ALTERATIONS IN HOST DEFENSE MECHANISMS OF SURGICAL AND TRAUMA PATIENTS WITH PARTICULAR REFERENCE TO POLYMORPHONUCLEAR LEUKOCYTE FUNCTION AND PREDISPOSITION TO MAJOR SEPSIS AND MORTALITY

by

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A thesis submitted to the Faculty of Graduate Studies and Research of McGill University in Partial Fulfillment of the Degree, Doctor of Philosophy in Experimental Surgery.

Montreal August, 1979

ABSTRACT

Host defense mechanisms in 1776 patients have been studied using the delayed hypersensitivity response and assessment of polymorphonuclear cell (PMN) function. There were 1172 normal (N) skin test responders with a 7.5% major sepsis rate and 2.1% mortality compared to 604 patients with relative anergy (RA) and anergy (A) who had 43% sepsis and 29% mortality. Pre-operative N patients (n=503) had 7.5% sepsis and 4.6% mortality compared to pre-operative RA/A (n=224) with 30% sepsis and 23% mortality. Sequential skin test data were helpful in predicting prognosis. Lymphocyte chemotaxis of N controls (n=13) was 106.4 ± 1.1 μ whereas in A patients (n=35) this was decreased to 89.9 ± 2.8 μ ($p < 0.001$). Neutrophil adherence was 71.5 ± 0.5% in N controls (n=54) compared to an increased value in those with A (n=45, $p < 0.001$) PMN chemotaxis (CTX) was decreased in patients with A (n=244) to 101.9 ± 0.8 compared to those with RA (n=139) 105.9 ± 1.0 and to hospitalized N (n=329) 112.3 ± 0.7 μ ($p < 0.001$). N laboratory controls (n=95) had PMN chemotaxis of 127.4 ± 0.2 μ ($p < 0.001$). PMN chemotaxis was inversely correlated with adherence ($Y = 137.7-0.51X$, $r=0.81$, $p < 0.001$) and directly correlated with lymphocyte chemotaxis ($Y = 22.4 \pm 0.7904X$, $r=0.79$, $p < 0.0005$). PMN phagocytosis was increased in the patients with decreased PMN CTX ($r=0.47$, $p < 0.05$) but bactericidal

function was not altered between normal controls and those with altered skin test response. The PMN CTX defect was attributed to inhibitors circulating in the serum. One was found to occur in normal serum. The decreased PMN CTX observed in surgical patients was due to the de novo appearance of a larger chemotactic inhibitor in the serum. In traumatized patients studied immediately post injury, up to five PMN CTX inhibitors were identified. PMN CTX inhibitors from one surgical patient were found to have the following physico-chemical properties: $S_{20,W}$ = 5.3 and 9.4, pI = 6.3 and 4.6 and MW = 110,000 and 310,000. A good correlation was made between depressed PMN function and major sepsis. It was concluded that alterations of phagocytic cell immunity occur concurrently with those in cell mediated immunity. These alterations are mediated via a complex system of serum modulators. This alteration of host defense mechanisms places afflicted patients at risk to develop major sepsis and mortality from sepsis.

RESUME

Les mécanismes de défense de l'organisme ont été étudiés chez 1776 patients, à l'aide de la réaction d'hypersensibilité retardée et de l'évaluation fonctionnelle des cellules polymorphonucléaires. Des 1172 patients ayant des réponses cutanées normales (N), 7.5% présentaient un taux de septicémie élevé et il y eut 2.1% de mortalité, comparativement à 43% de septicémie et 29% de mortalité chez les 604 patients montrant une anergie relative (AR) ou complète (A). Les patients N en phase pré-opératoire (n=503) furent sujets à 7.5% de septicémie et 4.6% de mortalité, ceux RA ou A en phase pré-operatoire (n=224), à 30% de septicémie et 23% de mortalité. Les résultats de tests cutanés séquentiels furent utiles dans l'établissement de pronostics. Le chimiotactisme lymphocytaire des témoins N (n=13) était de 106.4 u ± 1.1, tandis que celui des patients A (n=35) n'était que de 89.9 u ± 2.8 ($p < 0.001$). L'adhérence des neutrophiles, 71.5 ± .5% chez les témoins N (n=54) augmentait significativement chez les patients A (n=45, $p < 0.001$). Le chimiotactisme des polymorphonucléaires (PMN) diminuait selon le degré d'anergie: 101.9 μ ± 0.8 chez les patients A (n=244), 105.9 u ± 1.0 chez les patients RA (n=139), 112.3 u ± 0.7 chez les patients N hospitalisés (n=329, $p < 0.001$), 127.4 μ ± 0.2 chez les témoins N (n=95), $p < 0.001$). Le chimiotactisme des PMN était inversement proportionnel à l'adhérence ($Y=137.7 - 0.51 X$, $r=0.81$, $p < 0.001$),

et directement proportionnel au chimiotactisme des lymphocytes ($Y = 22.4 + 0.7904\ X$, $r=0.79$, $p < 0.0005$). La phagocytose par les PMN était plus élevée chez les patients à faible chimiotactisme ($r=0.47$, $p < 0.05$) main la fonction bactéricide n'était pas modifiée entre les témoins N et les patients ayant une réponse cutanée altérée. La déficience du chimiotactisme des PMN a été attribuée à des inhibiteurs circulant dans le sérum. L'un d'entre eux fut trouvé dans le sérum normal. La diminution du chimiotactisme polymorphonucléaire observée chez le patients opérés était due à l'apparition de novo dans le sérum d'un inhibiteur chimiotactique plus volumineux. On a identifié jusqu'à cinq inhibiteurs chimiotactiques des PMN chez les blessés étudiés immédiatement après le trauma. On a déterminé chez un patient opéré, que les inhibiteurs chimiotactiques des PMN présentaient les propriétés physico-chimiques suivantes: $S_{20,W} = 5.3$ et 9.4, pI = 6.3 et 4.6, et PM = 110,000 et 310,000. Une bonne corrélation fut établie entre la déficience fonctionnelle des PMN et la septicémie.

En conclusion: les altérations de l'immunité phagocytaire se produisent concurrement avec celles de l'immunité à médiation cellulaire; ces altérations se font par l'intermédiaire d'un système complexe de modulateurs sériques; de telles modifications des mécanismes de défense de l'organisme engendrent chez ces patients un risque plus élevé de septicémie et de mortalité due à la septicémie.

ACKNOWLEDGEMENTS

The author is grateful to the following persons who in many ways have helped with this work.

- Dr. L.D. MacLean, Surgeon-in-Chief, Royal Victoria Hospital who gave me the opportunity to work in this Department.
- Dr. A.P.H. McLean for continued encouragement and support.
- Dr. H.M. Shizgal for helpful advice on statistical analysis.
- Dr. R.A. Forse and Dr. A. Daniel for helpful discussions.
- Dr. H. Rode for very helpful discussions.
- Dr. R. Guttmann for access to his laboratory.
- Dr. R. McGreggor for advice with the adherence assay.
- Ms. I. Hughes and Ms. D. Charette for expert technical assistance.
- Ms. D. Gotto and Ms. C. Hallè for performing the skin tests and help with exhaustive chart reviews.
- Ms. G. Eustace for typing the manuscript in all its forms.

and finally

- to Dr. J.L. Meakins who convinced me to carry out this work, and in a careful but subtle way guided my learnings on host defense and shaped my future. His continued support, and extensive discussion on this and many general subjects were invaluable. For this I am grateful.
- to my wife Ekaterina and sons Venizelos and Jonathan for their patience and understanding.

PREFACE

The experimental work and compilation of this thesis were carried out while the candidate was training for certification in General Surgery at McGill University and the Royal Victoria Hospital, Montreal, Quebec, Canada. Use is made of the literary style recommended by the journal, Annals of Surgery. All work contained in this thesis is that of the candidate. Skin testing of surgical patients was carried out under the candidate's direct supervision by two trained nurses, Mrs. D. Gotto and Mrs. C. Hallè. All chemotactic readings were carried out in a blind fashion by Ms. I. Hughes. Ms. D. Charette, carried out the technical manipulations for the phagocytosis and bactericidal function under the daily direct supervision of the candidate. Help was also received from Mrs. I. Psykwit with some of the lymphocyte E-rosettes and PHA stimulation experiments.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ABBREVIATIONS

A	Anergy
A_{280}	absorbance at 280 nanometers
cfu	colony forming units
CTX	chemotaxis
CL	confidence limits
DH	delayed hypersensitivity
DNCB	dinitrochlorobenzene
EDTA	ethylenediaminetetraacetic acid
FCS	fetal calf serum
g	gravitational force
ISCO	instrumentation specialists Co.
I.U.	inhibitory units
K_{av}	partition coefficient
MEM	minimal essential medium
MW	molecular weight
N	normal
NHS	normal human serum
PHA	phytohemaglutinin
PBS	phosphate buffered saline
PPD	purified protein derivative
pI	isoelectric point
PMN	polymorphonuclear leukocyte
RES	reticuloendothelial system

RA	relative anergy
RPMI-1640	lymphocyte tissue culture medium
$S_{20,W}$	sedimentation coefficient
UV	ultraviolet
V_o	void volume
V_e	elution volume
V_t	total volume
WBA	whole blood adherence

INTRODUCTION

From the beginning of time, man has been plagued by infection. While their nature has changed, infectious problems haunt the hospitalized patient. Since the demonstration of the pathogenicity of bacteria by Pasteur and Koch and their relationship to infectious diseases, much work has been carried out on the natural defences of the human host against these miniscule organisms. A fervor of scientific activity at the turn of the century established most of the present concepts of host defence consisting of humoral and cellular immunity, the interrelationships between complement, phagocytes, opsonins, the value of vaccination, and the efficacy of serum therapy. With the advent of antibiotics in the 1940's, enthusiasm switched from studies on host defence to studies of antibiotic sensitivity of the infectious agents. The advent of aseptic techniques in surgical practice also contributed to decreased sepsis. However, despite scrupulous aseptic techniques, a myriad of antibiotic drugs and better understanding of their use, infections, a major source of morbidity and mortality are still encountered in surgical practice. A study by the National Research Council in 1964 indicated an overall incidence of in-hospital wound infections equal to 7.5%[186]. In Canada in 1973, this has been estimated at 4.8%[66]. The average cost of operative sepsis to the community is estimated at U.S.$7,000.00 per infection[1]. Multiplied by the estimated 140,000 surgical infections in Canada annually, and adjusting

for the devalued Canadian dollar, the burden per year to the taxpayer comes to Can.$1,127,000,000.00. These figures apply now, the era of availability of broad spectrum antibiotics and aseptic surgical techniques. Antibiotics have not solved the infectious complications of surgery. Interest in preventing these costly complications which are disastrous to the individual patient, is returning to investigation of host defence mechanisms[14,5,4,219].

In order to place in perspective the role of host defences in the development of an infectious process, it is useful to examine the determinants of any infectious process[166]. These are the infecting organism, the local environment in which the organism produces the infection,and the host defences which represent the systemic response to the process. The infecting organism has variable properties of virulence, therefore,differences in local and systemic determinants would allow or prevent infection by a modestly pathogenic organism. The features that characterize the local environment are biological, mechanical and chemical forms of decontamination and the objectives of these functions are Prevention of Lodgement of invasive bacteria. The skin for example,presents a mechanical barrier to invasion of bacteria, a biologic control by the presence of its own abundant and entrenched flora which compete with the invader, as well as a chemical barrier due to its content of fatty acids which are bactericidal to non-resistant flora. Other environmental barriers are bronchial ciliary action, tears and the normal flora of the gastrointestinal tract.

The systemic responses to an infecting organism or immunologic challenge are considered to be the classical host defense mechanisms. Host defense is made up of a humoral component, cellular immunity, phagocytic systems and complement. Each of these components has its own complexities, however, in many respects they have a final common pathway, that is, their effects are mediated by an appropriate inflammatory response.

Alterations in host defense can be considered of two types. First the primary or congenital defects, which may involve an isolated defect or a combination of defects. These are always present in the host throughout the usually shortened lifespan. Second are the acquired defects in host defense, and these seldom involve an isolated component. This thesis is concerned with these acquired defects of host defense specifically in the surgical host who is most susceptible to such alterations in natural immune defenses. A detailed, complete study, of the four components of host defense is beyond the scope of a single PhD thesis. Herein the author concentrates on the cell mediated and phagocytic components of host defense. The relationship of alterations in cell-mediated immunity and phagocytic cell function to surgical infection is demonstrated and extensive studies are presented on serum factors which modulate phagocytic cell function. A discussion of the data and future directions of research in this field complete this dissertation.

REVIEW OF THE LITERATURE

1. HISTORICAL ASPECTS

This literature review will briefly consider developments in host defense mechanism prior to 1950 which are of historical interest. In 1952 Colonel Bruton described a young boy with antibody mediated primary immunodeficiency disease[38]. This opened the "modern era" of studies in primary immune defects soon followed by studies on secondary or acquired defects in host defense mechanisms.

At the turn of the century there were two schools of thought on host defense, the humoral school championed by Von Behring and the phagocytic school championed by Metchnikoff[40]. Wright and his colleagues[263] put some aspects of this controversy into perspective with the concept of the "phagocytic index" and the "opsonic index" and the necessity of "buttering the microbes" with opsonins before they were acceptable for phagocytosis. In the first third of this century, there was a considerable amount of work delineating the mechanisms and variables in the processes of phagocytosis. The work of Wright was extended by many, including Meakins, who demonstrated specific opsonins were required for Staphylococcus and Streptococcus and that these opsonins could be artificially induced to treat recurrent staphylococcal abscesses[164]. Fenn clearly demonstrated that phagocytosis was proportional to the chances of collision[86]. Early work on the phagocytic component of host defense was primarily concerned

with phagocytosis per se, since the processes of margination, chemotaxis, degranulation and digestion of bacteria were not well studied. The description of Maaløe[151] in 1946 and the further development by Cohn and Morse[56] of the neutrophil bactericidal assay laid groundwork for the present day tests of neutrophil function.

