	
3	E+IC' (F)	0.137	- 0.044	= 0.093	17.4	28.7%
4	E+IC'total	0.580	- 0.049	= 0.531	100	
5	C' (F)	0.194	- 0.097	= 0.097	14.6	37%
6	C' total	0.766	- 0.102	= 0.664	100	
7	IC' (F)	0.107	- 0.044	= 0.063	9.5	37.1%
8	IC' total	0.715	- 0.049	= 0.666	100	
9	E (F)	0.060	- 0.038	= 0.022	3.38	31%
10	E total	0.695	- 0.043	= 0.652	100	
11	L (F)	0.052	- 0.038	= 0.014	1.43	27%
12	L total	1.02	- 0.043	= 0.977	100	
13	Substrate only	0.031				
14	C' + Substrate	0.328				
15	IC' + Substrate	0.061				

Table XII

Group V Acid Phosphatase Assay Replication No. 2

Tube No.	Procedure	Recorded O.D. Units	Correction Factor	Corrected O.D. Units	% Enzyme Release	Cell Mortality
1	E+C' (F)	0.172	- 0.102	= 0.070	13.6	45.6%
2	E+C' total	0.618	- 0.106	= 0.512	100	
3	E+IC' (F)	0.106	- 0.036	= 0.070	12.1	21.4%
4	E+IC' total	0.618	- 0.040	= 0.578	100	
5	C' (F)	0.171	- 0.102	= 0.069	14.1	30.2%
6	C' total	0.672	- 0.106	= 0.566	100	
7	IC' (F)	0.108	- 0.036	= 0.072	11.0	30.8%
8	IC' total	0.698	- 0.040	= 0.658	100	
9	E (F)	0.047	- 0.035	= 0.012	1.7	27.2%
10	E total	0.745	- 0.039	= 0.706	100	
11	L (F)	0.059	- 0.035	= 0.024	2.92	22.9%
12	L total	0.860	- 0.039	= 0.821	100	
13	Substrate only	0.028				
14	C' + Substrate	0.364				
15	IC' + Substrate	0.032				

Table XIII

Group V Acid Phosphatase Assay Replication No. 3

Tube No.	Procedure	Recorded O.D. Units	Correction Factor	Corrected O.D. Units	% Enzyme Release	Cell Mortality
1	E+C' (F)	0.214	- 0.121	= 0.093	9.95	18%
2	E+C'total	1.060	- 0.125	= 0.935	100	
3	E+IC' (F)	0.143	- 0.047	= 0.096	8.0	24%
4	E+IC'total	1.250	- 0.051	= 1.199	100	
5	C' (F)	0.313	- 0.121	= 0.192	17.2	25%
6	C' total	1.250	- 0.125	= 1.125	100	
7	IC' (F)	0.148	- 0.047	= 0.101	11.1	27%
8	IC' total	0.960	- 0.051	= 0.909	100	
9	E (F)	0.062	- 0.039	= 0.023	2.1	22%
10	E total	1.140	- 0.043	= 1.097	100	
11	L (F)	0.066	- 0.039	= 0.027	2.52	26%
12	L total	1.260	- 0.043	= 1.217	100	
13	Substrate only	0.032				
14	C' + Substrate	0.440				
15	IC' + Substrate	0.070				

Table XIV

Group V Acid Phosphatase Assay Replication No. 4

Tube No.	Procedure	Recorded O.D. Units	Correction Factor	Corrected O.D. Units	% Enzyme Release	Cell Mortality
1	E+C' (F)	0.284	- 0.110	= 0.174	18.7	30.8%
2	E+C' total	1.044	- 0.115	= 0.929	100	
3	E+IC' (F)	0.138	- 0.032	= 0.106	13.5	20.0%
4	E+IC'total	0.821	- 0.037	= 0.784	100	
5	C' (F)	0.236	- 0.110	= 0.126	20.5	30.4%
6	C' total	0.724	- 0.115	= 0.609	100	
7	IC' (F)	0.099	- 0.032	= 0.067	11.6	22.6%
8	IC' total	0.616	- 0.037	= 0.570	100	
9	E (F)	0.054	- 0.035	= 0.019	2.9	27.4%
10	E total	0.695	- 0.040	= 0.655	100	
11	L (F)	0.057	- 0.035	= 0.022	2.6	16.1%
12	L total	0.890	- 0.040	= 0.850	100	
13	Substrate only	0.028				
14	C' + Substrate	0.402				
15	IC' + Substrate	0.062				

Table XV

Group V Acid Phosphatase Assay Replication No. 5

Tube No.	Procedure	Recorded O.D. Units	Correction Factor	Corrected O.D. Units	% Enzyme Release	Cell Mortality
1	E+C' (F)	0.162	- 0.060	= 0.102	17.6	28%
2	E+C' total	0.645	- 0.065	= 0.580	100	
3	E+IC' (F)	0.114	- 0.035	= 0.079	13.4	14.8%
4	E+IC'total	0.625	- 0.040	= 0.585	100	
5	C' (F)	0.139	- 0.060	= 0.079	17.8	24.0%
6	C' total	0.508	- 0.065	= 0.443	100	
7	IC' (F)	0.105	- 0.035	= 0.070	11.6	24.8%
8	IC' total	0.645	- 0.040	= 0.605	100	
9	E (F)	0.058	- 0.035	= 0.023	3.75	18.3%
10	E total	0.655	- 0.040	= 0.615	100	
11	L (F)	0.049	- 0.035	= 0.014	2.15	41.4%
12	L total	0.690	- 0.040	= 0.650	100	
13	Substrate only	0.021				
14	C' + Substrate	0.180				
15	IC' + Substrate	0.055				

Table XVI

Group V Acid Phosphatase Assay Replication No. 6

Tube No.	Procedure	Recorded O.D. Units	Correction Factor	Corrected O.D. Units	% Enzyme Release	Cell Mortality
1	E+C' (F)	0.129	- 0.061	= 0.068	12.5	31.4%
2	E+C' total	0.608	- 0.065	= 0.543	100	
3	E+IC' (F)	0.140	- 0.038	= 0.102	21.2	22.0%
4	E+IC' total	0.523	- 0.043	= 0.480	100	
5	C' (F)	0.155	- 0.061	= 0.094	16.0	28.5%
6	C' total	0.652	- 0.065	= 0.586	100	
7	IC' (F)	0.141	- 0.038	= 0.103	29.8	18.3%
8	IC' total	0.388	- 0.043	= 0.345	100	
9	E (F)	0.047	- 0.034	= 0.013	2.29	25.6%
10	E total	0.606	- 0.039	= 0.567	100	
11	L (F)	0.049	- 0.034	= 0.015	2.28	43.4%
12	L total	0.695	- 0.039	= 0.656	100	
13	Substrate only	0.027				
14	C' + Substrate	0.164				
15	IC' + Substrate	0.048				

the P.M.N. group will be discussed later.

A consideration of the mean enzyme release by each group, regardless of procedure, shows that all the cell types, with the exception of P.M.N.'s in group VI, release a similar mean percentage of their acid phosphatase. Lymph node cells release a higher percentage of beta glucuronidase than do other cells, and are the only cells which release a greater percentage of their beta glucuronidase than their acid phosphatase.

The effects of the individual procedures on each of the experimental groups can be seen in Table XVII or Figures 10-15. In general, guinea-pig serum released more enzyme than heat-inactivated serum, which in turn had a greater effect than endotoxin alone or the control cells only. The addition of endotoxin to the incubation mixtures containing G.P.S. or inactivated G.P.S. made little difference to the pattern of enzyme release. The reason for the variations in the mean percentage acid phosphatase released in the "E + C" procedures and group VI (mentioned above) can be traced to a higher release of acid phosphatase by 500.0 ug/ml endotoxin in the "E + C" procedure of group VI (figure 15) compared with 1.0 ug/ml endotoxin in the same procedure and cell type (figure 12). Thus P.M.N.'s are the only cells whose lysosomes are susceptible to interaction between endotoxin and the heat-labile components of guinea-pig serum.

Also of interest is the different dependence of each cell type on the heat labile components of G.P.S. for the release of their lysosomal enzymes. Peritoneal Transudate cells do not release as high a percentage of their AP and BG when subjected to the "E + IC" and "IC" procedures compared with the "E + C" and "C" procedures (figures 10 and 13). Lymph node cells however release the same percentage of their enzymes in the

KEY TO TABLE XVII

Mean enzyme release from Groups 1-6

Standard Errors: 1 = ± 1.631

2 = ± 1.457

3 = + 1.371

4 = ± 1.342

5 = ± 0.942

6 = ± 0.841

7 = ± 0.791

8 = ± 0.775

This table contains the percentages of acid phosphatase and beta-glucuronidase released by each procedure and by each cell group. Each value is the mean calculated from the six experimental replications.