It was evident to students of leukocytes at the turn of the century that white cells other than polymorphonuclear neutrophils were present in the circulation. However, their function was not known and work with these cells was restricted to staining and classification. The work of Koch with tuberculous guinea pigs, and the later extension of the "Koch phenomenon" to humans who had exposure to tuberculosis, was the earliest work on classical delayed hypersensitivity. Injection of tuberculin into the skin of tuberculous patients in an attempt to cure them, caused a gradual reddening at the injection site followed by induration, with the whole process reaching a maximum at 48 to 72 hours[24]. The demonstration by Landsteiner and Chase, that delayed hypersensitivity could be transferred by cells, confirmed the impression that this was a cell-mediated reaction[137]. This confirmed Zinser and Mueller's proposal that the mechanisms of delayed hypersensitivity were different from serum mediated reactions[269].

The importance of the lymphocyte and its role in host defense became clear after intensive reasearch from the 1950's to the present which is discussed later.

2. HOST DEFENSE MECHANISMS AND METHODS OF STUDY

Intensive research into host defense mechanisms to the present, has identified four major components of host defense. The humoral components, the cell mediated component, the phagocytic component and the complement system. This division is somewhat artificial but useful for purposes of studying host defense. All four components must interact in a precise predetermined sequence for adequate host defense.

2.1 Humoral Immunity

Humoral immunity is that component of host defense which is mediated via circulating antibodies, the immunoglobulins. It is involved in protection against bacteria, viruses, parasites, allergens, and possibly, malignant cells. For the humoral component of host defense to be effective there must be close interaction between this and the other three components of host defense. Glick et al observed that removal of the bursa of Fabricius in chickens led to failure of antibody production in these animals[100]. The work of Cooper et al confirmed the concept of two types of lymphocytes - a thymus dependent lymphocyte population related to allograft immunity and a bursa dependent or B-lymphocyte population related to humoral immunity[62,63]. There are 20-30% B-cells in the total circulating lymphocyte pool, identifiable by surface membrane markers. B-lymphocytes can

transform into plasma cells whose function is to produce specific antibodies. Each B cell produces a highly specific antibody molecule and these molecules taken collectively are termed the immunoglobulins. Each immunoglobulin is composed of two heavy chains and two light chains linked together by disulfide bonds. Each crystalizable portion of the heavy chain molecule or the F_c portion has a constant amino acid sequence distinct to the particular immunoglobulin. It is on the basis of this sequence that immunoglobulins are divided into five distinct classes. More recent work has identified several subclasses within each class.

Immunoglobulin G or IgG comprises 70-80% of the total immunoglobulins, has MW 150,000 daltons and a mean adult concentration of 1,200 mg/dl. It is distributed intravascularly and throughout the interstitial spaces. There are four subclasses of IgG. Antibodies to most bacteria, virus neutralizing antibodies, precipitating antibodies, hemaglutinins, and incomplete hemolysins are among the types of antibody found in this class.

Immunoglobulin M or IgM makes up 5-10% of the total immunoglobulins, has a MW 900,000 with a mean adult concentration of 150 mg/dl. The majority of antibodies formed against polysaccharide and gram-negative bacterial antigens are in the IgM class. There are two IgM subclasses.

Immunoglobulin A comprises 10-15% of total immunoglobulins, has a MW 180,000 and has a mean adult concentration of 300 mg/dl.

This immunoglobulin is found in the secretions of man such as tears, saliva, bronchial secretions, and mucous, and is responsible for local immunity. There are also two IgA subclasses.

Immunoglobulin D or IgD is present in the serum at a mean adult concentration of 3 mg/dl, has MW 180,000 and constitutes less than 1% of the total immunoglobulins.

IgE or immunoglobulin E is the fifth class and is present in the serum of adults at the mean concentration of 0.03 mg/dl. It has a MW 200,000 daltons and accounts for 0.01% of the total immunoglobulins. It can be detected in the plasma by radio-immunoassay and is also bound to mast cells and basophils.

The biologic function of IgG is to activate complement and together coat bacteria to make them suitable for phagocytosis by appropriate cells. IgM is a powerful activator of the complement system and is the predominent antibody formed in response to gram negative bacteria. It is also the antibody produced during the primary response to a new antigen whereas IgG is involved in secondary responses. Antibodies of IgG and IgM are also responsible for neutralization of bacterial toxins, and direct bacterial killing of strains containing lipopolysa-charide coats, through the action of complement mediated "holes" in their membrane in conjunction with serum lysozyme.

Attempts to measure humoral immune functional capacity have not been pursued as vigorously as those of cellular responses. The principle method of assessing for an intact humoral response is challenge with an antigen to which an individual has not been

previously exposed. Viruses, diptheria toxin ("Shick test") and tetanus toxoid have been used,but these are not very reliable. In assay of the primary immune response, the best antigen is flagellin, a protein from the flagella of Salmonella adelaide[200]. Mercaptoethanol fractionation of the serum will identify the type of antibody produced, IgM or IgG. First injections of 5 µg of this protein in buffered saline pH 7.0 subcutaneously is followed by increased titers of IgM detected by hemaglutination of tanned sheep red blood cells which peak at 2 wks. Challenge injections 4 wks following the first injection lead to an antibody response primarily consisting of IgG. Keyhole limpet hemocyanin is another antigen for assessing the humoral response[67]. This is less suitable than flagellin because it may not be a primary antigen in man[179]. In vitro tests,involve quantification of the numbers of circulating B-cells, antibody production by peripheral blood lymphoid cells after stimulation, or assay for the presence of antibody producing cells by the Jerne plaque technique[125].

2.2 Cell Mediated Immunity

This is the component of host defense whose effects are mediated directly by cellular elements. A pluripotent stem cell in the bone marrow may replicate giving rise to unipotent committed stem cells. These are destined for development along one or another hematopoietic pathways: erythroid, granulocytic, megakaryocytic or lymphoid[264]. Cells in the lymphoid pathway are

then caused to differentiate along two lines depending under which specific organ's influence they fall during this differentiation. The microenvironment of the thymus causes cell differentiation into the thymous dependent or T-lymphocyte line. The microenvironment of the "bursa" equivalent in man will cause the unipotent stem cells to become bursa dependent or Bone Marrow dependent B-lymphocytes[53]. The function of the B-lymphocyte has been discussed earlier.

2.2.1 General Aspects of T-Lymphocyte Function

Lymphocytes are the major cell constituents of normal lymph nodes, splenic white pulp, and the unencapsulated lymphoid tissue of the alimentary tract and lungs. They also comprise a substantial portion of the leukocytes in normal blood and are the predominant cell type in lymph. Small round cells that qualify as lymphocytes by morphological criteria are prominent in the bone marrow, submucosa of the intestine, and in chronically inflammed tissue. Lymphocytes were first described by Hewson[109] in 1777. They range in size from approximately 5μ to greater than 15μ. It is a common, if somewhat arbitrary, practice to group them as small (5 to 8μ), medium (8 to 12μ), and large (12 to 15μ) lymphocytes. The small-lymphocyte population is heterogeneous and comprises at least two major subpopulations: the long-lived small lymphocyte population, and the short-lived. The former is relatively homogeneous and is primarily of thymus derived T-cells.

The short lived population is more heterogeneous and derived in part from bone marrow lymphoid precursors, the B-cells. The long-lived T cells are concerned with antigen reactivity and form the memory cell population. They constitute the bulk of cells found in the recirculating lymphocyte pool migrating between blood, lymph fluid and various organs and tissues. The short-lived lymphocytes may also migrate into various lymphoid organs such as the thymus and lymph nodes. Small lymphocytes have both a glycolytic pathway, with the lowest rate of oxygen consumption in unstimulated states compared to neutrophils[139], as well as an intact hexose monophosphate shunt[158]. Lymphocytes stimulated *in vitro* by specific and non specific antigens such as phytohemagglutinin enlarge, synthesize DNA and undergo mitosis within 48 to 72 hours[187]. However, changes in carbohydrate metabolism such as increased glucose utilization, pyruvate and lactate production, and glycogen synthesis can be detected within 4 hours of PHA stimulation[108].

There are three groups of cellular immune reactions. First, specific cytotoxic reactions, in which sensitized lymphocytes respond to foreign antigen present on or absorbed by the target cell surface. Prototypes are allograft rejection, antitumor immunity and the tuberculin reaction. The second cellular immune reaction involves lymphoid cells and mononuclear cells which are activated by antibody or complement components bound to target cells (in a sense "opsonized" cells). This reaction is non

specific. The third type of cellular immune reaction is induced by lymphocytes nonspecifically activated by a variety of agents [211,178,191]. Though we have come a long way from the view of Rebuck[188] written in 1947 that, "Not only are the lymphocytes one of the important sources of macrophages, but they perform a second equally important function in the production of antibodies", the study of lymphocyte function is still in its infancy[37].

2.2.2. Delayed Hypersensitivity

Delayed hypersensitivity is a name applied by Hans Zinsser in 1921 to the erythematous indurated reactions elicited in specifically sensitized subjects by intracutaneous challenge with bacterial antigens[268]. Delayed hypersensitivity reactions of which the tuberculin reaction is the prototype, develop at a significantly slower pace than the well-known forms of antibody-mediated reactions. They are characterized by a null period of several hours after the skin test, during which little gross evidence of a developing immunological process is apparent. They do not achieve maximum intensity for 1 or more days. In contrast, antibody mediated reactions such as local anaphylaxis, or the arthus reaction, begin promptly after skin test and have a time course measured in minutes or hours. One of the first scientific observations of DH response was made by Jenner[124] in 1798: the injection of vaccinia virus into an individual who previously had been immunized against vaccinia was followed by the appearance

of vesicles and erythema at the injection site, reaching a peak at 24 to 72 hours. In 1890 Koch[132] injected live tubercle bacilli intradermally into guinea pigs previously infected with tubercle bacilli and observed erythema and induration at the infecting site at 24 hours which progressed to tissue necrosis; in contrast, he did not observe this response in uninfected animals. A prolonged time course is only one feature of delayed hypersensitivity. A more fundamental property is the dependence of these reactions on sensitized lymphocytes, a fact established by Landsteiner and Chase in 1942 in one of the more crucial experiments in immunology[138,50]. In man, lymphocyte extracts, one being transfer factor[142], and the other RNA extracted from sensitized lymphocytes which differs from transfer factor[77], have been shown to transfer cell mediated immunity.

The basis of the DH response is the ability of the body, in particular the T lymphocyte, to recognize an antigen as foreign. Entry of a foreign antigen into the body of an immunocompetent individual initiates a series of events that are independent of the nature of the antigen. Locally, the antigen combines with tissue proteins and cellular elements to produce a vasoactive factor. This permits the escape of leukocytes as well as plasma proteins from the local vessels, with subsequent migration to the area of the antigen. Opsonization of the antigen and phagocytosis by macrophages follows. Following the macrophage-lymphocyte interaction, regional lymph nodes develop pyroninophilic cells

or immunoblasts in the mid to deep cortex. These cells proliferate over the next 4 to 5 days, then divide to form small lymphocytes sensitized to the antigen, which are then released into the systemic circulation. If the antigen is placed upon an isolated island of skin[136], or a stalk of isolated tissue[90], sensitization occurs only if the lymphatics are patent. If, at a later time, there is contact with the same antigen, a different response occurs. The antigen is "recognized" by a few of the circulating sensitized lymphocytes that may come in contact with it in a random manner. Once the antigen binds to membrane surface receptors of the sensitized lymphocyte, the cell responds by releasing various chemical mediators or lymphokines, such as neutrophil and monocyte chemotactic factors, blastogenic factor, and migration inhibition factor. These lymphokines cause many non sensitized cells to migrate to the area, and to dispose of the antigen and resulting cellular debris. The resulting induration, interpreted as a DH response, peaks at 24 to 48 hrs.

In accord with classical descriptions[21,30,45,233], cell mediated reactions of delayed hypersensitivity type began as perivascular infiltrations of lymphocytes 4 to 8 hrs after testing which subsequently extended into the intervascular dermis and epidermis, reaching a maximal intensity at 2 days. Blood-derived monocytes/macrophages were quite rare in these reactions, plasma cells were infrequent, and PMNs were prominent only in severe reactions accompained by tissue necrosis. However, recent

investigators[83] have demonstrated the prominent role played by inflammatory cells other than mononuclears, particularly basophilic leukocytes; degranulation of both basophils and mast cells; increased vascular permeability with consequent vascular compaction, papillary dermal edema, and some degree of erythrocyte extravasation;mast cell replication; microvascular alterations affecting endothelial cells and pericytes, with compromise of vessel lumens and basement membrane thickening; and activation of the clotting system with deposition of fibrin in a characteristic intervascular pattern in the reticular dermis[82].

The origin and specificity of the cells participating in delayed hypersensitivity reactions is of interest. Perhaps as many as 95% belong to a rapidly dividing cell population, derived from the blood and ultimately from the bone marrow. The bone marrow origin, was demonstrated by experiments involving passive transfer of various cell types from immunized donors, to unsensitized thymectomized and heavily irradiated rats[150]. Reactions could be transferred only when sensitized lymph node cells were supplemented with bone marrow cells from normal animals. McLuskey et al[163] showed that the great majority of cells which comprised the delayed hypersensitivity reaction in guinea pigs were not specifically sensitized cells. In addition,the cells which made up the infiltrate had recently proliferated and consisted mainly of medium (8 to 12 μ) and large (12 to 15 μ) lymphocytes, plus the other elements discussed above.

If delayed hypersensitivity response is to be used as a measure of immunologic competence, it is necessary to use antigens to which the subjects under study can reasonably be expected to have been sensitized. In hospitalized patients the use of at least five recall antigens will result in a positive response to two or more antigens in over 90% of patients tested [190,111,223,191]. Other agents such as dinitrochlorobenzene (DNCB), a hapten, have been used to assess both arms of the delayed hypersensitivity immune response[240]. Meticulous technique and a strict uniform definition of anergy must be used if data between different centers are to be compared[218].

Impaired cell mediated immunity measured by skin test anergy (failed DH response) or by in vitro measurements of lymphocyte function has been reported in: chronic infection[42,260]; measles, syphilis and infectious mononucleosis[128]; alcoholic liver disease[238]; leukocytosis (mostly secondary to acute infection)[235,236]; severe atopic dermatitis[196]; cancer[195,185,226,184]; progressive age[43,240]; after controlled surgical trauma[198] and uncontrolled accidental blunt and/or thermal injury[112,57,156, 171,168,123];and in patients undergoing thoracic duct drainage[155]. Manick and his coworkers have been able to demonstrate lymphocyte immunosupressive activity in human plasma due to a circulating peptide of MW less than 10,000 daltons[64,188]. Similar immunosupressive activity was found in the serum of patients with cancer[98,99] and burns[57,106]. Most recently, Manick and coworkers

correlated the appearance of serum immunosupressive activity with skin test anergy in post operative surgical patients[161].

2.2.3 Lymphocyte Chemotaxis

In 1888 Metchnikoff demonstrated that in his animals with experimental tuberculosis, the lymphocytes of the blood migrated into the tuberculous areas[177]. However, the German group at the Berlin Medical Congress in 1890 opposed his views because they believed that lymphocytes were incapable of ameboid motion and hence could not migrate from the vessels to the area of inflammation. Lewis and Webster[147,148,265] studied lymphocyte locomotion extensively, and found that lymphocytes were the first cells to migrate from explanted lymph node pieces into a plasma clot. Despite vigorous investigation of PMN chemotaxis after the report of Boyden's method in 1962, *in vitro* lymphocyte chemotaxis was not studied until 1971 when Ward *et al*[249] reported that thymic and lymph node cells from rats would show chemotactic migration towards "lymphokines" obtained from culture filtrates of antigen stimulated lymphoid cells. A long incubation of 18 hrs was used and CO_2 was reported as a requirement. A chemotactic index was used. Snyderman *et al*[216] reported a technique in 1972 for measuring chemotaxis of mononuclear cells but those were found to be monocytes. Using this technique, these authors demonstrated depressed mononuclear leukocyte chemotaxis in thermally injured patients[16] and characterized the lymphokines produced by

human T and B cells to which these monocytes would respond[15]. The experiments of Schreiner and Unanu[205] did not confirm lymphocyte chemotaxis using 5 to 8 μ pore size filters though Higushi et al[113] demonstrated that a PMN protease could cleave a chemotactic peptide of about 14,000 MW from a parent IgM that was chemotactic for rat thoracic duct lymphocytes. Though their incubation time was 3 hrs they confirmed Ward's earlier observations. Russell et al[202,258] were the first to report chemotaxis of lymphoblasts obtained from firstly cloned human lymphoblast cell lines. They used casein and endotoxin activated serum as attractants. A 3 hr incubation with 8 or 12 μ filters was used with the leading front technique. Wilkinson and his coworkers later applied this technique to study the effects of anesthetic agents[180], and membrane active enzymes[257] on human lymphocyte chemotaxis. The repeated observation made by these authors was that lymphocytes had to be transformed in some way into lymphoblasts either by PHA or in vitro culture before appreciable chemotaxis could be observed.

2.3 PHAGOCYTIC CELL IMMUNITY

2.3.1 General Aspects of Phagocytic Cell Immunity

Phagocytosis is the process by which cells ingest replicating and non-replicating agents encountered in the environment in order to contain or destroy them. In the human host, there are "circulating" phagocytic cells in the blood

stream and "fixed" phagocytic cells of the reticuloendothelial system (RES). The phagocytic cells of the body can also be separated into the microphages of Metchnikoff made up of the polymorphonuclear leukocytes and eosinophilic leukocytes; and macrophages, cells which collectively and in a functional sense make up the reticuloendothelial system. These cells types are: the histiocytes of the connective tissue and lung derived from blood monocytes; the microglial cells of the central nervous system; the sinus lining cells of the spleen, liver, bone marrow and anterior pituitary; and the reticulum cells of the lympho-reticular tissues, spleen, lymph nodes, bone marrow and thymus. Certain areas such as the "tâches laiteuses", the whitish areas of the surface of the omentum next to blood vessels, have a high concentration of histiocytes. Some of these cells emigrate into the free peritoneal cavity during infectious challenge accounting for the increased numbers of macrophages[122]. There must be very close interaction between the humoral component and complement and the phagocytic component, for host defense to be effective. Without opsonization of bacteria, the phagocytic component is relatively ineffective.

Methods of study of the phagocytic component of host defense are designed to study each component separately and collectively. Most studies on the circulating phagocytes, the PMN, utilize the uptake of radioactively labelled bacteria, suitably opsonized, and differential centrifugation to separate the PMN from non-phagocytosed extracellular bacteria. Counting

of radioactivity within the sedimented cells gives a percentage of bacteria phagocytosed compared to the original numbers available to the cells. Similar mixtures of PMN and bacteria can be utilized to assess bactericidal capacity. Aliquots of such mixtures plated after appropriate time intervals can give an indication of the numbers of bacteria killed from the total numbers available at time zero. Comparison of phagocytic and bactericidal function between control and study PMNs allows for differences to be detected. Once such differences are detected then further studies can be carried out to determine the precise etiology, be it an abnormality of cellular metabolism[20] or of opsonization and uptake[203].

Transient bacteremias occur in everyday medical practice such as after passage of a catheter, uterine curetage, prostatectomy, dental procedures and brushing of teeth. The reticulo-endothelial system is responsible for clearing the blood of such particles. Thus in vivo studies of RES function employ clearance of bacteria, radiolabelled denatured proteins or "vital dyes" from the circulation in animals[122]. The importance of the RES in host defense is becoming increasingly apparent as indicated by recent studies[208].

2.3.2 Polymorphonuclear Leukocytes

Polymorphonuclear neutrophils form the bulwark in host defense against bacterial pathogens. This was first recognized

by Panum in 1871 though Virchow felt that leukocytes were responsible for the spread of infection rather than its control[41,261] Metchnikoff's observations in 1882 on the phagocytes of starfish larvae led to the first concrete data on the role of leukocytes in host defense[80].

As mentioned earlier a pluripotent stem cell in the bone marrow replicates giving rise to unipotent cells which develop into several lines depending on maturational influences of the microenvironment. One such line involves passage of unipotent stem cells into a "mitotic compartment" yielding a myeloblast which divides and further differentiates into a promyelocyte and then a myelocyte. Further differentiation in a "maturating compartment" and passage through a metamyelocyte stage yields the polymorphonuclear neutrophil which is released in the blood stream[51].

The circulating blood granulocyte pool is 3.1×10^8 cells/kg at any one time with a half life of 6.7 hrs. The marginated granulocyte pool is estimated at 3.9×10^8 granulocytes/kg body weight. There is also a bone marrow reserve of $6.5 - 13 \times 10^9$ cells/kg in the human[47]. As PMNs go through various maturation stages, they become more specialized in their functions and lose the ability to replicate. The composition and metabolism of PMNs has been reviewed by Cline[52]. The primary role of PMNs in the economy of the body is localization

and removal of microorganisms. Several integrated functions are necessary for the achievement of these goals. First the PMNs must marginate or adhere to the endothelium of the vessel wall. Next PMNs must reach the site of invasion by chemotaxis along chemotactic gradients set up by local factors and/or the invading organisms. Once at the site of infection, they must phagocytose or otherwise immobilize the organisms and finally they must kill or inhibit the replication of the invaders.

2.3.2.1 Polymorphonuclear Leukocyte Adherence

The first observation of margination or adherence of leukocytes to the small vessels of the tail of tadpoles was made by Dutrochet in 1824. Accurate observations on the margination and emigration of leukocytes in inflammation were made between 1833 and 1850 by Wagner, Waller, Addison, Wharton-Jones and later by Cohnheim and Adami as reviewed by Grant[103]. Garvin was the first to design a system for measurement of leukocyte adhesiveness to glass beads[92]. Kvarstein made improvements in the method by regulating the rate of flow of the leukocyte suspension over the glass beads by means of a pump[134]. Those early methods required thermoregulation of columns, specially treated glass beads and constant flow rates. This made measurements difficult, though some basic observations, such as, adherence was an energy-dependent phenomenon were recorded. Bryant and Stucliffe introduced a new system for measurement of leukocyte adhesiveness to glass capillary tubes[39]. The adverse

effects of low temperature, sodium arsenite, divalent cation chelation, and hyperosmolarity, upon leukocyte adhesion was demonstrated.

Adhesion was enhanced by increased concentration of Mg^{2+} or suspension of leukocytes in a plasma free environment. McGreggor et al utilized glass columns packed with scrubbed nylon fiber to measure the adherence of PMN in vitro[154]. Ethanol was found to markedly inhibit granulocyte adherence when added in vitro directly to blood as well as given parenterally to rabbits. Acetylsalicylate and prednisome added directly to blood in vitro failed to alter adherence to granulocytes. In contrast both drugs given in vivo to volunteers in large doses, significantly decreased the adherence of granulocytes.

Extending these studies in patients with acute inflammation, (defined as an erythrocyte sedimentation greater than 80mm/h or rectal temperature greater than 38°C secondary to bacterial infection), Lentrek et al[146] demonstrated increased adherence of granulocytes in these patients. A factor was detected in the plasma of these patients which increased the adherence of normal granulocytes. This factor was not found in serum and was heat labile. It appeared that a heterogeneous system of molecules ranging in MW between 30,000 and 400,000 daltons was responsible for the adherence augmenting effect as shown by Sephadex G-200 chromatography of inflammatory plasma. Further work by MacGregor[152] showed that plasma from volunteers given salicylates or glucocorticoids was capable of inhibiting the

adherence of normal granulocytes. This factor was heat stable, non-dyalizable and not present in serum. When this was mixed with the adherence augmenting factor found in inflammatory disorders, the effects were neutralized and normal adherence of control granulocytes resulted. The correlation between increased adherence and transient granulocytopenia in hemodialysis, endotoxemia and epinephrine administration, was also demonstrated[153].

McGillan and Phair[162] in a recent study of normal PMNs showed that zymosan activation of normal plasma was effective in augmenting control PMN adherence. Incubation of activated plasma with antiserum to C3 neutralized this effect, implicating the complement system in these reactions. The failure of serum to alter adherence despite the presence of complement indicates that the adherence augmenting effect is more complex.

2.3.2.2 Polymorphonuclear Leukocyte Chemotaxis

Chemotaxis of leukocytes is a unidirectional migration in response to a diffusing gradient of a chemical attractant[242]. This is incontrast to random migration exhibited in living tissues in steady state conditions[11]. A third type of leukocyte motion is chemokinesis, an accelerated random motion secondary to a stimulus. Leber first reported leukocyte chemotaxis in 1888 after studying cellular responses and subsequent accumulation of leukocytes following introduction of various substances in

the anterior chamber of rabbit eyes. Metchnikoff, already well advanced in the study of phagocytic protective function, recognized the importance of these findings and incorporated them into his phagocytic theory[173]. Early studies of neutrophil chemotaxis involved attempts at in vivo measurements as reviewed by Harris[107]. The Rebuck skin window[190] became the most popular, though modifications by Feurer et al[87] using collection chambers were also used.

In 1962, Boyden described a new method for assessing leukocyte chemotaxis by use of a chamber separated into an upper and lower compartment by means of a filter with small pore size. Neutrophils to be tested placed in the upper chamber were allowed to migrate towards the bottom chamber where a chemoattractant was placed. Counts of cells on the bottom side of the filter compared to the top side allowed for estimation of chemotactic migration. Several modifications of this technique have been made[130,91,131,25], the most elegant being the introduction of the leading front technique by Zigmond and Hirsch[267]. This technique allowed for quantitation of cell migration by measuring the distance travelled by the leading front of cells, using incubation times that did not permit complete penetration of cells into the opposite side of the filter. This method gives an integer value for chemotaxis, is not dependent on ratios, unlike the chemotactic index proposed by Boyden, and allows more experimental flexibility.

Locomotion by human leukocytes is temperature dependent but independent of pH between 6.5 - 7.5[184]. Anaerobic glycolysis is the major source of energy involved in leukocyte motility, though a respiratory burst and activation of the hexose monophosphate shunt occurs in response to chemotactic stimulii[101]. Adenyl cyclase activity regulating levels of intracellular AMP, and guanidine cyclase controlling GMP, seem involved in regulation of chemotaxis. Na^+ and K^+ dependent ATPase enzyme systems may also be required[27].

Cytoplasmic flow is continuous in moving cells and occurs throughout the entire width of the cell. "Lamellipodia" are randomly produced; however, chemotactic movement is associated with preferential flow of cellular contents into lamellipodia on the side of the cell nearest the attractant[182,183]. This movement, like the amoeba, may be mediated by contractile protein[199]. Chemotaxis does not involve a change in the speed of the leukocytes, merely a change in their directional movement[182,183].

Complement-derived factors are the most important substrates for the generation of leukocyte chemotactic activity. Some examples are C5a and peptides derived from C5 C3a and related peptides derived from C3 and the activated trimolecular complex $\overline{C567}$ [150,201,248]. Proteases from bacteria[244], may attack C3 or C5 or both yeilding chemotactic factors. Complement-independent chemotactic factors for leukocytes include bacterial and viral factors, enzymes, or tissue breakdown products[241,245,246]. Derivatives of structural tissue, namely split products of

collagen and fibrin also show chemotactic activity[48,221]. Exogenous proteins with leukocyte chemotactic activity are whole casein, α_s-casein, B-casein and gluten[256]. Material present in culture supernatents from stimulated lymphoid cells is also chemotactic for leukocytes[251], and is distinct from other lymphokines such as migration inhibition factor[250]. The method utilized by PMNs in sensing the chemotactic gradient is one of a "spatial mechanism", where the cell compares the gradient at two or more points on its "body" according to Zigmond[266].