TABLE XVII

PROCEDURE

Experimental Group	E+C'	C'	E+IC'	IC'	E	B	Mean Enzyme Release
P.T.C. 1.0 ug Endotoxin	19.883[1] 13.000[2]	20.800[1] 14.583[2]	13.417[1] 11.350[2]	13.183[1] 9.217[2]	3.383[1] 1.617[2]	3.300[1] 1.717[2]	12.328[3] 8.589[4]
L.N.C. 1.0 ug Endotoxin	14.250[1] 26.850[2]	17.983[1] 31.667[2]	12.633[1] 24.483[2]	12.400[1] 23.250[2]	4.467[1] 2.200[2]	4.150[1] 2.133[2]	11.892[3] 18.431[4]
P.M.N. 1.0 ug Endotoxin	25.667[1] 13.317[2]	21.400[1] 10.533[2]	6.267[1] 3.967[2]	6.267[1] 4.483[2]	5.333[1] 1.083[2]	6.417[1] 1.033[2]	10.981[3] 5.736[4]
Mean Enzyme Release	19.935[5] 17.722[6]	20.061[5] 18.928[6]	10.772[5] 13.267[6]	10.617[5] 12.317[6]	4.394[5] 1.633[6]	4.622[5] 1.644[6]	11.733[7] 10.919[8]
P.T.C. 500 ug Endotoxin	21.633[1] 14.583[2]	18.117[1] 13.500[2]	8.167[1] 9.817[2]	6.400[1] 9.267[2]	5.867[1] 2.183[2]	6.067[1] 2.183[2]	11.042[3] 8.589[4]
L.N.C. 500 ug Endotoxin	13.650[1] 28.600[2]	16.700[1] 30.800[2]	14.267[1] 30.350[2]	14.100[1] 28.250[2]	2.700[1] 0.200[2]	2.317[1] 0.000[2]	10.622[3] 19.700[4]
P.M.N. 500 ug Endotoxin	34.233[1] 12.483[2]	28.567[1] 12.683[2]	12.917[1] 6.800[2]	7.100[1] 7.133[2]	7.450[1] 2.033[2]	7.550[1] 2.217[2]	16.303[3] 7.225[4]
Mean Enzyme Release	23.172[5] 18.556[6]	21.128[5] 18.994[6]	11.783[5] 15.656[6]	9.200[5] 14.883[6]	5.339[5] 1.472[6]	5.311[5] 1.467[6]	12.656[7] 11.838[8]

Top figure in each square represents percentage AP release.
Bottom figure in each square represents percentage BG release.

TABLE XVIII

This table contains the percentages of each enzyme released by a cell type irrespective of the concentration of endotoxin used. Each value is the mean calculated from the six experimental replications.

TABLE XVIII

PROCEDURE

Experimental Group	E+C'	C'	E+IC'	IC'	E	B	Mean Enzyme Release
P.T.C.	20.758[1] 13.792[2]	19.458[1] 14.042[2]	10.792[1] 10.583[2]	9.792[1] 9.242[2]	4.625[1] 1.900[2]	4.683[1] 1.975[2]	11.685[3] 8.599[4]
L.N.C.	13.950[1] 27.725[2]	17.342[1] 31.233[2]	13.450[1] 27.417[2]	13.250[1] 25.750[2]	3.583[1] 1.200[2]	3.233[1] 1.067[2]	10.801[3] 19.065[4]
P.M.N.	29.950[1] 12.900[2]	24.983[1] 11.608[2]	9.592[1] 5.383[2]	6.683[1] 5.808[2]	6.392[1] 1.558[2]	6.983[1] 1.625[2]	14.097[3] 6.481[4]
MEAN ENZYME RELEASE	21.553[5] 18.139[6]	20.594[5] 18.961[6]	11.278[5] 14.461[6]	9.908[5] 13.600[6]	4.867[5] 1.553[6]	4.967[5] 1.556[6]	12.194[7] 0.548[8]

Top figure in each square represents percentage AP release.
Bottom figure in each square represents percentage BG release.

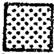
* Key to Table II (Appendix):

1 = ± 1.154	2 = ± 1.031	3 = ± 0.969	4 = ± 0.949
5 = ± 0.666	6 = ± 0.595	7 = ± 0.560	8 = ± 0.548

Mean Enzyme Release from Each Cell Type

KEY TO FIGURES 10-15

Acid Phosphatase

 Beta Glucuronidase

E+C' = Endotoxin + 10% Complement + Leucocytes

C' = 10% Complement + Leucocytes

E+IC' = Endotoxin + 10% Inactive Complement + Leucocytes

IC' = 10% Inactive Complement + Leucocytes

E = Endotoxin + Leucocytes

L = Leucocytes only

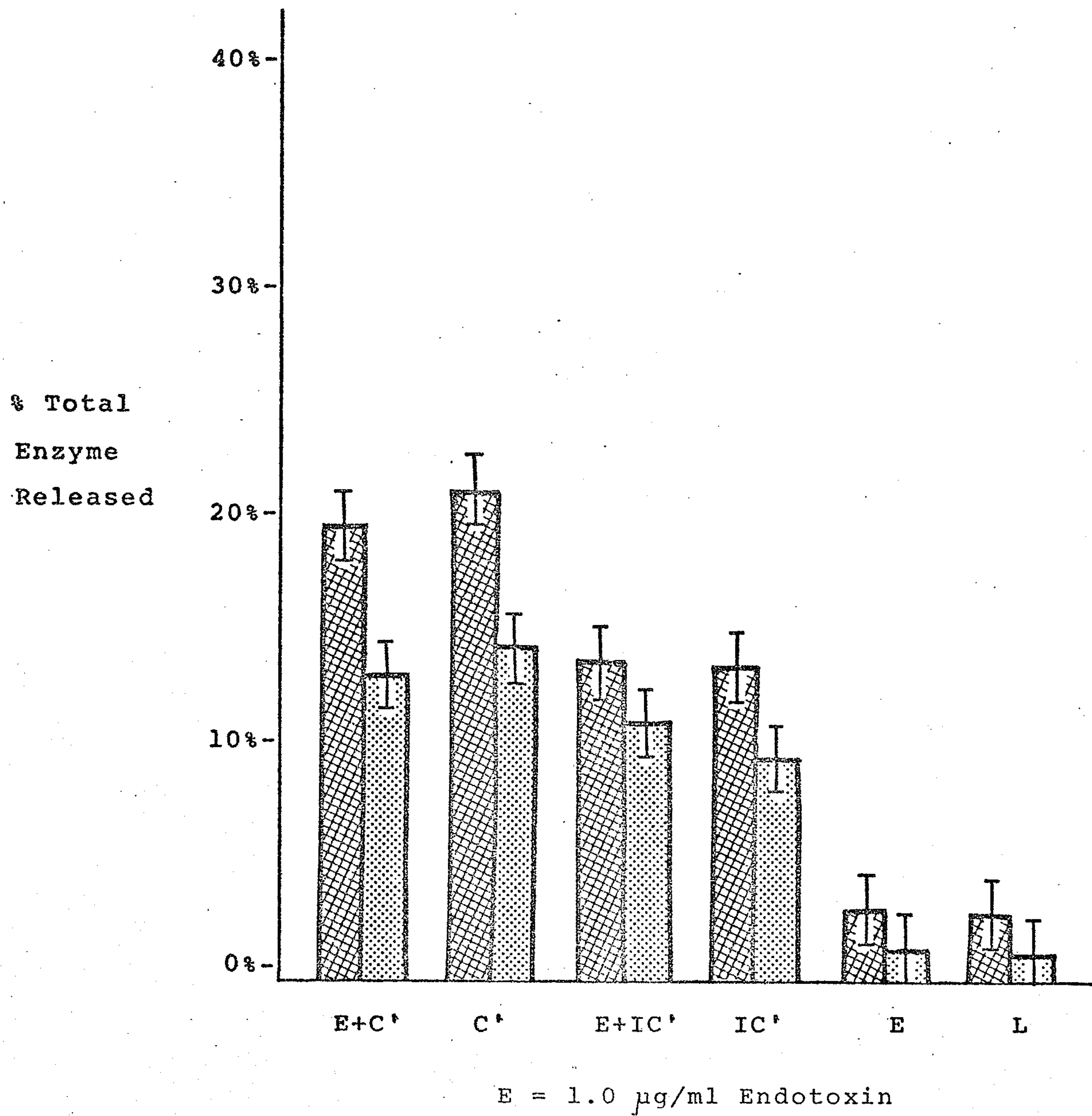
I = Standard Error

FIGURE 10

Peritoneal transudate cells incubated with guinea-pig serum released quantities of acid phosphatase and beta-glucuronidase which were similar to those released by endotoxin and serum. Heat treatment of the serum only partially inhibited the release of acid phosphatase and beta-glucuronidase.

GROUP I

PERITONEAL TRANSUDATE CELLS (P.T.C.'s)