The application of in vitro chemotactic measurements of human leukocytes has uncovered two types of leukotactic abnormalities: cellular and humoral. Intrinsic leukocyte defects either primary or secondary have been found in the Chediak-Higashi syndrome[58]; in newborn infants[175]; in patients with rheumatoid arthritis[183]; diabetes mellitus[181]; and in children with the "lazy leukocyte syndrome"[177]. Smith et al[214] described a child with recurrent purulent rhinitis, otitis media, pneumonias and pyoderma, in whose serum was found an inhibitor of leukocyte chemotaxis that was stable at 56°C, exerted a direct effect on neutrophils, and did not alter normal cell viability, adhesiveness to glass, phagocytosis or bactericidal function. A similar case was reported by Ward and Schlegel[247]. Two more reports of PMN chemotactic defects are associated with redheadedness and recurrent infection[260] and the other associated with a cryoglobulin[69], established primary chemotactic defects as

distinct disease states. Secondary defects of PMN chemotaxis in patients with infections were reported by Mowat and Baum[182]. De Meo and Andersen[74], Gewurz et al[95] and Ward and Berenburg[243] have also reported defective chemotaxis due to serum inhibitors of PMN function. Van Epps et al[236] identified chemotactic inhibitors in the serum of patients with leukocytosis $>$ 12,000 WBC/mm^3 and skin test anergy. These are not cell directed inhibitors, according to the authors, but rather inactivated serum derived chemotactic factors. Similar chemotactic factor inactivators have been identified in normal serum after appropriate manipulations by Ward and coworkers[28,231]. Van Epps et al substantiated their original observations of PMN chemotactic factor inactivators in followup studies[234,237]. Maderazo et al first reported a cell directed inhibitor in the serum of a patient with an autologous leukotactic defect[157]. Other reports followed, linking depressed neutrophil chemotaxis with other immunologic abnormalities[195,259,192], but it was not until the reports by Meakins et al[170,169] that depressed neutrophil chemotaxis was associated with sepsis and mortality from sepsis. Concurrently several reports of depressed neutrophil chemotaxis in patients sustaining burn trauma appeared in the literature, though the nature of the defect, i.e., intrinsic to the cells or secondary to inhibitors in the serum is controversial[253,104,88].

2.3.2.3 Polymorphonuclear Leukocyte Phagocytosis and Bactericidal Function

Phagocytosis and intracellular killing of bacteria by PMNs are essential functions in host defence, and numerous attempts have been made to relate abnormalities in these functions to the development of sepsis. However, difficulties of methodology and lack of standarization of techniques have resulted in divergent interpretations of the experimental results. The original technique of Maaløe using differential centrifugation for the separation of extracellular and intracellular bacteria has been adapted and modified by several investigators[59,119,181,212]. Several developments of interest in this area follow. The interaction between bacteria and neutrophils in the test tube was described[55], phagocytosis was visualized and photographed[117], the original reports of Robineaux and Frederick[194] on lysosomal degranulation were confirmed[116], and PMN granules were isolated and their bactericidal properties were demonstrated[54,115]. Phagocytosis[36,102] and the degranulation process[277,165] were studied by electron microscopy demonstrating that phagolysosomes form by blending of lysosome and phagosome membrances and discharge of lysosome contents into phagosomes.

Several modifications have been made to leukocyte phagocytosis and bactericidal assays mostly in attempts to standarize the system and separate adequately phagocytosis from bactericidal function[9,228], determination of optimum phagocyte to bacteria ratio[217], opsonization requirements[239], and achievement of reproducibility. Most recent advances utilized radioactivity labelled

bacteria and differential centrifugation along with lysostaphin, to separate the processes of bacterial attachment to leukocyte membranes, internalization, and subsequent killing[239,78,227]. Microassay methods, and use of opsonized oil emulsions to assess phagocytosis have also been described[204,224] as well as the influence of heat killing of bacteria on phagocytosis[73], use of different strains of bacteria[199], metabolic events subsequent to phagocytosis, and the effect of drugs on both functions[72,19, 144,180,187,118].

2.4 Complement

The complement system consists of serum proteins that act sequentially to amplify the effect of antibody-mediated immunity, cell-mediated immunity and phagocytosis. They comprise the classical complement pathway now designated - complement (C), and the alternative complement pathway which involves an additional four serum proteins. Complement components are designated with numerals in order of their sequence of activation. Activated components are designated by an overlying bar (eg. $\overline{C1q}$), and cleavage products by letters of the lower case (eg. C3b). The complement pathway is divided into recognition (C1q, C1r, C1s), activation (C2,C3,C4) and attack portions (C5,C6,C7,C8,C9)[160]. C3 is the pivotal molecule in the complement sequence. It can be activated by either the classical pathway or the alternative pathway. This alternate pathway (or properdin) involves factor D,

factor B, properdin, initiating factor and perhaps properdin convertase. Activation of the classical pathway of complement usually requires an antigen - antibody reaction. Only IgM and three of the four subclasses of IgG are known to activate the classical pathway. The union of specific antibody to its corresponding antigen produces or reveals a site in the Fc portion of the heavy chain that combines with Clq to fix the trimolecular complex of Cl (Clqrs) in the presence of calcium ion and start the activation process.

A single molecule of IgM or at least two molecules of IgG spaced about 7000 Å apart on the cell surface can initiate activation. The fragments of complement activation have several biological functions. C4a is involved in immune adherence. C3a is a potent chemotactic factor whereas C3b involves opsonization, immune adherence and release of platelet factor 3. C5a is also a potent chemotactic agent for leukcoytes as is $C\overline{567}$. $C\overline{8}$ initiates lytic processes once inserted into a cell membrane whereas $C\overline{9}$ completes lysis. Through these powerful activities complement plays a vital role in immunologic and nonimmunologic defense against bacteria of both high grade and lower grade pathogenicity, fungi, viruses, protozoa and helminth invaders. It is also involved in the elimination of foreign cells, initiation and control of inflammatory reactions and coagulation of blood.

Methodsof assessing adequate compelment function are available in the clinical laboratory. All 11 proteins of the classical pathway are required for hemolysis of sensitized

erythrocytes, thus measurement of total hemolytic complement remains the functional C assay of greatest value. Individual proteins such as C2, C4 and Clq in the classical pathway and factor B and properdin in the alternate pathway can be measured with radial immunodifussion using specific antisera. Identification of cleaved or otherwise altered components of complement remain the function of specialized laboratory[96].

3. CONGENITAL DEFECTS IN HOST DEFENSE

Primary or congenital immunodeficiencies are varied and their description and classification in the literature is continuously changing. Part of the reason is that present techniques used to study the various aspects of immunity were not available to the investigators at the time that the actual cases were studied and published. Fudenberg and his coworkers[89] classified primary immunodeficiencies as those of stem cells, B-cells or T-cells though a cell line cannot be the end product of a defective gene.

Primary immunodeficiencies are divided into five main categories. They are all summarized in Table 1. There are six types of antibody mediated primary immunodeficiencies, the most common being selective IgA deficiency affecting 1:600-800 neonates. Most common symptoms is recurrent sino-pulmonary viral and/or bacterial infections. IgA levels in the serum are less than 5mg/dl the other immunoglobulins are normal or increased.

Total B-cells are normal as are B-cells bearing surface IgA. The disorder is probably due to abnormal synthesis or release of IgA. Prognosis is guarded but patients can live to the seventh decade with careful management.

Pure isolated T-cell abnormalities are rare. Usually when T-cell function occurs there are associated B-cell defects. Presently only two pure cell mediated immunodeficiencies are recognized in the literature. One is thymic hypoplasia with hypocalcemia (Di-George Syndrome)[75] and the other is chronic mucocutaneous candidiasis[143].

Primary phagocytic immunodeficiencies may be divided into extrinsic defects, including suppression of total numbers of phagocytic cells, opsonin deficiencies, and abnormalities within the phagocytic cell resulting in impaired bactericidal capacity. Chronic granulomatous disease[127] is an inherited defect in neutrophil bactericidal function, which manifests itself with recurrent severe infections with unusual organisms. Phagocytosis is normal, however bactericidal function is abnormal with intracellular bacteria remaining alive up to 120 min. The cells lack NADP-oxidase and NADPH-oxidase or glutathione peroxidase. Hydrogen peroxide production is thus abnormal resulting in decreased bacterial killing. The Lazy-leukocyte syndrome[177] is characterized by defective chemotactic response of patient PMN to normal chemotactic stimuli. There is also associated neutropenia. Five more primary phagocytic cell defects are

listed in Table 1, as are four acquired disorders of the complement system.

Immunodeficiencies of both T and B lymphocyte systems lead to abnormalities of both the humoral component as well as the cell-mediated component. The most lethal immunodeficiencies fall in this group (Table 1).

4. ACQUIRED DEFECTS IN HOST DEFENSE

4.1 General Considerations

Acquired defects of host resistance are by implication a result of some other influence usually secondary to a disease process, an event, or drug therapy. The list of factors which can produce these defects is an impressive and continually growing one. Characteristically,these defects tend to be variable in severity and only occasionally are they as complete as the congential absence of a cell function. Malnutrition, sepsis, trauma, surgery, burns, advanced age, diabetes, advanced cancer and combinations of these problems may all produce acquired immunodeficiency. When patients on immunosuppressive drugs and drug regiments are added to the previous group, the high incidence of nosocomial infection and deaths associated with infection secondary to suppression of host defense is not surprising.

TABLE 1

Primary Immunodeficiencies

A. ANTIBODY MEDIATED IMMUNODEFICIENCY DISEASE

1. Sex-linked infantile hypogammaglobulinemia[197]
2. Transient hypogammaglobulinemia of infancy[65]
3. Sex-linked immunodeficiency with hyper-IgM[222]
4. Selective IgA deficiency[17]
5. Selective IgM deficiency[121]
6. Selective deficiencies of IgG subclasses[206]

B. CELL-MEDIATED IMMUNODEFICIENCY DISEASES

1. Thymic hypoplasia with hypocalcemia (Di George Syndrome)[75]
2. Chronic mucocutaneous candidiasis (with or without endocrinopathy[14]

C. CHEMOTACTIC PHAGOCYTIC AND BACTERICIDAL DYSFUNCTION

1. Chronic granulomatous disease[127]
2. Lazy leukocyte syndrome[177]
3. Tuftsin deficiency[58]
4. Chediak-Higashi syndrome[225]
5. Glucose-6-phosphate dehydrogenase deficiency[60]
6. Myeloperoxidase deficiency[145]
7. Elevated IgE, defective chemotaxis, eczema and recurrent infections[114]

D. DISORDERS OF THE COMPLEMENT SYSTEM

1. Familial C5 dysfunction[176]
2. C3 deficiency (Type 1, Type II)[12]

D. (cont'd)

3. C2 deficiency[13]

4. ClQ, ClR, ClS deficiency[70]

E. COMBINED CELL-MEDIATED AND HUMORAL MEDIATED IMMUNODEFICIENCY DISEASES

1. Severe combined immunodeficiency[120]

2. Cellular immunodeficiency with abnormal immunoglobulin synthesis[141]

3. Immunodeficiency disease with enzyme deficiency[97]

4. Immunodeficiency with eczema and thrombocytopenia (Wiskott-Aldrich Syndrome)[32]

5. Immunodeficiency with Ataxia-Telangiectasia[192]

6. Immunodeficiency with short-limbed dwarfism[18]

7. Episodic lymphocytopenia with lymphotoxin (Immunologic Amnesia)[13]

4.2 Burns

Most of the early work on acquired depressed host defense was on burn patients. The thermal injury is the most severe form of trauma and the high rate of infection in this group makes them ideal for study. Balch was the first to study neutrophil function in burns[23]. He was able to show normal or increased neutrophil phagocytic and bactericidal activity. However, the leukocyte response to superficial skin trauma using a modified Rebuck skin window test was decreased[189]. Both the rate of delivery of PMNs and lymphocytes to the superficial skin abrasion was decreased and this could not be explained by decreased perfusion. Alexander and his colleagues studied host defenses following burn injury and were able to show that the primary response to an antigen such as alligator erythrocytes was depressed following burn injury, but the secondary or anamnestic response to tetanus toxoid was enhanced[6]. Using a rat burn model they were able to correlate the degree of burn to the depressed primary antibody response. Similar support for an intact or enhanced secondary antibody response has been presented by Balch[22]. Decreased neutrophil bactericidal activity in burned patients, using a different *in vitro* assay, was reported by Alexander *et al*[3]. Furthermore, this decreased bactericidal activity was correlated to development of invasive sepsis[10]. A peculiar variation of normal neutrophil bactericidal function at periodic intervals of 10 and 40 days, observed by these workers, has yet to be

explained. In more recent studies, Alexander et al[7,8] confirmed that in burned patients and patients at high risk for infection, decreased neutrophil bactericidal activity against Staphylococcus aureus 502A was present as was abnormal neutrophil chemotaxis and failure of a delayed hypersensitivity skin test response. However, they could not correlate the latter two to infectious episodes. The bactericidal activity of burn neutrophils towards E. coli was normal. Opsonic index of serum was depressed early after the burn but returned to normal by the fourth to fourteenth postburn day. Serum levels of IgG, properdin and C3 while initially low remained within the normal range after the nineth post burn day. They could not show any association between bacteremia and low levels of C3, IgG, factor B or properdin, though C3 factor B and IgG rose following bacteremia as acute phase proteins. Defective opsonization was associated with a high risk of bactermia only when there was coexisting abnormality of neutrophil function.

Macrophage and reticuloendothelial system function is difficult to study in humans and most data from animals studies are conflicting. Cell mediated immunity is markedly altered following thermal injury. Man and experimental animals will accept allografts from unrelated donors following burns, with grafts lasting up to eight months[129]. Delayed hypersensitivity skin test response is absent in patients with severe burns, and the sequential measurement of skin test response has been shown

predictive of mortality in burn patients[112]. Immunosuppressive activity of normal lymphocyte response to PHA in the serum of burned patients has also been demonstrated[57]. The response of lymphocytes to PHA using a whole blood assay system has been shown to be depressed[8].

Animal studies support the above findings. Rappaport and his colleagues showed that 5-40% total body surface burns in guinea pigs led to loss of delayed hypersensitivity to purified protein derivative (PPD), after 2 wks post burn[186]. Macrophages harvested from the peritoneal cavity however, responded like normals in capillary migration tests incidating serum factors extrinsic to cell function itself may have been interfering with cell-mediated immunity *in vitro* in this model.

4.3 General Surgical Patients

Burned patients are intuitively thought of as susceptible to infection because of the obvious breakdown of the natural defense barrier afforded by the skin. There are, in addition, multiple acquired defects in host defense. Until recently, identification of patients who appear normal physiologically but who have acquired defects of host resistance and thus are prone to infection was not possible. MacLean *et al*[156] reported that failed delayed hypersensitivity skin test response to five recall antigens or to dinitrochlorabenzene sensitization and challenge was depressed in 55 seriously ill and injured patients.