E = 1.0 μg/ml Endotoxin

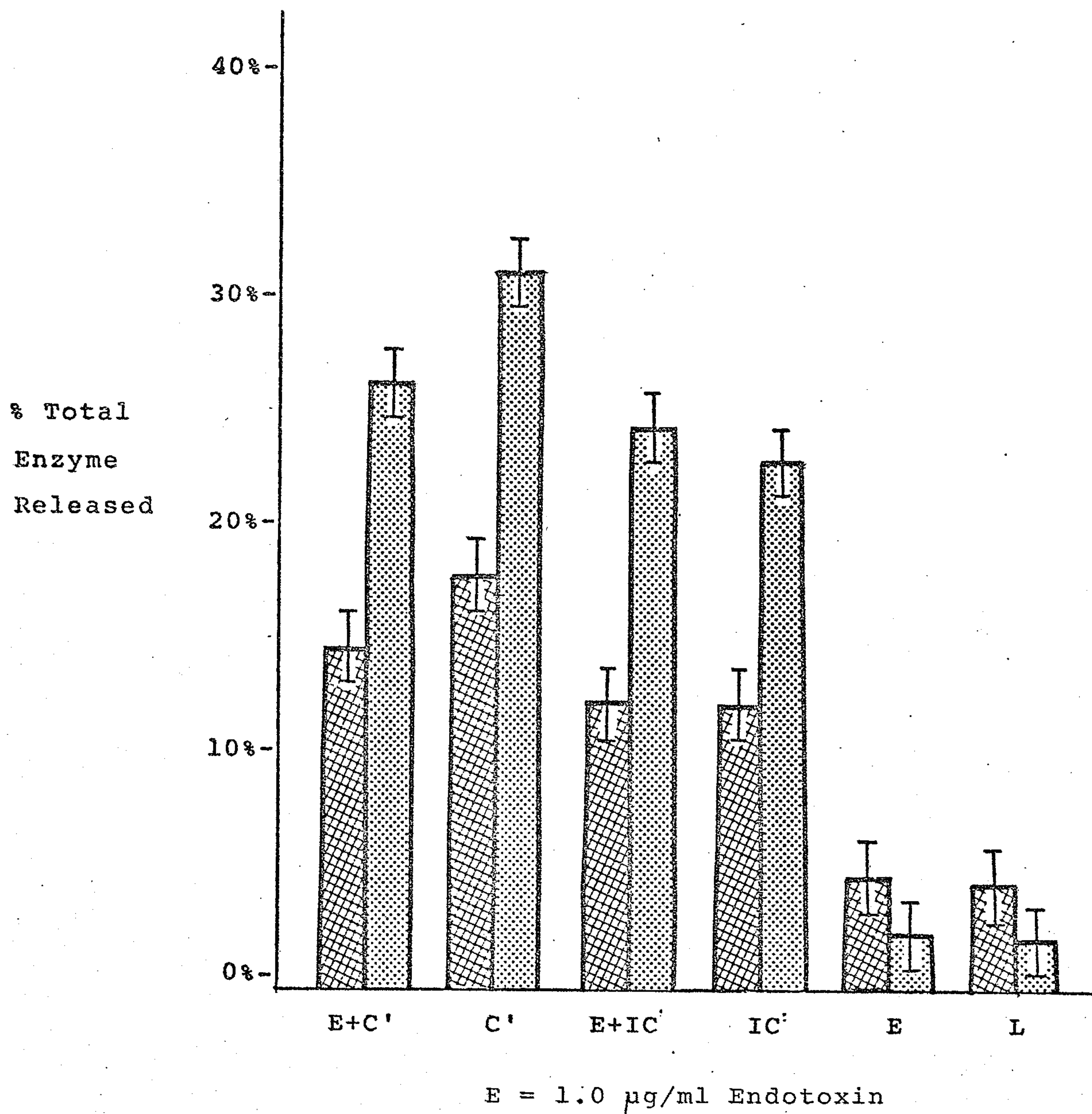
FIGURE 10

FIGURE 11

The effect of each of the six experimental procedures on lymph node cells. Endotoxin did not affect the release of enzymes from cells incubated with guinea-pig serum. Heat treatment of the serum did not inhibit release of acid phosphatase.

GROUP II

LYMPH NODE CELLS (L.N.C.'s)



E = 1.0 μg/ml Endotoxin

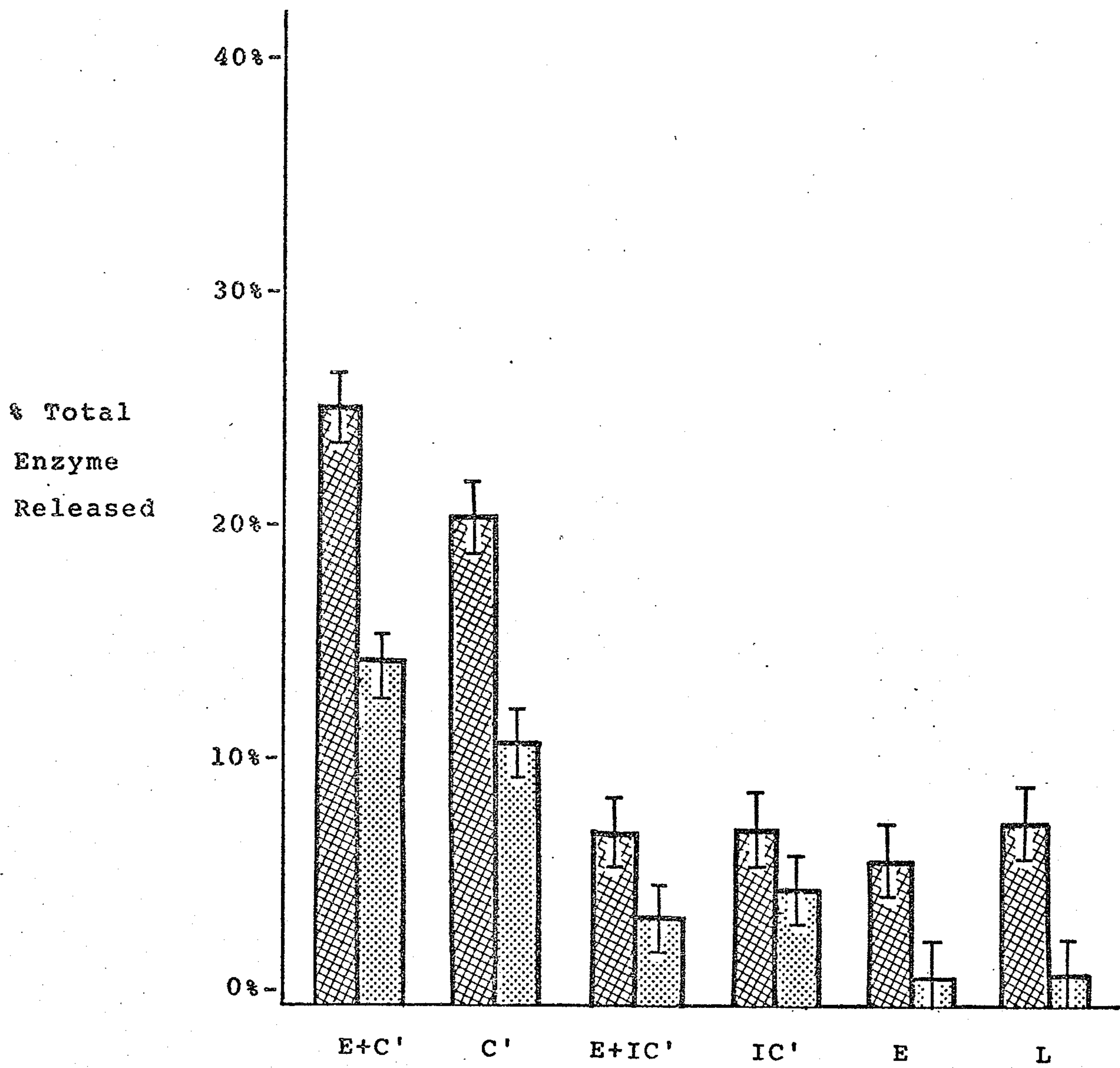
FIGURE 11

FIGURE 12

Peritoneal Exudate Cells incubated with endotoxin and guinea-pig serum released slightly more acid phosphatase than cells incubated with guinea-pig serum alone. Heat treatment of the serum reduced the release of both enzymes to control levels.

GROUP III

PERITONEAL EXUDATE CELLS (P.M.N.'s)



E = 1.0 µg/ml Endotoxin

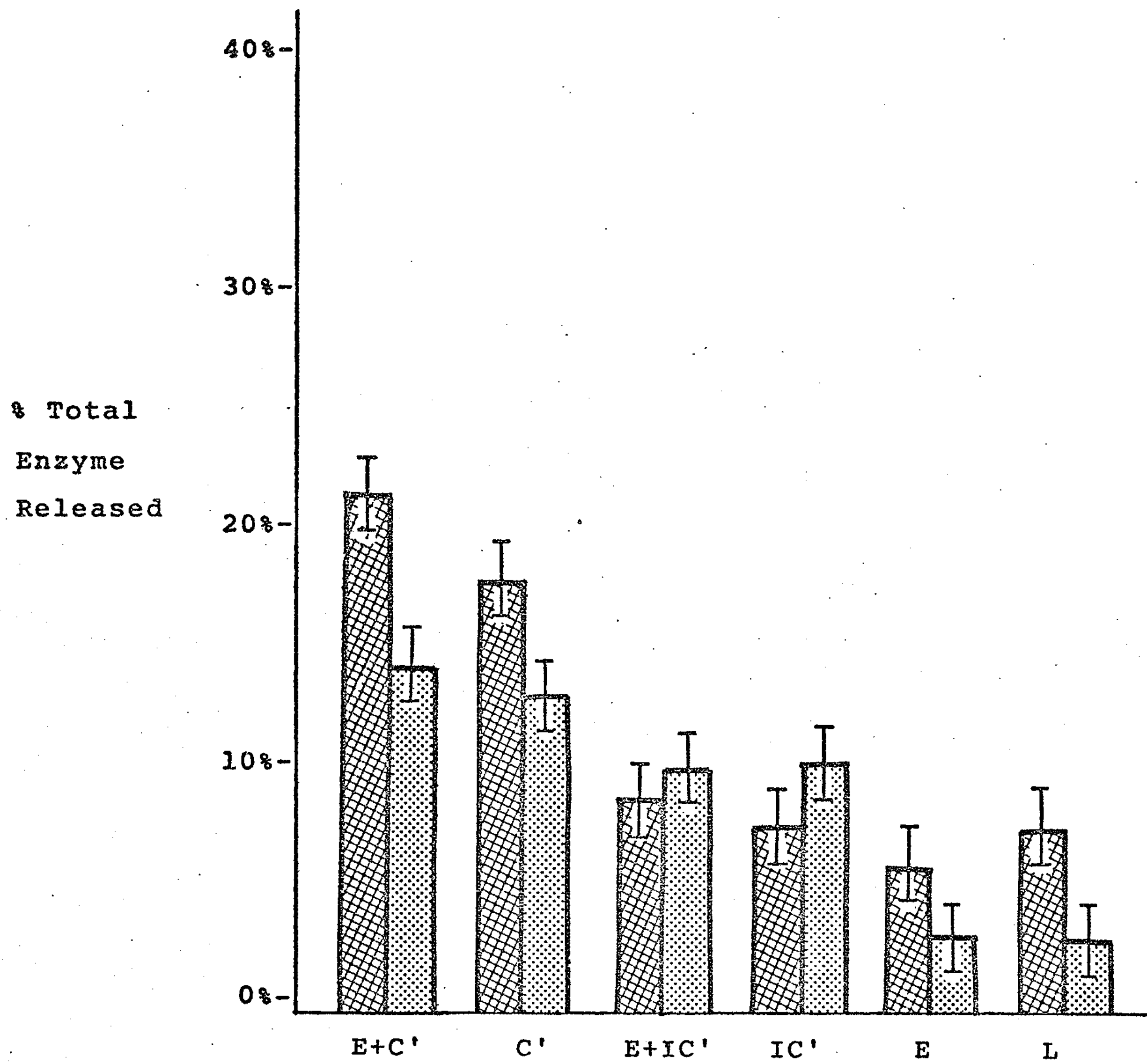
FIGURE 12

FIGURE 13

The release of acid phosphatase from peritoneal transudate cells was not altered by changing the concentration of endotoxin to 500.0 ug/ml.

GROUP IV

PERITONEAL TRANSUDATE CELLS (P.T.C.'s)



E = 500.0 μg/ml Endotoxin

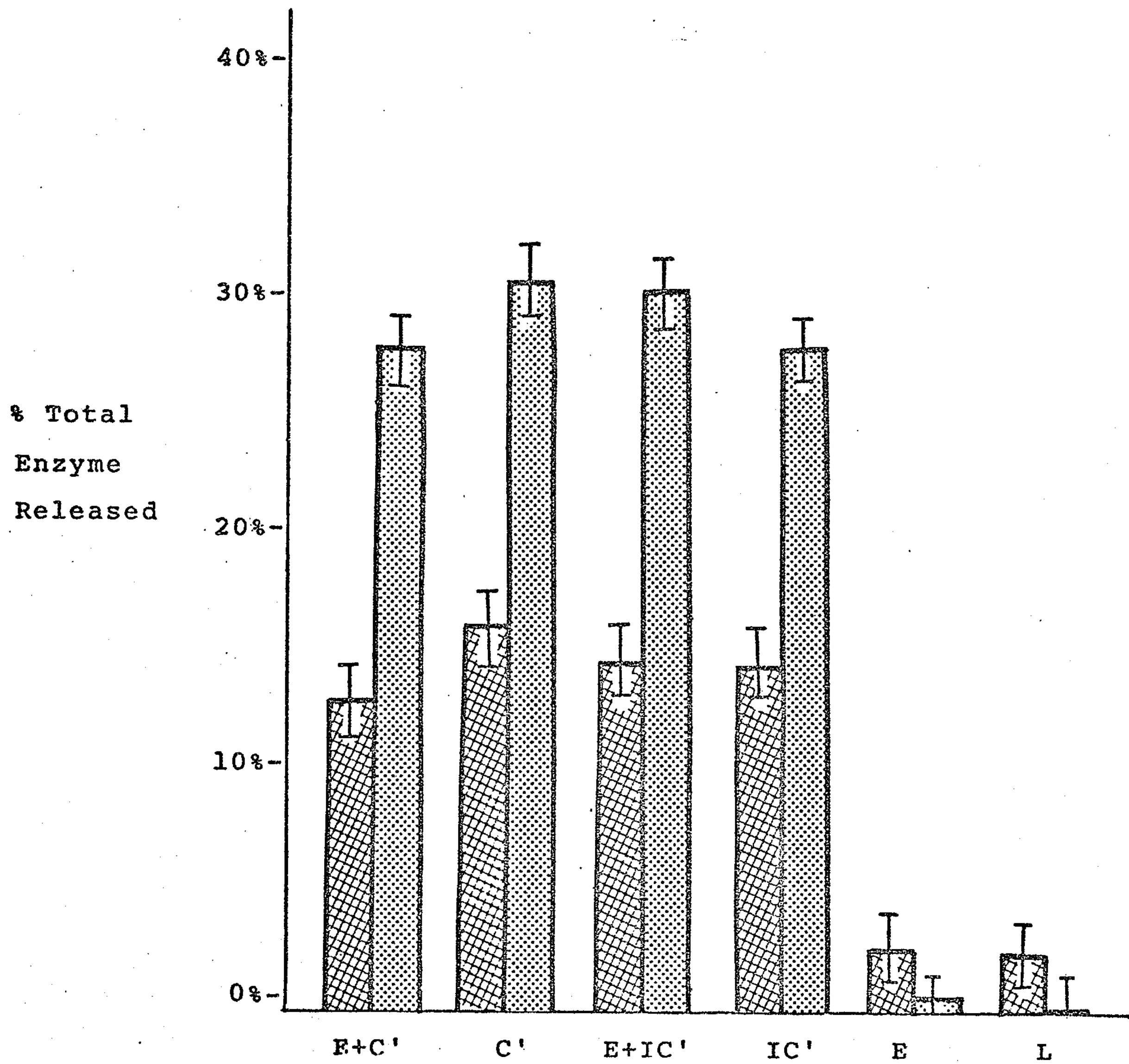
FIGURE 13

FIGURE 14

Lymph node cells released markedly greater proportions of their total beta-glucuronidase compared with the acid phosphatase release. Heat treatment of the serum did not inhibit enzyme release.

GROUP V

LYMPH NODE CELLS (L.N.C.'s)



E = 500.0 μg/ml Endotoxin

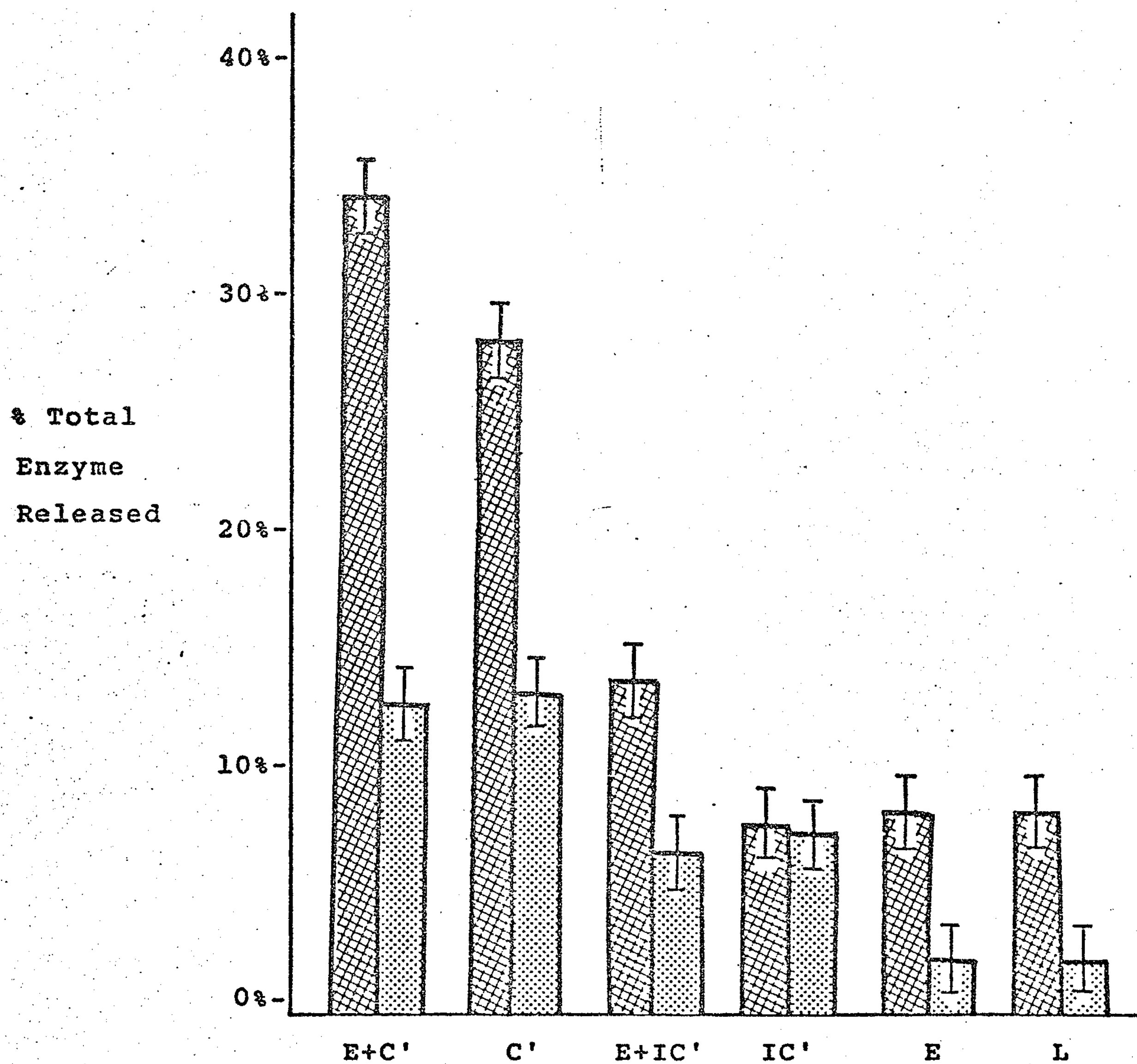
FIGURE 14

FIGURE 15

Peritoneal exudate cells released more acid phosphatase when they were incubated with guinea-pig serum and 500.0 ug/ml endotoxin than they did when incubated with serum alone. Heat treatment of the serum reduced the release of the enzymes to near the control levels.