The skin reactions were classified as normal if there was a response to more than two antigens. Relative anergy if there was response to one antigen and anergy if there was no response. A response was defined as induration of 5mm or greater at 24 or 48 hrs. Of the 9 anergic patients, all of them died. There were 18 patients with relative anergy and 28% died. Only two (7%) of the 28 normal responders died. Fifty patients studied preoperatively demonstrated 37 normal responders with 5% minor sepsis (wound infections) and no major sepsis (intracavity abscess or positive blood culture) or death. In 13 patients with anergy/relative anergy, six episdoes of major sepsis occured with one death. Immunosuppressive activity of the mixed lymphocyte reaction was found in the serum of four patients. The groups were matched for age. There was a greater proportion with cancer in the anergic group. Pietsch and Meakins[196] confirmed these observations in studies of 354 surgical patients. They showed that pre-operative anergy predisposed to a high rate of morbidity due to sepsis. Sequential skin test measurements were also found useful in predicting eventual clinical outcome.

Meakins et al[171] expanded studies on host defense in 110 anergic and relatively anergic patients. Of those who failed to convert to normal reactivity,74.4% died compared to only 5.1% in the group whose skin test response became normal. Mixed lymphocyte culture, cell mediated lympholysis and blastogenic

factor were normal in all patients and not clinically helpful. Neutrophil chemotaxis was depressed in anergic patients compared to controls. Furthermore neutrophil chemotaxis improved to normal as skin test response returned to normal. A cell directed serum factor that would cause normal neutrophils to demonstrate decreased chemotaxis was also shown. The total lymphocyte counts were decreased in the anergic group as was the number of T-lymphocytes. Meakins et al[168] further demonstrated the development of skin test anergy and decreased neutrophil chemotaxis following severe blunt trauma, and placed in perspective "the cart and the horse". In this group of patients, skin test anergy and depressed neutrophil function occurred first, followed by major sepsis in the patients at risk. The degree and severity of injury correlated with the presence of anergy and depressed neutrophil function as well as the duration of the defects. Restoration of host defense followed resolution of trauma.

The above findings have been substantiated by other investigators[128]. Johnson et al[126] skin tested 197 male surgical patients admitted to a VA Medical Center for major general, vascular or thoracic surgery. Pre-operative anergic patients suffered a 43% incidence of major complications, compared to 17% in relatively anergic and 14% in normal patients. Mortality was 16% in the anergic group, compared to 2% in normals. With their method of assessing neutrophil chemotaxis, they were not able to detect differences between the two groups.

The variation in their assay was too great to draw any valid conclusions. These authors stress the value of utilizing the full battery of five recall antigens in order to score the patients correctly. For example, if streptokinase was eliminated from their skin test antigens, they would have classified 23% of normal patients as anergic or relatively anergic. Gerther et al[94] carried out anthropometric measurements, serum complement, serum immunoelectrophoresis, lymphocyte T-rosette formation, neutrophil migration and delayed hypersensitivity skin testing in 64 consecutive surgical patients upon admission. There were 62% anergic patients with 33% rate of sepsis compared to 14% rate of sepsis in the normal group. Alexander et al[8] did not find delayed hypersensitivity useful in identifying patients at risk for sepsis, a conclusion not supported by Hiebert et al[112].

4.4 Blunt Trauma

Blunt trauma has been shown to produce depressed host defense detected by delayed hypersensitivity skin test response and neutrophil chemotactic migration. Bjornson et al[34] showed that complement consumption could reduce the opsonic capacity of a patient's serum for certain microorganisms and not for others. Furthermore, the classical complement pathway was activated during septicemia in burns and this occurred preferentially due to inhibition of the alternative pathway. However,in one burn trauma study, there were abnormalities detected in serum immuno-

globulins (IgM) and in the alternative and classical pathways of complement[33]. Bauer et al[26] found significant decrease in the numbers of circulating T-lymphocytes 24 to 48 hrs following severe blunt trauma. The counts dropped from 1,748 + 600 cells/mm^3 to 600 or less. A similar drop was noted following controlled surgical trauma, i.e. elective, major surgery. The decrease in both cases was transient lasting up to five days.

4.5 Surgery

Slade et al[213] found that a standard donor nephrectomy which lasted 3 hrs 20 mins led to depression of delayed hypersensitivi skin test response immediately post-operatively and the effect lasted up to 2-3 wks. In vitro tests of T-cell and B-cell lymphocyte function were also depressed immediately following induction of anesthesia and stayed depressed up to five days. The correlation between in vitro and in vivo assessment of cell mediated immunity was found to be poor. However, McCloughlin et al[161] showed excellent correlation between the appearance of immunosuppressive activity in the serum and skin test anergy in patients following major surgical trauma. Pietsch et al[197] studied 52 surgical patients 75% of whom had major surgery, 1-8 days post operatively and found no significant difference in the post operative skin test response when this was compared to the pre-operative control value. Roth et al[198] studied

in vitro lymphocyte function in 35 cancer patients undergoing surgery. They detected depressed function that returned to normal by the fourth week. Though there are discrepancies between these studies in large part accounted for by differences in methodology, it is clear that trauma, either controlled or uncontrolled causes transient depression in host defense.

Other conditions are associated with depressed host defense as indicated by skin test anergy. One interesting association is leukocytosis as indicated by Heiss and Palmer[110]. These authors studied 32 patients with leukocytosis greater than 15,000/mm^3 with a variety of clinical conditions. Eight of 32 or 25% had complete anergy. Interestingly 75% of these had bacterial infection as the cause of the leukocytosis. This would indicate that sepsis was precipitating the anergic state.

4.6 Advanced Age

Age is associated with anergy. Pietsch *et al*[195] showed that the incidence of skin test anergy increased above the 8th decade. Burgio *et al*[90] showed that the intensity of the reaction depended upon the age of the patient based on over 600 skin tests. Waldorf *et al*[240] were able to show that both arms of cell-mediated immunity, involved in the establishment of a delayed hypersensitivity response were defective in patients over 70 years of age. In this group DNCB sensitization was achieved in only 69% compared to 94% sensitized to DNCB in those 69 years or less.

4.7 Neoplastic Disease

Neoplastic disease may amongst its many effects, alter the immune system as measured by DH skin test response. It is not clear however if the failed response is due to the tumor load per se or to associated conditions due to the cancer, one of which may be malnutrition. Lamb et al[135] studied 57 patients with Hodgkin's disease, 59 with cancer, 41 with non-Hodgkin's lymphoma and 35 patients with leukemia using 5 recall antigens. Their control group was 208 male patients with no cancer. They showed that 1% of controls had anergy whereas 58% of patients with Hodgkin's lymphoma were anergic. Patients with cancer had 20% anergy, leukemia 22%, and those with non-Hodgkin's lymphoma had 34% anergy. Subdivision of the patients into those in "good clinical condition" and "poor clinical condition" (including cachexia) showed a greater proportion of anergic patients in the poor clinical condition group. No correlation with clinical outcome was made. Confirmation of depressed or absent DH in patients with cancer has been presented by Pinsky and coworkers[195]. Rao et al[185] studied 237 patients with carcinoma of the colon and rectum for their ability to react to a new antigen DNCB and to four recall antigens. They found that patients with Dukes' A had 75% positive response to DNBC compared to 56% in those with Dukes' B lesions, 61% Dukes' C and 42% in those with Dukes' D. The data with recall antigens showed no difference between the four stages of disease

though the presence of cancer did predispose to skin test anergy. No controls were included. There was a correlation between response to DNCB and outcome. Those with positive DNCB response tended to have more favourable recurrence and survival rates with each stage of the disease after 12 months observation. No data on 5 year survival rates were presented.

4.8 Malnutrition

The relationship between malnutrition and infection has been observed for a long time. Studley[226] found that in patients undergoing surgical treatment for peptic ulcers, there was a 33% mortality rate in those with a preoperative weight loss of more than 20% of body weight as compared with a 3.5% mortality in those with better nutrition. Animal studies have demonstrated a striking increase in susceptibility of animals fed suboptimal diets to bacterial infections[81,174]. Both B-cell and T-cell immunity have been shown depressed *in vivo* and *in vitro* animal models[44,262]. The most convincing documentation of the interaction of malnutrition and infection in human beings, has been made in children from underdeveloped countries where nutritional deficiencies are common[207]. Most studies on humeral immunity have shown that protein calorie malnutrition is not associated with depressed levels of serum immunoglobulins[185]. Actually, the level of gamma-globulin synthesis may be increased in

children with Kwashiokor[49]. The response to primary antigens is disputed, with some authors detecting a diminished response[93] and others in more recent studies detecting no differences between controls and malnourished patients[29]. The effect of protein and calorie malnutrition on the complement system in man is not known. Studies of PMN function during malnourished states have demonstrated normal phagocytic activity[29,229]. PMNs from malnourished patients demonstrate lower glycolytic activity at rest or in response to particle challenge, stimulating phagocytosis. There is also low hexose monophosphate shunt activity[210]. Controversy exists whether malnutrition in general or just protein deficiency is responsible for the observed defects. Cooper et al studied the effects of chronic protein deficiency in a successive faster-litter model in mice and rats and found that primary immunity was unaffected when Brucella abortus was the challenge antigen, whereas if sheep erythrocytes were used a depressed primary antibody response was observed. Furthermore, there was enhanced graft-vs-host reactions, accelerated rejection of skin allografts, enhanced resistance to viral infections and enhanced PHA response to spleen cells. Interestingly, the resistance to challenge with bacterial infection in the form of streptococcus was depressed[61]. Strivastana et al observed that although protein depletion of rats resulted in atrophy of the lymph nodes, the protein,

ribonucleic acid and deoxyribonucleic acid contents of various lymphoid organs from protein-depleted rats were significantly higher than in the rats fed regular diets[220]. This indicates that biosynthetic functions of individual lymphocytes from protein-depleted rats remain intact and may partially explain the enhanced *in vitro* T-lymphocyte function seen in protein depleted rats. In contrast, Daly *et al* showed that protein depletion in rats led to loss of DH response to PPD, indicating that this *in vivo* assessment of cell mediated immunity becomes depressed following malnutrition[68]. More recent data by Dionigi *et al*[76] indicated that dogs made progressively malnourished by protein deprivation have deficiencies of IgG and C3 as well as decreased primary antibody response to sheep red blood cells, lymphocyte counts, lympocyte response to PHA, and neutrophil chemotaxis. The significance of these observations in susceptibility to infection is not clear. It is difficult and hazardous to extend observations in animal models to man, though there is evidence in the literature suggesting that malnutrition is associated with depressed host defense. Good reviews of the subject have been presented by Law *et al*[140] and Ota *et al*[189].

MATERIALS & METHODS

5. SKIN TESTS AND PATIENT SELECTION

5.1 Patient Selection

Patients on the surgical wards and the Intensive Care Unit of the Royal Victoria Hospital were used in this study. Verbal or written informed consent was obtained. Initially patients undergoing all types of operations were studied, but in the later phase of this research, skin tests were performed on a more selective patient population which included patients that were to undergo operations more complex than a cholecystectomy. Some patients were transferred to this hospital, after complications had developed at a peripheral hospital, for specialty care, and all these patients were studied as soon as possible after admission. Sequential testing was carried out in all patients demonstrating an altered initial response, and whenever possible in patients with initial normal responses.

5.2 Skin Test Methodology and Definition of Anergy

Skin testing was carried out by the intradermal injection of the following five recall antigens: Mumps skin test antigen, (undiluted, Eli Lilly Co., Indianopolis, IN); Candidin (1:100 dilution, Hollister-Stier Laboratories, Mississauga, Ont.); Purified Protein Derivative (PPD), 5 T.U./0.1 ml, Connaught

Laboratories, Willowdale, Ontario); trichophytin, and streptokinase-streptodornase (Varidase, 100 u/cc, Hollister-Stier Laboratories, Mississauga, Ontario). In addition, a control solution of buffered diluent (Hollister-Stier Laboratories) was used. Syringes were prepared with 0.1ml of each antigen solution immediately prior to use. Tests were administered and interpreted by the same person, with random cross-checks by an independent observer in a blind fashion. The average diameter of induration at 24 and 48 hrs was measured and recorded, and a positive response was defined as induration greater than 5mm in diameter at either reading. The patients were then classified on the basis of their response as follows: Normal (N), two or more positive responses to any of the five antigens; Relative Anergy (RA), one response; Anergy (A), no response to any of the five antigens.

5.3 Classification of the Degree of Surgery

In order to have a measure of the type and severity of operations performed on these patients, five operative classes progressively increasing in severity and complexity were arbitrarily created. Class 1: breast biopsy, inguinal herniorhaphy, varicose vein stripping, closure of colostomy. Class 2: laparotomy and biopsy, Cholecystectomy (±CBDE), thyroidectomy. Class 3: hemicolectomy, pneumonectomy, vagotomy and antrectomy,

Abdomino-perineal resection, small bowel rection. Class 4: aortofemoral bypass, resection of abdominal aortic aneurysm, partial hepatectomy, small bowel fistula closures with resection. Class 5: total pancreaticoduodenectomy, porto-systemic shunts, esophagogastrectomy, total gastrectomy, total proctocolectomy.

6. CLINICAL DATA COLLECTION

6.1 General Data Collection - All Patients

All patients skin tested had the following basic data collected and recorded. Sex, age, admission diagnosis, past medical history, medications, and initial skin test response. Their hospital course was closely followed thereafter and abstracted to include: febrile episodes, operation (type, duration, findings, pathology reports, transfusion requirements, serial hemoglobins and hematocrits, serial albumins and abnormal SMAC parameters, serial CBC determination; requirement for Total Parenteral Nutrition (TPN) and duration; presense of major sepsis defined as a positive blood culture, an abscess detected at surgery or autopsy or fatal pneumonia; and mortality, including autopsy findings if available, or discharge. Temporal measurements of neutrophil function were also recorded along with skin testing to correlate adequacy of host defense and clinical outcome.

6.2 Clinical Data Collection - Special Areas

6.2.1 Cancer Patients

In addition to the general data collection stated above, all patients with an admission diagnosis of malignancy had the following data collected. History of recent weight loss and quantification. Type of cancer and duration of symptoms. Extent of disease proven by pathologic examination of surgically removed specimens, autopsy findings, or visual observation at the time of laparotomy.

6.2.2. Patients with Gastrointestinal Hemorrhage

Patients admitted with the endoscopically or angiographically proven diagnosis of gastrointestinal (GI) hemorrhage had the following data collected. Skin test response as soon as possible upon admission. Measurement and recording of admission pulse, blood pressure and hematocrit. Accurate recording of blood and crystalloid transfused, before and after initial skin tests. The need for operative intervention and the outcome.

7. LYMPHOCYTE FUNCTION STUDIES

7.1 Circulating Total Lymphocyte Counts

Circulating total lymphocyte counts were obtained from data supplied by the hospital clinical laboratory and are based on total leukocyte counts and differential leukocyte counts

obtained from either manual or automated determinations using an electronic particle counter (Coulter Counter, Coulter Electronics Inc., Hialeck, FL).

7.2 T-Lymphocyte Counts

Estimation of the number of circulating T-lymphocytes in normal laboratory controls and hospitalized patients was carried out by modifications of the sheep erythrocyte rosette (T-rosette) technique. Both total[265] and active[265,215] E-rosetting cells were measured. Leukocyte rich plasma (see later) was centrifuged for 10 min at 200 x g. All but 1 ml of the supernatent was discarded and 1 ml of physiologic saline (normal saline) was added to the tube. The cell pellet was resuspended and then layered carefully onto a 3 ml of ficoll-hypaque (Pharmacia Fine Chemicals) in a 15 ml plastic tube. The tube was centrifuged at 480 x g for 25 min. The mononuclear cells collected at the interface were skimmed off with a pasteur pipette and transferred to another 15 ml tube. Two thirds volume of Hanks Balanced Salt Solution (HBSS, Microbiolgoical Associates) was added and mixed and the tube centrifuged at 200 x g for 10 min. The cells were washed two more times with HBSS and resuspended in RPMI-1640 (Microbiological Associates) supplemented with 25% decomplemented fetal calf serum (FCS, Microbiolgoical Associates) at a cell concentration of 6×10^6 cells/ml. Sheep red blood cells (SRBC) stored in Alserver's Solution not longer than 2 weeks at $4^{\circ}C$,

were washed thrice with normal saline and resuspended in RPMI-25% FCS at a concentration of 4.8×10^7 cells/cc. To obtain the total T-rosette forming cells, equal 0.1 ml volumes of lymphocyte suspension and SRBC suspensions were mixed in a small plastic tube in duplicate and left overnight at room temperature. The following morning the pellet was resuspended gently, and one drop of cell suspension was mixed with one drop of 0.03% toluidine blue (in normal saline) on a glass slide. The slide was covered with a coverslip and cells were allowed to settle. This was examined under a microscope and 300 cells were counted noting those that formed rosettes. A rosette was defined as a SRBC with 3 or more lymphocytes attached to its surface. The percent of total T-rosette forming cells was then determined from the following formula.

$$\% \ E_t\text{-rosettes} = \frac{\text{Number of SRBC rosettes}}{\text{Total number lymphoyctes counted}}$$

To obtain the active T-rosette forming cells, equal 0.1 ml volumes of SRBC and peripheral blood lymphocyte suspensions were mixed and centrifuged immediately at room temperature for 5 min at 200 x g. The pellets were then gently resuspended and the same procedure was followed as above to obtain the per cent of active T-rosette forming cells using the formula:

$$\% \ E_a \text{ - rosettes} = \frac{\text{Number of SRBC rosettes}}{\text{Number of total cells counted}}$$

7.3 PHA Stimulation of Lymphocytes

Lymphocytes were prepared as above and resuspended in RPMI 1640 supplemented with 15% normal human AB serum, 100 units penicillin and 100 micrograms gentamycin/ml, plus HEPES buffer pH 7.4. Four ml aliquots of 1 x 10^6 cells/ml were distributed into plastic tubes and 10 µg/ml of PHA was added (Grand Island Biochemicals). The tubes were capped tightly and incubated upright at 37°C for 3 days. After this, tritiated thymidine, 50 µC (0.2 mC/mmole), was added and the cells incubated for a further 18 hours. Appropriate nonstimulated controls were included. After determination of DNA incroporated radioactivity by collecting the DNA onto glass fiber filters, a stimulation index was calculated from the following formula:

$$\text{Stimulation Index (SI)} = \frac{\text{counts/min/PHA stimulated tube}}{\text{counts/min/PHA unstimulated tube}}$$

7.4 Lymphocyte Chemotaxis

7.4.1 General Considerations

In the initial experiments on lymphocyte chemotaxis, cells were prepared as in Section 3.3 and stimulated for 3 days with PHA, following which, the tubes were centrifuged and stimulated lymphocytes were collected and resuspended in Minimal Essential Medium (MEM, Microbiological Associates) supplemented with 10% decomplemented FCS to a concentration of 3 x 10^6 cells/ml. These cells were then used to measure lymphocyte chemotaxis. In later experiments, lymphocytes were prepared fresh and used without prior stimulation.

7.4.2 Chemotactic Chambers

Lymphocyte chemotaxis was measured as follows. Sykes-Moore tissue culture chambers were separated in two areas by means of a 2.5 cm diamter, 150 μ thick, 3 μ pore diameter nitrocellulose filter (Millipore Corp.). Rubber gaskets and round glass coverslips were used to create upper and lower compartments of about 0.7 ml volumes. Casein 5 mg/ml (Hamerstein, BDH Biochemicals) dissolved in MEM-10% FCS was added to the lower compartment as a chemoattractant and the lymphocyte suspension was added to the top compartment. The chambers were then placed flat in a dry air incubator at 37°C for 5 hrs. Following this the filters were removed, processed and chemotactic migration using the leading front technique was measured in a blind fashion as for PMN chemotaxis.

7.4.3 Effect of Time, Metabolic Inhibitors, and Filter Pore Diameter

To assess the optimum time for lymphocyte chemotaxis, chemotactic chambers were set up using fresh normal control lymphocyte Chemotactic migration was allowed to occur for one, two, three, four and five hours, following which chemotaxis was arrested by placing the chambers at 4°C. The filters were then processed for reading.

To assess the effects of metabolic inhibitors and heat on lymphocyte chemotaxis, cells were resuspended in MEM-10% FCS and then heated to 70°C for 30 min. The cell viability was

assessed as below. Separate aliquots of PBL were resuspended in MEM-10% FCS containing potassium fluoride (KF) 0.1 M or ethelenediaminetetraacetic acid (EDTA) 0.1%, and incubated for 30 min at room temperature. Chemotaxis was then assessed with 3 μ pore filters and 5 hr incubation.

The effect of pore size on lymphocyte chemotaxis was assessed by setting up chemotactic chambers using 3 μ, 5 μ, and 8 μ nitrocellulose filters. The chemotaxis of normal lymphocytes was then measured with these using a 5 hr incubation.

7.4.4 Random Lymphocyte Migration

Random lymphocyte migration was assessed using standard chemotactic chambers with 3 μ filters to which MEM-10% FCS was added in the lower compartment in place of casein. Incubation time of 5 hrs was used and filter processing and reading was the same as for assessement of chemotactic migration.

7.4.5 Lymphocyte Viability, Differential Counting and Special Staining

Lymphocyte viability was assessed by mixing equal volumes of the final lymphocyte preparation to be used for chemotactic measurements and 0.20% trypan blue[230] in phosphate buffered saline (PBS). After a 30 min incubation at 37°C, the tubes were centrifuged for 5 min at 200 g and the supernatent was discarded.

The pellet was resuspended in a drop of PBS and transferred to a glass slide. Two hundred cells were counted noting those stained blue, thus containing the dye.

Differential counts of the final lymphocyte suspension to be used for chemotactic measurements were performed as follows. Aliquots of the suspension were centrifuged 5 min at 200 x g and the cell pellet was resuspended in 3 drops of normal saline. Smears were made of this on clean glass slides and air dried. The slides were then stained with hematoxylin or Wright's Stain (Fisher Scientific) and examined under oil. The % PMN contamination was assessed by dividing the numbers of PMN, easily identified due to their characteristic nuclei, by the total numbers of cells counted. The rest of the cells were differentiated on the basis of nuclear morphology, nucleus to cytoplasm ratio, and cell diameter. Cells with dense dark nuclei, high nucleus to cystoplasm ratio and under 8 μ diameter were classified as small lymphocytes, and all the others (excluding PMN) as large cells. The large cells were further separated into large lymphocytes and monocytes-macrophages using special stains.

Air dried smears were stained for myeloperoxidase activity as follows. Incubation mixture: 30% ethanol 100 ml; benzidine dihydrochloride 0.3 gm; 0.132 M Zinc Sulfate, 1 ml;sodium acetate ($NaC_2H_3O_2$-$3H_2O$) 1.0 gm; 3% H_2O_2 0.7 ml; NaOH 1.0 N, 1.5 ml; safranin 0, 0.2 gm. This was made up by adding the reagents in order shown and stored tightly capped up to 6 months. The

smears were fixed in 10% formol-ethanol for 60 seconds at room temperature and then washed for 15 seconds with running tap water. The wet slides were placed in the incubation mixture for 30 seconds, washed for 10 seconds in tap water, dried and examined. The myeloperoxidase containing cells were identified by their content of black granules. PMN were easy to identify and thus the % monocytes was obtained by subtracting the numbers of PMN from the numbers of myeloperoxidase containing cells and dividing by the total number of cells counted. Chemotactic filters were fixed in formal-ethanol and similarly stained. They were dried and cleared with xylene before being examined with a microscope.

The non-specific esterase staining method[232] was also used to identify monocytes. All reagents were commercially obtained from Technicon Instruments Corporation, Tarrytown, N.Y. In tube I was added 6 drops nitrate (T01-0530) and 6 drops dye (T01-0529) and mixed well, followed by 18 drops buffer (T01-0678) and 3 drops of substrate (T01-0680). In tube II, 2 drops of cell suspension (prior to making smears) 2 drops FCS, 2 drops lyser (T01-0554) and 4 drops fixative (T01-0679) were added and allowed to stand for 40 seconds at room temperature. Then tubes I and II were mixed and incubated for 8 min at 37^{o}C. The cells were pelleted,resuspended in buffer,and a wet smear made. Examination under the microscope showed the monocytes stained deeply red. The percentage monocytes was then obtained by dividing the red cells by the total number of cells counted excluding PMN and multipled by 100.

Once the number of monocytes was known in the total population of large cells, the percentage of large lymphocytes in the initial cell preparation used for chemotaxis was obtained from the formula:

$$\% \text{ large lymphocytes} = \frac{\text{numbers of large cells - numbers of monocytes}}{\text{total number of mononuclear cells}}$$

Staining of chemotactic filters for non-specific esterase was done as follows: Tube I was set up as before, Tube II was replaced by a staining dish and a filter replaced the cell suspension. Fixation was for 10 mins. After mixing, the staining was allowed to progress overnight at room temperature[57]. The following day the filters were dried, cleared with xylene and examined.

7.4.6 Separation of Chemotaxis from Chemokinesis

To establish whether this system measured chemotaxis or chemokinesis of lymphocytes, chemotactic chambers were set up where the concentration of casein was varied from 0, 0.5, 1.0, 2.5 and 5.0 mg/ml in the upper and lower compartments of the chamber. Normal lymphocytes were used and cell migration proceeded for 5 hrs in 3 μ pore diameter filters. After examining the filters and obtaining the migration distances in the zero gradient chambers, the technique of Zigmund and Hirsch[267] was utilized to calculate the predicted migration distance in each chemoattractant combination and these were then compared with the measured distances within each filter.

7.4.7 Standarized System for Measurement of Lymphocyte Chemotaxis

The standard system used to measure lymphocyte chemotaxis from normal controls and hospitalized patients was as follows. Lymphocytes were obtained from peripheral blood following ficoll-hypaque centrifugation and a differential count as well as percentage of large lymphocytes per preparation was carried out. No sample with PMN contamination greater than 5% was used. Casein 5 mg/ml in MEM-10% FCS served as chemoattractant. Filter pore size was 3 μ. Incubation time was 5 hr. Filter staining and reading was as for PMN chemotaxis.

8. POLYMORPHONUCLEAR LEUKOCYTE STUDIES

8.1 Adherence

8.1.1 Whole Blood Adherence

The method of McGreggor et al[54] was modified and used to measure the adherence of leukocytes in whole blood. The standard system is depicted in figure 1. A 1 cc tuberculin syringe was fitted with a 44.5 mm 25 G needle and packed with 30 mg of scrubbed nylon fiber (Leuko-Pak Leukocyte Filter, Code 4CZ401, Fenwall Laboratories, Deerfield, Illinois). The fiber was prewetted with MEM and the syringe was allowed to stand in a 12 x 75 mm borosylicate tube in a test tube rack. The whole assembly was prewarmed to 37°C. Each blood sample was assayed in triplicate. To perform the assay, 1 ml of whole blood was added to the syringe by means of a 1 ml tuberculin syringe fitted with a 14 G,

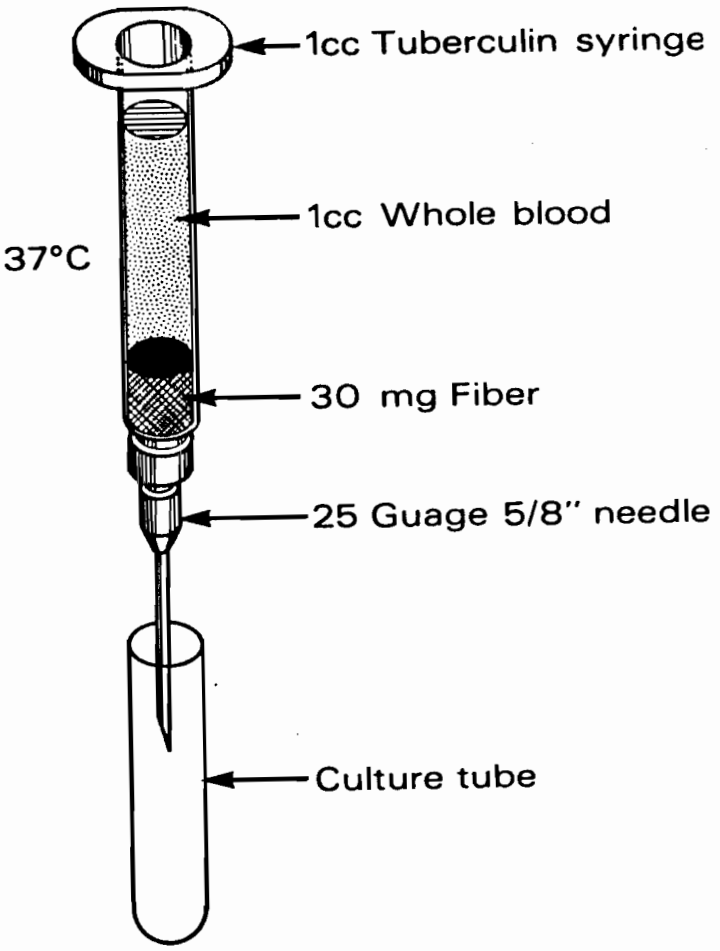


Figure 1. A graphic representation of the neutrophil adherence assay system.