GROUP VI

PERITONEAL EXUDATE CELLS (P.M.N.'s)



E = 500.0 μg/ml Endotoxin

FIGURE 15

"E + C", "C'", "E + IC'" and "IC'" procedures (figures 11 and 14). This lack of dependence on the heat labile components of G.P.S. is almost completely reversed by P.M.N.'s which, when treated with inactivated serum, do not release percentages of enzymes in significantly greater proportions than those procedures not incorporating G.P.S. at all (figures 12 and 15).

C. Analysis of Cell Mortality

The differences in mortality between experimental procedures and control procedures were subjected to an analysis of variance.

The mean mortalities for P.T.C. were 16.02% for the experimental procedures and 14.2% for the controls. For L.N.C. experiments the mortalities were 27.1% and 26.68% and for PMN experiments the mortalities were 4.80% and 4.48% for experimental and control mortalities respectively. The mortalities for each cell type were significantly different ($P < 0.005$), however there was no significant difference between experimental and control procedures in any cell type.

D. Correlations Between the Filtrable Enzymes, Cell Concentration and Mortality:

The correlation studies were performed to examine the relationships which existed between AP and BG under the different experimental conditions and to determine whether the other variables of cell mortality or cell concentration bore a relationship to the percentage of either enzyme which was released into the medium.

(1) The correlation of AP with BG was examined since a strong positive relationship was necessary to support the view that the release of each enzyme represented the same physical event, i.e. lysosome rupture. See Table IXX

The P.T.C.'s released percentages of AP and BG which correlated positively at the 0.01 level of significance. The enzymes released from L.N.C.'s and P.M.N.'s correlated positively ($P < 0.001$) and this significance was maintained when the correlation analysis was applied to the combined cell types. These findings supported the view that these enzymes were released in close mutual association, and are therefore a reasonable indication of lysosomal rupture.

(2) The relationship between the percentages of AP and BG released and the cell concentration in each of the incubation tubes was examined to consider the possibility of cell concentration affecting the release of the lysosomal enzymes. See Table IXX

P.T.C. concentration did not relate with the percentages of AP or BG released. The amount of filtrable AP, however, correlated positively at the 0.01 level with the L.N.C.

concentration in group II, but there was no corresponding relationship with BG in the same group. Group VI P.M.N.'s released AP and BG both of which correlated negatively with the cell concentration at the 0.02 and 0.05 levels of significance respectively. This reflected the negative correlations found only in the E+C' and E+IC' procedures within this group (see below).

The enzymes released by the experimental groups were examined for correlations with cell concentrations in the procedures containing C' or IC'. See table XX A negative correlation between cell concentration and enzyme release would suggest that there is a limiting factor in the reaction and that this limit would most likely be imposed by inadequate complementation of the system. In group IV the cell concentrations in the E+C' and E+IC' procedures correlated negatively with BG at the 0.02 and 0.05 levels respectively, but there was no correlation between cell concentration and the corresponding AP values. However both AP and BG released from the group VI E+C' procedure correlated negatively with cell concentration at the 0.05 and 0.01 levels of significance respectively. In the same group the IC' procedure released AP which correlated negatively with the cell concentration ($P < 0.02$), but there was no corresponding relationship with BG.

Since the E+C' procedure was the only group which released both AP and BG in percentages which correlated

TABLE IXX

Correlation matrices showing the correlation coefficients between the percentages of acid phosphatase (AP) and beta-glucuronidase (BG) released and the corresponding cell concentrations in each incubation mixture. Each matrix represents the relationship existing within one cell type.

Each cell contains the correlation coefficient between the parameters shown at the top of the vertical column and to the left of the horizontal column.

PERITONEAL TRANSUDATE CELLS

	AP	BG	CELLS
AP	1.00		
BG	0.49***	1.00	
Cells	0.07	-0.10	1.00

LYMPH NODE CELLS

	AP	BG	CELLS
AP	1.00		
BG	0.86****	1.00	
Cells	0.41***	0.19	1.00

POLYMORPHONUCLEAR NEUTROPHILS

	AP	BG	CELLS
AP	1.00		
BG	0.75****	1.00	
Cells	-0.40***	-0.37***	1.00

TABLE XX

CORRELATION COEFFICIENTS BETWEEN CELL CONCENTRATIONS AND ENZYME RELEASED FROM PROCEDURES INVOLVING COMPLEMENT OR HEAT INACTIVATED COMPLEMENT

Experimental Group	Enzyme	C'	E+C'	IC'	E+IC'
I. P.T.C.	AP	0.21	0.14	-0.29	0.12
	BG	0.17	-0.03	-0.47	0.8
II. L.N.C.	AP	0.92***	0.76*	0.32	0.40
	BG	0.10	-0.01	0.18	0.24
III. P.M.N.	AP	0.11	-0.33	0.54	0.86*
	BG	0.15	-0.62	0.44	0.85*
IV. P.T.C.	AP	0.13	0.23	-0.51	-0.07
	BG	-0.58	-0.84**	-0.06	-0.80*
V. L.N.C.	AP	-0.31	-0.11	-0.21	0.70
	BG	-0.65	-0.01	-0.43	0.24
VI. P.M.N.	AP	-0.34	-0.72*	-0.82**	-0.46
	BG	-0.51	-0.89***	-0.37	-0.02

Key to Figures IXX, XX and XXI

* = Significant at the 0.05 Level.

** = Significant at the 0.02 level.

*** = Significant at the 0.01 level.

**** = Significant at the 0.001 level.

TABLE XXI

Correlation matrices showing the correlation coefficients between percentages of released AP and BG, cell concentration and cell mortality (Mort.). The matrix is interpreted in the same way as before.

PERITONEAL TRANSUDATE CELLS

	AP	BG	CELLS	MORT.
AP	1.00			
BG	0.55***	1.00		
CELLS	0.01	-0.11	1.00	
MORT.	0.48*	0.49*	-0.01	1.00

LYMPH NODE CELLS

	AP	BG	CELLS	MORT.
AP	1.00			
BG	0.90****	1.00		
CELLS	0.140	0.09	1.00	
MORT.	0.08	0.02	0.19	1.00

POLYMORPHONUCLEAR LEUKOCYTES

	AP	BG	CELLS	MORT.
AP	1.00			
BG	0.70****	1.00		
CELLS	-0.41**	-0.37*	1.00	
MORT.	0.14	-0.07	-0.20	1.00

negatively with cell concentration, it was concluded that an active constituent in this incubation mixture may not have been present in sufficient quantity to allow optimal enzyme release. The most likely limiting factor of this nature would have been complement.

(4) Since rupture of lysosomal enzymes is often associated with cell injury or death the relationship between the released enzymes and mortality was examined.

Both the AP and BG released from P.T.C. correlated positively with mortality at the 0.05 level of significance. In L.N.C.'s however, neither enzyme correlated with mortality and in the P.M.N. groups only AP correlated with mortality at the 0.01 level of significance. It was concluded that after one hour incubation P.T.C.'s released enzymes which were associated with cell mortality and therefore may have been the most sensitive to the action of lysosomal enzymes.

(5) The effect of cell concentration on mortality was determined in the event that excessive cell numbers in an incubation mixture may have made increased demands beyond the capacity of the supporting medium, with resultant cell death. However when each cell type was examined there were no correlations between cell concentrations and mortality. See table XX It was concluded that the medium could always maintain the number of cells incubated in it.

Summary of Results:

1. Endotoxin in 500.0 mg/ml concentrations affected the AP released from P.M.N.'s by guinea-pig serum. With the exception of P.M.N.'s endotoxin had neither a synergistic nor inhibitory effect on the release of lysosomal enzymes by guinea pig serum from the cells.

2. P.M.N.'s were totally dependent on the heat labile components of guineq pig serum for the release of its lysosomal emzymes and heat inactivation also inhibited most of the release of AP and BG from P.T.C.'s. Lymph node cells, however, were reliant on guinea pig serum to release the enzymes but the effect was independent of the heat labile components of the guinea pig serum.

3. The interaction of 500.0 ug/ml endotoxin with complement produced a negative correlation of P.M.N. concentration with the percentages of both AP and BG released. This was the only combination or cell type that resulted in this negative correlation.

4. The release of AP correlated with BG throughout the study at the 0.001 level of significance.

5. L.N.C.'s were more sensitive to experimental manipulation than P.T.C.'s which in turn were more sensitive than P.M.N.'s. There was no difference in mortality between control (L) procedures and experimental procedures in any of the cells studied.

6. P.T.C.'s were the only cell type whose percentage mortality correlated positively with the release of both enzymes studied.

7. The cell concentration in incubation mixtures did not relate to the mortality of the cells after incubation.

PART IV

DISCUSSION

Mice provided accessible pools of leukocytes from a large population sample. The inbred strain limited the possibility of immunological interaction between cells from different animals which were included in the same cell pool.

The source of complement required careful consideration. The serum from the mice used in cell harvesting would not have posed problems of cell toxicity or contain heterophile antibodies, but the haemolytic activity of mouse serum is very low - 7.5 $C'H_{50}$ per ml compared with 200 $C'H_{50}$ per ml in guinea-pig serum (Gewurz, et al, 1968). Guinea-pig serum has a well-defined complement system and was available in convenient quantities which could be easily stored at -20°C. Reconstituted lyophilized complement retains its haemolytic activity at this temperature for at least 48-62 days, and longer when stored at -70°C, (Osler, et al, 1952; Levine, 1967). Guinea-pig complement and human complement have similar reactions to immune complexes (Ward, et al, 1966) and the interactions of guinea-pig complement and endotoxin have been extensively studied (Bladen, et al, 1966; Gewurz, et al, 1968A; Mergenhagen, et al, 1968; Gewurz, et al, 1968B; Snyderman, et al, 1968; Lichtenstein, et al, 1969).

Although a cytotoxic effect of guinea-pig serum in mouse cells could not be ruled out initially, it was known that the majority of mouse tissues, with the exception of thymic lymphocytes and lymphoma cells, are unaffected by guinea-pig serum (Batchelor, 1967). The finding that the

mortality of all cell types was the same in incubation mixtures containing complement as in control incubation mixtures would support the view that guinea-pig serum is not cytotoxic for the cells under investigation.

Heterophile antibodies against xenogeneic tissue or tissue fluid antigens occur in a wide range of species (Humphrey & White, 1970) although only a few of these systems have been investigated in detail, the Forssman antigen being the best known example. The guinea pig serum used in this investigation could lyse mouse red blood cells. The red cell lysis was inhibited by absorbing the guinea-pig serum overnight with mouse leukocytes at 6°C or by heating the serum to 57°C for thirty minutes thus providing further evidence that an antigen antibody reaction occurs between G.P.S. and mouse cells. As guinea-pig serum is used as a source of complement in this system then the heterophile antibodies reacting with mouse cell-bound antigens must be considered as part of the reaction. Other species have been shown to contain natural antibodies against mouse leukocytes, e.g. calf serum (Cohn & Parks, 1968) which could induce hydrolytic enzyme and lysosome formation; and human serum which could facilitate stimulation of mouse lymphocytes by phytohaemagglutinin (Adler, et al, 1970).

There is considerable evidence that antibodies are produced by cells at the site of chronic inflammation (Askonas and Humphrey, 1958), in periodontal disease (Brandtzaeg and Kraus, 1965; Platt, et al, 1970) and in experimental periodontal disease (Rizzo and Mitchell, 1966;

Cowley, 1969; Ranney, 1970; Berglund, et al, 1970). Conversely granulomata have been produced by antigen-antibody complexes (Spector and Heesom, 1969). Spector and Willoughby (1963) suggested that antibody on a lymphocyte surface may attach to a local antigen at an inflammatory site. The action of low grade antigen-antibody reactions at a cell surface when xenogeneic serum is added to the incubation mixtures in these experiments may be similar to events in certain in vivo conditions.

When antigen-antibody reactions involving the complement fixation are occurring in an in vitro system, consideration must be given to the availability of the reactants. If the heterophile antibodies of the guinea-pig serum were insufficient to react with all the available antigenic sites on the cell membranes or if the system were inadequately complemented, then decreasing concentration of G.P.S. would be represented experimentally by a decreased percentage release of AP and BG. Assuming there is a finite number of antigenic determinants on a cell surface, or a point of maximal lysosome activation there must be a point beyond which an increase in G.P.S. concentration would not cause increased percentage release of AP and BG. Dilutions of 1:5, 1:10 and 1:15 G.P.S. were tested on P.T.C.'s for differences of release of AP and BG. In all dilutions the percentages of enzymes released were similar, so a 1:10 dilution of G.P.S. was chosen for the experimental system.

The availability of G.P.S. was also checked by testing relationships between cell concentrations and the percentage of each enzyme released. In cells incubated with G.P.S. alone there were no negative correlations between cell concentrations and enzyme released with the exception of AP and cell correlation ($p < 0.1$) in PMN's. Since this relationship did not pertain for both enzymes it could not be considered that the effect was representing lysosome rupture. Otherwise the incubation mixtures were considered to contain adequate quantities of G.P.S.

The two concentrations of endotoxin were chosen to disclose any effects which may have been dose dependant. Many different effects in vivo can be seen following administration of varying doses. An injection of 1.0 ug Veillonella endotoxin intramucosally in rabbits is sufficient to prepare the site for a Schwartzman reaction and to cause a rise in temperature of 1.8°F, (Rizzo & Mergenhagen, 1964). Concentrations of 4.0 ug/ml up to at least 300.0 ug/ml have been shown to consume guinea-pig complement at 37 C. Maximal complement fixation occurred at concentrations of 100.0 ug/ml. although concentrations higher than 100.0 ug/ml did not inhibit the complement fixing activity of endotoxin, (Bladen, et al, 1967). It has also been shown that 500.0 ug/ml endotoxin will fix complement in maximal amounts (Gewurz, et al, 1968). Thus it was considered that 1.0 ug/ml endotoxin would be suitable to show any effects due to low

endotoxin concentrations while 500.0 ug/ml endotoxin would cause maximal complement fixation if the experimental conditions were otherwise conducive to this reaction.

Although all the reactants were dispensed in the incubation tubes within a few seconds of each other, the complement was always added last so that the endotoxin and cells would have access to it simultaneously.

After incubation the enzyme activity of the filtrate of the cell suspension was to be expressed as a percentage of the enzyme activity of the whole cell suspension. This method of expressing the effects of endotoxin and complement on cells would have the advantage of not directly comparing enzyme units released by different procedures. Direct comparisons of enzyme activity expressed in enzyme units could have been a source of inaccuracy since the different components in each incubation tube may have affected enzyme activity to different degrees. However the accuracy of the total enzyme assay had to be consistent to allow comparisons between procedures. Triton X-100, a non-ionic detergent has been used to solubilize membrane-bound acid phosphatase in disrupted rabbit alveolar macrophages with greater effect than French press treatment, homogenization or freeze-thaw treatment, and the enzymes so released were considered to represent 100% activity (Axline, 1968). Studies demonstrating the effect of Triton X-100 on bound acid hydrolases showed that concentrations of Triton X-100 greater than 0.1% produced maximal release of acid phosphatase, beta glucuronidase

and other acid hydrolases, suggesting that the 5% concentration used by Axline was unnecessary. The detergent had no inhibitory or denaturing effect on the enzymes (Wattiaux and de Duve, 1956; Allen and Gockerman, 1964). The experiments of Wattiaux and de Duve and Axline were performed with disrupted cells and so the ultrasonication was used in this study to rupture the cells and thus render the lysosomes accessible to the action of Triton X-100. The effect of ultrasonification on whole cell integrity is discussed in the materials and methods section.

The percentage of enzyme released from the cells into the filtrable phase required consideration before the effects of the experimental procedures could be assessed. Since filtration excluded whole lysosomes the enzyme activity which was measured represented what was in solution or what was bound to filter permeable membrane fragments. Evidence has accumulated which suggests that many hydrolases are membrane bound. Axline (1968) demonstrated 40% of total acid phosphatase activity was bound to the lysosomal membrane, β-glucosidase is membrane bound (Beck and Tappel, 1968) and β-N-Acetylglucosaminidase is almost completely membrane bound (Conchie & Hay, 1963; Weissman, et al, 1967), while similar affinity has also been demonstrated for α-mannosidase and β-galactosidase (Conchie & Hay, 1963). Thus it is obvious that the percentage of filtrable lysosomal enzyme does not represent the percentage of ruptured lysosomes. The different membrane affinities of lysosomal enzymes would explain why different relative quantities of each enzyme

entered the filtrable phase.

When the cells were sonicated to free the lysosomes, the enzymes which were released into the medium were contained in the volume of the suspending medium plus the volume of the cell cytoplasm. Thus the filtered total enzyme was in a slightly greater dilution than the enzyme in the filtrate of the non-sonicated cells. The change in dilution is estimated by centrifuging equal volumes of sonicated and non-sonicated cells at 1000 r.p.m. for 30 minutes; both volumes being derived from the same cell suspension. This procedure was repeated for each cell type. The packed debris of the sonicated cells was compared with the packed cell volume of the non-sonicated cells. The difference in volume between the two sediments was too small to measure precisely or reproducibly and so it was concluded that accuracy of the calculations was not improved by considering this error.

The enzyme assay system for acid phosphatase has been discussed by Allen and Gockerman (1964). The rate of reaction is linear over a wide range of enzyme activity and the rate of substrate hydrolysis is not dependant on the concentration of sodium alpha naphthyl phosphate in the assay mixture. Thus the calculation of acid phosphatase released from the cells during incubation as a percentage of the total enzyme activity from the same cell suspension would appear to be valid. Similarly the effect of phenolphthalein

glucuronide on the assay of beta glucuronidase activity has been demonstrated to be critical up to 1.5×10^{-3} M but not above that concentration (Bowers, et al, 1967). For this reason the phenolpthalein glucuronide substrate in this study was in a final concentration of 1.5×10^{-3} M and not at 1.25×10^{-3} M as recommended by Gianetto and de Duve (1955).

Acid phosphatase and beta-glucuronidase have been strongly associated with lysosomes (de Duve, 1967; Strauss, 1967) and have been in widespread use as markers for lysosomal enzyme activity. There are several reasons why a cell pool would not release identical percentages of each enzyme after any single procedure: 1) The inherent differences in the assay techniques for each enzyme. 2) The differing membrane affinities of each enzyme would determine how much is released into the filtrable phase. 3) There may be several populations of lysosomes at least in spleen and lymph node cells, each with its characteristic mechanical stability and complement of enzymes (Bowers and de Duve, 1967). However, the positive correlation in this study of AP with BG ($P < 0.001$) in L.N.G. and P.M.N. groups would suggest that any of the experimental procedures used would affect the release of these enzymes in a similar manner, which in turn is an indication of lysosomal damage. That the positive correlation between AP and BG was reduced to the 0.01 level of significance in the P.T.C. groups may imply that one of the above sources of variation in the enzyme

analysis was taking effect. Nevertheless the strong overall correlation between AP and BG strengthened the assumption that patterns of release of all the lysosomal contents would parallel those found for AP and BG.