88.9 mm needle and the blood was allowed to percolate through the fiber at 37°C under gravity flow. Subsequently, the leukocyte count was determined in the effluents and in the original sample, by means of an electronic particle counter (Coulter Counter, Model Z_f, Coulter Electronics). The percentage adhering leukocytes was calculated from the following formula:

$$\% \text{ whole blood leukocyte Adherence} = \frac{A - B}{A}$$

where A = initial leukocyte count

B = average leukocyte count in the three effluents

To determine the optimum fiber weight required for the whole blood adherence (WBA) assay, syringes were packed with 2.5 mg, 5, 10, 20, 30, 40, 60, 80 and 100 mg fiber and the adherence of normal whole blood determined in triplicate.

The day to day variation in control adherence was determined by measuring the adherence in four subjects (2M, 2F) on several different days during this study. Blood samples were usually drawn in the morning.

The effect of metabolic inhibitors was assessed by adding directly to whole blood enough solid KF to reach a concentration of 0.1 M and EDTA at 0.1% following which WBA was measured.

8.1.2. Purified Leukocyte Adherence

Purified leukocytes at a concentration of 3-5 x 10^6 cells/ml in MEM were obtained as described below. The assembly for measurement of the purified leukocyte adherence was the same as for whole blood adherence except that the fiber weight was 5 mg. The assembly and purified leukocyte suspensions were prewarmed to 37°C and the adherence determined as for whole blood adherence determinations.

Day to day variation in control purified leukocyte adherence was assessed in four different subjects. The effect of metabolic inhibitors was assessed by incubating the cells in 0.1 M KF in MEM or 0.1% EDTA in MEM, and subsequently measuring the adherence. A separate aliquot was also heated at 70°C for 30 min and after assessing cell viability by trypan blue, the adherence of this preparation was measured. The effect of pH on leukocyte adherence was assessed by resuspending the cells in MEM at various pH from 6-8.5.

8.1.3 Purified Polymorphonuclear Neutrophil Adherence

Purified PMN were obtained as described under the section PMN Phagocytosis and bactericidal functions. They were resuspended in MEM at 3-5 x 10^6 PMN/ml. The adherence of this cell type was measured using the purified leukocyte system. Independent confirmation was obtained by performing differential cell counts on the effluents and the original sample after carrying out a whole blood adherence assay.

8.1.4 Patient Studies

The whole blood adherence system was used to study the adherence of patient leukocytes in most cases. Specialized studies employed the purified leukocyte adherence assay. Heparinized blood was drawn in the morning and kept at 37°C before use. To assess for effects of patient serum or plasma on normal neutrophil adherence, additional blood was obtained in heparinized and non-heparinized tubes. After clotting at room temperature for 2 hrs the clots were rimmed and the serum was collected and stored at 4°C after centrifugation of the clot at 200 x g for 10 min. Plasma was collected by centrifugation of heparinized whole blood at 200 x g for 10 min and stored at 4°C until use. Patients for study were selected on the basis of the skin test response, blood being drawn as soon as the skin test results were available.

8.1.5 Effects of Serum and Plasma on Leukocyte Adherence

Purified leukocytes were obtained from normal controls and resuspended in MEM, patients serum or patient plasma. After a 30 min incubation at 37°C the adherence was measured using 5 mg columns in the purified leukocyte adherence system. The whole blood adherence of the normal control leukocyte donor as well as the patient donating the serum and plasma were also measured.

8.1.6 The Effects of Drugs on Leukocyte Adherence

The desired drug concentration was achieved by adding appropriate small volumes of high concentrations of the drug in PBS directly to whole blood. After mixing and incubation at 37°C for 30 min. the leukocyte adherence was measured using the whole blood adherence system and compared to a control blood sample to which was added PBS only. To study the effect of drugs in the absence of blood components, the drug was dissolved in MEM which was then used to resuspend normal leukocytes before measuring their adherence in the purified leukocyte adherence system.

8.2 Polymorphonuclear Neutrophil Chemotaxis

8.2.1 Preparation of Purified Leukocyte Suspension

Whole blood was obtained by venopuncture in heparinized vacutainer tubes or in plastic syringes with 10 U Heparin per ml blood. A 2 ml volume of Dextran-70 (Pharmacia Fine Chemical) was added per 10 ml blood and mixed. The erythrocytes were allowed to sediment at 1 x g at room temperature for 1 hr. Subsequently, the leukocyte rich supernatant was pipetted into 17 x 100 mm plastic disposable tubes (Falcon®, Becton Dickinson) and centrifuged at 200 x g for 10 min. The supernatant was discarded and the cell pellet was washed three times with HBSS

and resuspended in MEM at a concentration of 3-5 x 10^6 cells/ml. This preparation was used for PMN chemotactic measurements, or in the purified leukocyte adherence assay.

8.2.2 Preparation of Casein as Chemoattractant

Casein (Hamersten, BDH Biochemicals) was dissolved in MEM at 5 mg/ml by alkali hydrolysis. This involved adding enough 1N NaOH to approximately 90 ml MEM to bring this to pH 12.0, adding 500 mg Casein and stirring, until all the casein had dissolved. The pH was then reduced to 7.2 by dropmise addition of 0.5 M $NaHPO_4$ and the volume adjusted to 100 ml with fresh MEM. This was filter sterilized using a 0.45 μ pore diameter millipore filter and stored in 10 ml aliquots at 4°C until use. Fresh casein solution was prepared every 2 weeks.

8.2.3 PMN Chemotactic Assay

Sykes-Moore tissue culture chambers were divided into upper and lower compartments by means of a 2.5 cm diameter, 3 μ pore size, 150 μ thick nitro-cellulose filter (Millipore Corp.), neoprene rubber gaskets and round glass coverslips. By means of a 25 G needle as a vent and a tuberculin syringe, casein 5 mg/ml in MEM was used to fill the bottom compartment ($\sim$ 0.7 ml) and the leukocyte suspension was placed in the upper compartment. The chamber was then incubated for 90 min. at 37°C in a dry air incubator following which, cell migration was arrested by placing

the chambers at 4°C. Each sample was assayed in triplicate. The filters were processed and analyzed as described in the following sections.

8.2.4 Filter Staining Method

The method of Wilkinson[256] was used. Filters were fixed in absolute ethanol 2 min, and rinsed in distilled water for 2 min. Then they were placed in Harris' Hemotoxylin for 1 min, followed by distilled water for 2 min. Next followed placement in tap water for 10 min, 70% ethanol 2 min, 95% ethanol 3 min and 80% ethanol: 20% butanol for 3 min. The filters were cleared by placing in xylene for 10 min. They were then mounted on glass slides using permount: xylene::1:1 and examined immediately.

8.2.5 Method of Determining the Distance Travelled by the Leading Front Cells[267]

The mounted filters were examined under the 40X objective of an Olympus microscope equiped with a micrometer rack. A general appraisal of the slide was made noting cell appearance, migration and contamination. Attention was then directed to the starting side of the filter located by the main mass of cells. Focusing into the filter demonstrated the PMNs with their characteristic nuclei which had migrated into the filter. The depth of focus was adjusted to the opposite side of the filter noting the numbers of PMN that had reached this side. Focusing was

then reversed coming up towards the starting side, until the leading front two cells were in clear focus. A note was made of the micrometer reading. To ensure that this was indeed the leading edge of the cell gradient,the focus was adjusted upwards till more cells were visible within 10 μ of the leading front cells. If not, a new pair of leading cells was chosen. The focusing was adjusted until the starting side of the filter was located and the reading on the micrometer rack was noted. The distance from the starting side of the filter to the leading front cells was the chemotactic migration of that field. A new microscopic field was chosen at random and the procedure repeated. All slides were examined in a blind fashion. The mean migration distance of five random fields per slide was determined and each sample was assayed in triplicate. The migration distance was then reported as the mean ± SD of these readings.

8.2.6 Random PMN Migration

To assess random PMN migration, chambers were set up and assayed as for chemotactic migration except that casein was omitted from the bottom chamber which was filled with MEM only.

8.2.7 Patient Studies

Patients were chosen for study of their PMN chemotaxis on the basis of their skin test response or clinical course.

An attempt was made to study all patients as soon as their skin test result became available. Patients sustaining trauma were studied as soon as possible after admission. Serum was also collected whereever possible and stored at -70°C in a REVCO refrigerator for later analysis in the search for serum inhibitors of PMN chemotaxis. In some patients plasma was also stored for later analysis.

8.2.8 Effect of Serum, Plasma and Drugs on PMN Chemotaxis

To study the effect of patient serum or plasma on normal control PMN chemotaxis, a laboratory control was bled and leukocytes prepared as described. During the final wash they were resuspended in serum or plasma from patients whose autologous PMN chemotaxis at the time of serum or plasma collection was already known. The cells were then loaded directly on chemotactic chambers after a 15 min incubation or washed once in MEM, resuspended in MEM and assayed. The effect of drugs on PMN chemotaxis was studied by resuspending normal control PMN in MEM into which the drugs were dissolved at the appropriate concentration. In separate experiments, the drug was added directly to whole blood, the PMNs were isolated and their chemotaxis assessed.

8.3 Polymorphonuclear Neutrophil Phagocytosis & Bactericidal Function

The method of Quie and coworkers[181] was modified and used in these studies. All procedures were carried out using aseptic technique.

8.3.1 Preparation of Bacteria

The bacteria used was Staphylococcus aureus 502A, which was prepared in batches every 3 months. A 10 ml aliquot of an overnight culture of Staphylococcus aureus 502A was innoculated into 200 ml of Mueller-Hinton broth plus 5 mC of ^{3}H-thymidine (Swartz/Mann Biochemical 17 C/mmole) and incubated at 37°C for 2 days. Following this, the bacteria were washed three timeswith cold normal saline using centrifugation at 10,000 x g for 20 min in an International Preparative centrifuge (#870 Rotor), to collect the bacterial pellet. They were resuspended in phosphate buffered saline supplemented with 5% glycerol to a concentration of 5 x 10^{8} cfu/ml using an electronic particle counter (Coulter Counter, Model Z_f, 30 μ aperture) and a standard pour plate technique for confirmation. The bacteria were then stored at -70°C in 2 ml aliquots.

8.3.2 Bacterial Opsonization

A large store of normal human serum was obtained from ten hosptilized patients and diluted to 10% with MEM. This was distributed into 2ml aliquots and immediately stored at -70°C until use. All phagocytosis and bactericidal assays in this thesis were carried out using this source unless stated otherwise. Bacteria and 10% normal human serum (NHS) were thawed. The bacteria were centrifuged at 2000 x g for 20 min at 4°C and the suppernatent was discared. They were resuspended in an

equal volume of 10% NHS and incubated at 37°C for 15 min to achieve opsonization. This tube was then centrifuged at 2,000 x g for 20 min at 4°C, and the supernatent was discarded. The bacterial pellet was stored on ice in a capped tube until use.

8.3.3 Polymorphonuclear Neutrophil Preparation

Peripheral heparinized venous blood was used to prepare a leukocyte rich supernatent. This was centrifuged at 200 x g for 10 min, all but 0.5 ml of the supernatent was discarded, and 0.5 ml of normal saline was added to the tube. After resuspension, the cell pellet was loaded onto 3 ml of ficoll-hypaque (Pharmacia). Centrifugation at 480 x g for 30 min followed and the mononuclear and PMN cell fractions were isolated. The PMN pellet with some erythrocyte contamination was resuspended in 10 ml of sterile 0.87% NH_4Cl and placed on a tilting table aliquot mixer at 37°C for 20 min to lyse the red cells. The PMNs were collected by centrifugation at 200 x g and the process repeated. The PMN were then washed in MEM and resuspended to a concentration of 1 x 10^7 PMN/ml. Cell viability was assessed by means of trypan blue, and smears were prepared as before, and stained with Wright's stain and myeloperoxidase stain to assess the cell population utilized.

8.3.4 Phagocytic and Bactericidal Assay

The assay was set up as follows for each patient. The opsonized bacterial tube was made up to the original stored volume with warm MEM and mixed. Five 12 x 75 mm plastic, capped tubes were used and into each was placed 0.15 ml of the bacterial suspension, containing 7.5×10^7 bacteria. To tubes 1, 2 and 3 was added 0.75 ml of PMN suspension containing 7.5×10^6 PMN, while 0.75 ml of a heated PMN suspension was added to tube 4 and 0.75 ml of MEM to tube 5. Immediately upon mixing, aliquots of 100 µl and 50 µl were withdrawn using ependorf pipettes. The former was placed in 4 ml of cold PBS in an ice bucket and the latter into 5 ml of cold sterile distilled water. The assay tubes were then placed on a tilting table aliquot mixer (Fisher Scientific) at 37°C. Samples of 100 µl were again taken at 5, 10 and 20 min and placed in cold PBS. Samples of 50 µl were taken at 10, 20, 40, 80, 160 and 240 min and placed in cold distilled water. In separate experiments, the PMN: Bacteria ratio was varied by resuspending the starting PMN and/or bacteria to the required concentration so as to keep the same reaction volumes i.e. 0.9 ml.