The percentage of AP on BG assayed was the amount of free enzyme in a mixture which was filtrable through a Millipore filter after incubation. It is impossible to maintain whether this filtrate represented an extracellular release of enzymes only or if some cell rupture occurred under the pressure of filtration with subsequent inclusion of cytoplasm in the filtrate. Since the AP and BG released from the control mixtures were always low it would seem reasonable to assume that filtration did not damage the lysosomes excessively.

Lysosomal rupture within leukocytes has been closely associated with cell injury or death (Hirsch, et al, 1963; Zucker-Franklin, 1965; Quie & Hirsch, 1964). The relationships between filtrable enzymes released and cell permeability to Trypan blue were examined. Although the relationships measured were those which existed after one hour of incubation and may have changed with time, it is of interest to note that no strong correlation between the filtrable enzymes and L.N.C. and P.M.N. mortality was found, which suggests that these cells can release lysosomal enzymes without sustaining immediate injury. Peritoneal Transudate Cells were the only cells which released both AP and BG in amounts that correlated with Trypan blue permeability ($P < 0.05$), thus raising the possibility that P.T.C.'s are more sensitive to lysosomal

rupture than L.N.C.'s or P.M.N.'s.

The effects of endotoxin alone on the experimental system were assessed by the release of filtrable AP and BG from the isolated cells. Endotoxin did not stimulate enzyme release from any of the cells examined, which is in accord with the similar findings that rabbit P.M.N.'s incubated with 200.0 ug/ml endotoxin alone for three hours did not exhibit any observable lysosomal change (Hirsch & Cohn, 1960) and that acid phosphatase was not significantly redistributed in mouse peritoneal leukocytes when incubated in vitro with endotoxin (Weiner, et al, 1965).

As expected from an antibody-antigen reaction at a cell surface in a complement sufficient medium, the unheated G.P.S. induced the release of AP and BG from all the cell types examined. When guinea-pig serum is heated to 56°C the most sensitive components of the complement system are C'8, C'1, and C'2 all of which have a half-life of under two minutes while C'5, C'6, C'4, C'3 and C'9 have half-lives of 8, 11, 15, 29 and 40 minutes respectively (Nelson, 1965). The effect of the heat labile components of guinea-pig serum on the release of lysosomal enzymes could be assessed by considering the quantities of AP and BG which were released by each cell type by heated and unheated serum.

The release of AP and BG from PMN's was almost totally inhibited by the heat inactivation of the complement.

This supports the theory that the effect on the lysosomes was due to a complement-fixing antigen-antibody reaction, and that the discharge of lysosomal enzymes is mediated by the complement system. It is also very likely that the reaction took place on the cell membrane, which is the site of most xenogeneic reactions (Cohn & Parks, 1968; Adler, et al, 1970). Most of the mouse cells could probably carry similar antigens which would cross react with guinea-pig serum (Humphrey & White, 1970).

Peritoneal transudate cells release slightly greater quantities of AP and BG than the control mixtures when incubated with heater G.P.S. The failure of heat treatment of the serum to inhibit the release of enzymes to the extent that was observed in P.M.N.'s may suggest that P.T.C.'s release small amounts of lysosomal enzymes without the activation of complement after an immune reaction on the membrane. Macrophages have been shown to produce complement components, e.g. guinea-pig alveolar transudate cells (Olitzki & Gershon, 1966) primate peritoneal transudate cells (Stecher, et al, 1967) mouse peritoneal transudate cells (Thorbecke, et al, 1965) and rat in mice chimeras (Phillips & Thorbecke, 1966). It is possible that the P.T.C.'s in this study replaced some of the depleted complement components thus explaining the incomplete heat inhibition of the G.P.S. activity. Evidence that mouse complement components can interact with guinea-pig C' has been presented by Winn (1965). However a more likely explanation is the

presence of other cell types, perhaps lymphocytes, in the incubation mixture contributing to the enzyme content of the assay tube. It is interesting to note that the lysosomes of macrophages are not normally discharged into the environment, but remain in vacuoles for the life of the cell with digestion of the lysosomal contents and degradation of the enzymes (Cohn & Fedorko, 1969). In contrast P.M.N.'s can discharge their granules after phagocytosis (Zucker-Franklin and Hirsch, 1964) and the acid hydrolases are redistributed into the non-sedimentable fraction of transformed lymphocytes (Hirschhorn, et al, 1968). This may signify that the P.M.N.'s and lymphocytes are biologically adapted to survive rupture of their lysosomes since there was no correlation of the percentage of AP and BG released with cell mortality in these groups, but there was a positive correlation between both AP and BG released and P.T.C. mortality ($P < 0.05$). This effect may be of little practical importance, however, because there was not a significant difference in mortality between the P.T.C.'s incubated alone and in the experimental procedures. Cohn and Parks (1968) showed that increased lysosome formation was stimulated by natural antibody against mouse macrophages in bovine serum but immune cytolysis occurred when complement was added. This illustrates that natural antibodies can cause cell death and that the reaction in this system was probably less intense but qualitatively similar than that using bovine serum.

Heat-treated G.P.S. released AP and BG from lymph node cells in the same quantities as unheated G.P.S. This was the only cell type which released AP and BG independently of the heat labile components of guinea-pig serum. There are several possible explanations for this finding:

(1) The release of AP and BG from L.N.C.'s was the result of a different mechanism than that which operated between G.P.S. and peritoneal transudate or exudate cells. In view of the probable antigenic similarity of all the cells from one strain of animals it seems unlikely that L.N.C.'s would not be subject to cell-bound immune reactions in much the same way as the other cells.

(2) Lymphocytes may be sensitive to immune reactions on their cell membranes and sustain maximal release of AP and BG without added complement. This would contradict the observation that lymphocytes are more susceptible to immune cytolysis in the presence of complement than in a complement-free system (Ling, 1968).

(3) Lymphocytes may carry complement components which react similarly to those depleted by heat-treatment of the guinea-pig serum. Guinea-pig lymphocytes carry C'4 (Littleton, et al, 1970) and C'5 (Sayed, 1971) but evidence for the presence of complement components in mouse lymphocytes is lacking as yet. The fact that addition of complement increases immune cytolysis of lymphocytes may also signify that lymphocyte-borne complement would not act to release lysosomal enzymes.

(4) Lysosomal activation may be a functional rather than pathological consequence of immune reactions associated with the cell membrane, when it occurs in conditions favouring development of cell-mediated immunity. In support of this concept is the observation that lysosomal

activation of lymphocytes has been associated with premitotic activity (Allison and Malucci, 1964) and with mouse lymphocyte transformation (Hirschhorn and Hirschhorn, 1965; R. Hirschhorn, et al, 1967; R. Hirschhorn, et al, 1968; and Adler, et al, 1970).

The system of Adler, et al, (1970) is interesting because of its similarity to the one described here. They observed that a natural anti-mouse agglutinin in human sera in the culture medium affected both the background stimulation of mouse lymphocytes and the degree of stimulation by phytohaemagglutinin. Other workers have also demonstrated mouse lymphocyte transformation in the presence of xenogeneic serum (Granger and Williams, 1968; Ramseier, 1969). There is evidence suggesting that in vitro blast formation is the counterpart of cell-mediated immunity (delayed type hypersensitivity) in vivo (Ruddle and Waksman, 1968B; Humphreys and White, 1970). Although the lysosomal enzymes released in this study were measured after a one hour incubation period and lymphocyte transformation is usually measured after twenty-four to thirty-six hours, it is possible that the mouse lymphocytes treated with guinea-pig serum may have transformed during a longer incubation period.

Another interesting parallel between the system of Adler, et al (1970) and this study was the presence of phytohaemagglutin in the former and endotoxin in the latter. Phytohaemagglutinin is widely recognized for its ability to initiate lymphocyte transformation. Adler, et al (1970) demonstrated that the anti-mouse lymphocyte globulin in

human serum reacted with a receptor site on the cell wall to prepare the cell for stimulation by phytohaemagglutinin. Endotoxins are also capable of lymphocyte stimulation (Ling, 1968; Shivers and Cruse, 1971) which raises the possibility of whether they can mimic the effects of phytohaemagglutinin either independently or in combination with immune reactions on the cell wall *in vivo*. Evidence implicating endotoxins in delayed type hypersensitivity reactions in periodontal disease has been produced by Ivanyi and Lehner (1970). They showed that lymphocyte stimulation occurred in cells taken from patients with gingivitis or mild periodontitis when the cells were exposed to sonicates of gram negative bacteria associated with periodontal disease. Patients without periodontal disease exhibited a much lower lymphocyte stimulation index than the experimental group and the sonicates of gram negative bacteria associated with dental caries - but not periodontitis - and indiginous to the alimentary tract, both failed to stimulate lymphocytes above the control levels. A similar experiment showed that the sonicates of gram negative bacteria associated with ANUG stimulated the lymphocytes of patients suffering from this condition or who had a history of recurrent episodes of ANUG. Gram negative bacteria not associated with the condition failed to stimulate lymphocytes of the experimental groups and none of the bacterial sonicates stimulated lymphocytes of patients without a history of ANUG (Wilton, et al, 1971).

Sensitized lymphocytes are also susceptible to stimulation when incubated with the sensitizing antigens (Ruddle and Waksman, 1968A; Carpenter, et al, 1962) which in Carpenter's studies were gram-negative bacteria. In view of this evidence it is arguable that either the direct stimulation of lymphocytes by endotoxin, or stimulation by recognition of an antigen at a lymphocyte surface are both mechanisms which might plausibly be suspected of complicity in the pathogenesis of periodontal disease. This study has shown that endotoxin and an immune reaction will act on lymphocytes without a cytotoxic effect within the time limits of the experiment, which supports the theory that the cells may remain viable and therefore retain their potential to undergo blast formation. What may also be of importance is that an immune reaction will release AP and BG, and therefore probably all the lysosomal contents from lymphocytes into the environment apparently without the action of the heat labile components of guinea-pig serum. These hydrolytic enzymes could have a direct effect on the intercellular material of the gingivae.

Lymph node cells differ from the other cells in that they release a greater percentage of their total BG than do either of the other cell types and, in contrast to the other cells, they release more BG than AP. Although it would be difficult to investigate the possibility, the preferential release of BG by lymphocytes may at least in part explain why the levels of this enzyme in the gingival crevice fluid correlate with the severity of periodontal disease (Bang,

et al, 1970) while acid phosphatase levels do not (Sueda, et al, 1967). The finding that lysosomal enzymes are present in plasma cells in gingivitis of dogs (Cowley, 1969) supports the theory that lymphoid cells may represent a significant source of hydrolytic enzymes in periodontal disease. Plasma cells probably produce antibody to antigens in the gingival crevice (Mergenhagen, 1970) so it would seem possible that ingress of antigens could react with this antibody near the cell membrane with subsequent release of lysosomal enzymes.

The effects of endotoxin on the action of complement were investigated because any interaction between these agents may have provided a clue to the different action of endotoxin in peritoneal cells in vivo and in vitro. Two possible effects of endotoxin on complement were predicted. Firstly, if sufficient concentrations of endotoxin were added to this system the complement may be consumed by the endotoxin, as described by Gewurz, et al (1969), with subsequent diminished release of AP and BG. Alternatively, the finding that endotoxin can potentiate the fixation of C' by antigen-antibody complexes (Bladen, et al, 1967; Gewurz, et al, 1968A; Gewurz, et al, 1968C) may have resulted in potentiated activation of the complement system and consequently increased the number of complement components available to release lysosomal enzymes. However the results indicated that the addition of endotoxin to G.P.S. neither increased nor decreased the percentage of AP or BG released

from P.T.C.'s or L.N.C.'s. This is compatible with the finding that endotoxin cannot reduce the amount of complement available for lysis of sheep red cells unless it is pre-incubated with the complement (Bladen, et al, 1967). The failure of endotoxin to affect the action of G.P.S. on P.T.C. could be explained if a small amount of complement were required to stimulate maximal release of lysosomal enzymes from certain cell types, and that any depletion of complement by endotoxin is not sufficiently rapid or complete to prevent maximal enzyme release. Since L.N.C.'s were not dependent on the heat labile serum components the failure of endotoxin to affect lysosomal enzyme release is not surprising.

Polymorphonuclear leukocytes exhibited two slight variations of the pattern established by the other cells: (1) They released more AP in "E+C'" procedures than in "C'" procedures. (2) When 500.0 ug/ml endotoxin was present with G.P.S. a negative correlation between cell concentration and AP or BG existed ($P < 0.05$ and 0.01 respectively). This correlation would imply that if more complement or fewer cells were present in the system a greater percentage of the total AP and BG would have been released into the filtrable phase. This correlation did not exist when P.M.N.'s were incubated with G.P.S. alone or heat treated G.P.S. plus endotoxin so it would seem that the addition of endotoxin to the system made the relative concentrations of P.M.N.'s and G.P.S. a critical factor in enzyme release from

polymorphonuclear leukocytes. This is compatible with the finding that P.M.N.'s were the only cells completely dependent on the heat-labile guinea-pig serum components and therefore supports the hypothesis that endotoxin interacts with complement to affect the release of lysosomal enzymes. This raises the interesting question of whether endotoxin could interact with the complement system to effect the release of lysosomal enzymes from P.M.N.'s without the superimposition of a cell-bound immune reaction.

This experiment demonstrates that lysosomal enzymes can be released, probably by immunological mechanisms, from cell types whose morphological counterparts occur in inflamed human gingivae, and which can be induced in an infiltrate by administration of endotoxin. No evidence was found to support the hypothesis that endotoxin was capable of potentiating the release of lysosomal enzymes by guinea-pig serum from lymph node cells or peritoneal transudate cells. Five hundred micrograms per ml endotoxin increased the release of AP from polymorphonuclear neutrophils, but beta glucuronidase was unaffected by the same procedure.

Lymph node cells released AP and BG independently of the heat labile components of guinea-pig serum but the reaction was not sufficiently severe to increase trypan blue permeability after a one hour incubation period. The possible reasons for the lack of complement dependence were discussed. In contradistinction P.M.N.'s exhibited a total dependence on complement for the release of AP and BG, while P.T.C.'s

were partially dependent on the heat labile components of G.P.S. to achieve maximal release of AP and BG. The possible significance of the different patterns of enzyme release between these cells was discussed.

In view of the potential for inflammation and tissue damage that lysosomal enzymes possess, this study indicates that it would be relevant to define more precisely the role played not only by P.M.N.'s but by the chronic inflammatory cells within the tissue. The access of antigens to the gingival tissue may result in release of lysosomal enzymes from the cell types used in this study. It would seem justified to try to establish the intensity of immune reactions in the gingival milieu. The results of this study and the discussion related thereto indicate that it would be valuable to know whether immune reactions occured outside the soft tissue in the gingival crevice or within the gingival tissues. If the role played by each cell type in the immune reactions of periodontal disease were determined the host response to dental plaque could be more accurately assessed for its potential to effect tissue loss.

SUMMARY AND CONCLUSIONS

Summary and Conclusions:

This study was based on the hypothesis that bacterial endotoxins may interact with cell-associated immune reactions which fix complement with subsequent alteration of the release of lysosomal enzymes from the involved cells. A histological assessment of the inflammatory response to endotoxin injections in mouse mucosa was performed. An in vitro experimental system was then described to measure the release of lysosomal enzymes from the cell types which were represented in the inflammatory infiltrate.

This histological study investigated the qualitative and quantitative inflammatory response of the buccal mucosa of mouse after administration of single and multiple injections of endotoxin. The salient conclusions were:

(1) Multiple injections of 1.0 ug endotoxin elicited tissue reactions which did not exhibit haemorrhagic necrosis nor abscess formation, but were distinct from the control injection sites.

(2) The cell types in the response to multiple injections of 1.0 ug endotoxin were polymorphonuclear neutrophils which reached maximum density sooner than in the single injections, and plasma cells, lymphocytes and macrophages which were more persistent than in single injections. These cell types are also evident in the human periodontal lesion.

(3) Isolation of the cells in the tissue by enzymatic means was unsuitable for in vitro studies of lysosomal enzymes.

The in vitro study investigated the effects of guinea-pig serum, heat inactivated guinea-pig serum and endotoxin on the lysosomal enzymes of isolated mouse leukocytes. The following conclusions were drawn:

(1) Guinea-pig serum released acid phosphatase and beta-glucuronidase from polymorphonuclear neutrophils, lymph node cells and peritoneal transudate cells of mice, probably by immunological means.

(2) Endotoxin increased the percentage of acid phosphatase released from polymorphonuclear neutrophils by guinea-pig serum.

(3) Five hundred micrograms per ml endotoxin could make the relative concentrations of guinea-pig serum and polymorphonuclear leukocytes a critical factor in determining what percentages of the total AP and BG were released from these cells. Since P.M.N.'s were the only cells which were totally dependent on the heat-labile components of guinea-pig serum (below) this finding combined with (2) above supported the hypothesis that endotoxin can interact with complement to affect the release of lysosomal enzymes.

(4) Polymorphonuclear leukocytes were totally dependent on the heat labile components of guinea-pig serum for the release of its lysosomal enzymes. Peritoneal transudate cells released reduced amounts of AP and BG when incubated with heat-inactivated guinea-pig serum compared with unheated serum but greater amounts than control groups. Lymphocytes released enzymes when incubated with guinea-pig serum, but were not dependent on its heat labile components.

(5) Lymph node cells were more sensitive to experimental manipulation than peritoneal transudate cells, which were in turn more sensitive than polymorphonuclear leukocytes. There was no statistically significant

difference in mortality between control and experimental procedures in any of the cells studied.

(6) Peritoneal transudate cells, polymorphonuclear leukocytes and lymph node cells released percentages of their total acid phosphatase and beta glucuronidase which maintained a strong positive correlation with each other, thus indicating that they were suitable markers for the entire lysosomal contents of these cells.

These results must be extrapolated to human periodontal disease with great caution. They indicate that peritoneal transudate cells, lymph node cells and polymorphonuclear neutrophils, all of which are represented morphologically in the inflamed gingiva, are potential sources of lysosomal enzymes which may be released into the extracellular environment, *in vitro*, probably by an immune reaction. The possible significance of the activation of lysosomes from each cell type has been discussed.

Polymorphonuclear neutrophils proved to be the only cell type which was useful for investigation of the effects of endotoxin on the action of complement on lysosomal enzymes. This does not detract from the attractive theories implicating the plogogenic capacity of the endotoxins in periodontal disease nor their suspected role in the stimulation of delayed type hypersensitivity reactions.

These findings indicate that recognition of the site and type of immune reactions in the gingival milieu should be investigated before their protective or destructive roles can be defined in relation to the periodontal disease process.

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