8.3.5 Analysis of the Phagocytic Assay

Analysis of tubes of phagocytosis was as follows. Tubes taken at time zero were centrifuged at 2,000 x g for 20 min and the supernatent discarded. The pellet was washed once with cold

PBS and then resuspended in 5% trichloroacetic acid (TCA) and left overnight in the cold room. The tubes obtained at 5, 10 and 20 min were centrifuged at 160 x g for 5 min, the supernatent discarded and the PMN-bacteria pellets were washed twice with cold PBS. The pellets were resuspended in 5% TCA and left overnight in the cold room. The following day the precipitated DNA was collected by filtration of the samples through 2.4 cm glass fiber filters (GF/A, Whatman, Canlab Biochemicals) using an FH-200 ten place filter holder (Hoeffer Scientific Instruments, S.F., California). The filtrates were discarded. The filters were washed with 95% ethanol and dried with ether. They were placed in 15 ml glass scintillation vials with 10 ml of toluene based counting fluid (0.08 g POPOP and 16 g POP per 4 L toluene). Radioactivity was determined with a Packard, Tri-Carb, Liquid Scintillation Spectrometer using the tritium chanel with apperture 50-670. The results are expressed as follows. The zero time tube represented total available bacteria and the 5, 10 and 20 min tubes (#1, 2 & 3) the PMN - associated bacteria. Tube 4 represented the PMN-attached bacteria but not phagocytosed or internalized. Tube 5 represented any bacterial contamination of the PMN pellet due to free bacterial contamination by cosedimentation despite the low speed used. Thus phagocytosis was expressed as a per cent of initial labelled bacteria associated with the PMNs at time t using the formula:

$$\% \text{ phagocytosis}_t = \frac{A_t - (B+C)_t}{D_o - B_t}$$

where A = radioactivity associated with PMN pellet at time t

D_o = total radioactivity in tube at time zero

B = background radioactivity

C = bacterial contimation of PMN pellet

t = time (5, 10 or 20 min)

Phagocytosis and mere attachment to PMN membranes, but not internalization, could then be separated from the data given by tube 4 at 5, 10 and 20 min.

8.3.6 Analysis of the Bactericidal Assay

Analysis for bactericidal function was carried out as follows. The samples obtained at all time intervals including time zero were further diluted 1:100 taking 0.1 ml of material from the 5 ml distilled water tube into 9.9 ml of PBS. A 0.1 ml aliquot was then taken from this and plated on TSA - agar plates. The bacterial colonies were counted after incubation for 24 hrs at 37°C. Since the sampling volume and dilutions were the same for each time period, the results were expressed as a percentage of the initial inoculum killed at each time using the formula:

$$\% \text{ bacterial function}_t = \frac{A_o - B_t}{A_o}$$

where A_o = cfu in tube at time zero

B_t = cfu in tube at time t

t = 10, 20, 40, 80, 160 or 240 min.

9. CHARACTERIZATION OF THE CHEMOTACTIC INHIBITORS IN THE SERUM

9.1 Demonstration of a Cell Directed Inhibitor in Patient Serum

Hospitalized patients with decreased autologous PMN chemotaxis served as serum donors. Serum was used fresh or stored frozen at -70°C. Normal laboratory personnel served as PMN donors. The leukocytes were prepared as before and resuspended in either normal heterologous serum, patient serum and patient serum diluted with MEM. Separate aliquots were resuspended in normal and patient sera, incubated 30 min at 37°, washed twice with MEM and resuspended in MEM. The chemotactic migration of these cells was then measured.

9.2 Demonstration of Counterinhibiting Activity in Normal Serum

Patient leukocytes with decreased chemotactic migration were prepared, washed with MEM three times and resuspended in 100% pooled normal human serum or autologus serum. Their chemotactic migration was then measured. Various mixtures of normal and patient serum as well as patient serum and fractions of serum obtained following ammonium sulfate fractionation were also used to assess counterinhibiting activity.

9.3 Demonstration of Binding of Inhibitor onto Leukocytes

Patient and normal control leukocytes were prepared and washed in MEM up to eight times. After each wash, an aliquot of cells was withdrawn for measurement of chemotactic migration.

A 2 ml aliquot of inhibitory serum was also used into which were resuspended successive fresh batches of 5×10^6 normal control leukocytes. These were incubated for 10 min at 37°C, and then removed by centrifugation and washed in MEM. Their chemotaxis was then assessed. Twelve successive resuspensions with fresh neutrophils were used.

9.4 Isoelectric Focusing

Isoelectric focusing was performed using the method of Haglund[105]. A 100 ml capacity column (LKB, Sweden) with an outer and inner cooling jacket was used. Experiments were carried out at 2-4°C using 1% Ampholine pH 3-10 in a 0-50% anticonvection sucrose gradient. This was prepared from a 50% sucrose solution (W/V) in water containing 1% Ampholine (solution H), and a 1% Ampholine solution in water (solution L), in 24 2.6 ml portions as follows: portion number 1 contained 4.6 ml solution H, portion number 2 contained 4.4 ml of solution H and 0.2 ml solution L. In each subsequent portion the volume of solution H was decreased by 0.2 ml and that of solution L increased correspondingly so that portion # 24 contained solution L only. The serum was dyalized overnight against 1% glycine to reduce the ionic strength. A precipitate which formed was discarded, and 1% Amopholine was added. This was used to replace solution L in fractions number 11 and 12 for a total volume of 3.8 ml added to the column. The

anode (bottom of column) was protected with 1% monoethanolamine in 50% sucrose and the cathode (top of column) with 10% phosphoric acid.

Focusing was carried out for 72 hr at 300 V with an initial current of 7.5 ma resulting in a starting power of 5.3 Watts. Three days later,the current dropped to a constant 2.2 ma with power at 0.66 Watts. The column was fractionated into 24 x 5 ml fractions and the pH measured immediately at 4°C. The fractions were dialyzed against 0.1 M sodium phosphate buffer,pH 7.4. Subsequently, 1 ml aliquots were used to pre-incubate normal leukocytes for 20 min, the cells were washed, resuspended in MEM and their chemotaxis assessed.

9.5 Sucrose Density Gradient Centrifugation

Linear 10-30% sucrose density gradients were prepared by means of a manual gradient former of standard design. The gradients were buffered with 0.02 M sodium phosphate pH 7.4. After cooling to 4°C, samples of sera, 0.2 ml were loaded on the gradients along with marker proteins. These were: cytochrome C, $S_{20,W}$ = 2.1; human serum albumin, $S_{20,W}$ = 4.3; and ferritin, $S_{20,W}$ = 17.7 (Boehringer Mannheim Biochemicals). The gradients were centrifuged at 180,000 x g for 18 hr at 4°C using a Beckman model L-2 ultracentrifuge. They were fractionated using an ISCO Model D fractionator and the position of the marker proteins verified by A_{280} or A_{410} nm. The serum gradients were analyzed

for chemotactic inhibiting activity by mixing 200 µl of each fraction with 300 µl of MEM and using this to resuspend normal leukocytes before measureing their chemotaxis. Appropriate equiosmolar controls were included to allow for any effects of sucrose. Sedimentation coefficients were determined using the method of Martin and Ames[159].

9.6 Molecular Sieve Column Chromatography

Although various column sizes were employed in these experiments, the method of packing, calibration and developing was the same in all. For example, in a typical experiment, a 1.6 x 100 cm column (K16, Pharmacia) was fitted with a flow adapter at the bottom, and connected to a peristaltic pump (LKB, Model 12000, Sweden). The column was packed with Sephadex G-200 prevously swollen as per manufacturer's instructions[283]. Flow rate for packing was double that at which the column would be developed. After packing overnight, the top of the column was closed off with a second flow adaptor and the pump was switched to allow flow of the eluent, 0.02 M phosphate buffered saline, through the column from top to bottom. A sample introduction port and an air bubble trap were also included in the inflow assembly. The eluent was passed through a Model 260 UV monitor connected to a model 261-E linear servorecorder (Gilson Instruments) and the A_{280} nm monitored continuously. Fractionation was performed with an FC-100 microfractionator using the drop counting mode (Gilson Instruments). Serum to be fractionated was loaded on the

column and the column developed. The fractions were assayed by mixing 3 x 10^6 leukocytes with 0.5 ml volumes of each fraction, incubating 20 min, washing the cells, resuspending in MEM and measuring their chemotaxis. The columns were calibrated by running combinations of the following proteins through each column. Horse heart cytochrome C, MW = 12,500. Chymotrypsimogen A from bovine pancreas, MW = 25,000. Hen egg white albumin, MW = 45,000. Bovine serum albumin, MW = 68,000. Aldolase from rabbit muscle, MW = 158,000. Catalase from beef liver, MW = 240,000. Ferritin, MW = 450,000. Blue dextran, MW > 2x10^6 was used to determine the void volume (V_o), and acetyl-saliycilic acid determined the total column volume (V_t). Elution volumes of the markers were detected by A_{280} or A_{410}nm.

10. PARTIAL PURIFICATION OF THE LEUKOCYTE CHEMOTACTIC INHIBITORS IN SERUM USING AMMONIUM SULFATE FRACTIONATION

A typical experiment is described. All ammonium sulfate precipitions followed this scheme. Pooled serum, 35 ml, from a patient with decreased autologous PMN chemotaxis and serum inhibiting activity was thawed out and brought to 20% saturation with ultrapure ammonium sulfate (Swartz-Mann Biochemicals), stirred for 30 min at 4°C and the precipitate collected by centrifugation at 15,000 x g for 10 min in the #870 rotor of an ICE B-20 preparative centrifuge. The supernatent was removed,

1 ml was saved and the remainder was brought to 40% saturation of ammonium sulfate and again stirred at 4°C for 30 min. The precipitate was redissolved in PBS, using just enough volume of buffer required to achieve complete solubulization. This procedure was repeated to obtain cuts at 60%, 80% and 100% saturation of ammonium sulfate. Supernatents and precipitates were exhaustively dialyzed against PBS to remove the ammonium sulfate and assayed for chemotactic inhibition at various dilutions with MEM. The amount of protein in each fraction was determined by the method of Lowery et al[284]. An accurate count of the numbers of leukocytes per assay tube was made to allow for calculation of inhibition units.

II. STATISTICAL ANALYSIS OF DATA

Statistical analysis was carried out using basic programs on a Digital PDP11 computer. Two data groups were analyzed for significant differences by use of paired or unpaired t-tests with the Student Distribution. More than two data groups were evaluated by Analysis of Variance and both the Student Distribution and Scheffe's Test to determine significant differences of the means. Morbidity and mortality data were tested by X^2 analysis. All results are reported as mean ± S.E. unless otherwise indicated.

RESULTS

12. SKIN TEST RESPONSE AND CLINICAL OUTCOME

12.1 Overview

One thousand seven hundred and seventy six patients were studied and analyzed from July 1976 to the end of April 1979. The worst skin test recorded during the patients hospitalization and the eventual clinical outcome is shown in table 2. The rate of sepsis in normal responders was 7.5% and the death rate 2.1%, compared to a significantly higher rate in those with relative anergy, 32.0% and 15.0% respectively and those with anergy, 47.0% and 35.0% respectively. This analysis includes all patients studied, both surgical and trauma, operated or not operated upon and skin tested before surgery, after surgery, or following complications which developed elsewhere. Analysis of sequential skin test results on 790 surgical patients on whom data were available is shown in tables 3, 4 and 5. There was good correlation between a favourable prognosis if the skin tests improved or remained normal. If skin tests worsened or there was failure to recover or improve skin reactivity towards normal, the prognosis was less favourable.

12.2 Pre-Operative Surgical Patients

The reliability of the delayed hypersensitivity response in predicting patients at risk prior to surgery, was tested by

TABLE 2

The worst skin test result in 1776 surgical patients during their hospitalization and their clinical outcome

SKIN TEST	PATIENTS	SEPSIS	DEATH
N	1172	7.5%	2.1%
RA	178	32.0%	15.0%
A	426	47.0%	35.0%

TABLE 3

Sequential skin testing and clinical outcome in 618 surgical patients whose skin test response did not change during their hospitalization

SKIN TEST	PATIENTS	SEPSIS	DEATH
N→N	530	8%	1%
RA→RA	16	56%	69%
A→A	72	58%	65%

TABLE 4

Sequential skin testing and clinical outcome in 172 surgical patients whose skin tests improved during their hospitalization

SKIN TEST	PATIENTS	SEPSIS	DEATH
RA → N	67	37%	1%
A → N	88	47%	3%
RA → A	17	59%	29%

TABLE 5

Sequential skin testing and clinical outcome in 43 surgical patients whose skin tests worsened during their hospitalization

SKIN TEST	PATIENTS	SEPSIS	DEATH
N → RA	10	60%	50%
N → A	13	69%	77%
RA → A	20	70%	80%

analysis of 727 patients who were skin tested preoperatively, and followed prospectively and sequentially following their operations, until discharge or death. Six hundred and thirty eight patients were operated and 89 were not operated upon. These groups, being free of the influences of operative complications, trauma or burns were used to characterize the rates of response to the five antigens used, the effect of age, and the influence of disease on the delayed hypersensitivity response.

The age distribution of normal and anergic patients by decades is shown in figure 2, plotting the particular decade versus the patients of that age who demonstrated normal or failed responses as a percent of the total Normal or Anergic population. The normal patient age range was 18 to 91 years with male/female: 1.14/1.00 and a mean age of 58.1 ± 1.0 years, with median of 60.5 years and mode of 67 years. The anergic patients age range was 19-95 years with mean age 63.9 ± 1.0 years median of 65.8 years and mode of 60 years with male/female: 1.08/1.0. A plot of the percent of patients of each particular decade demonstrating normal or failed response which is not shown here

This indicates that a very young as well as an older patient population have a high proportion of anergy, especially in the 8th and 9th decades. Numbers are, however, small at these extremes to allow rigorous statistical analysis.

The percent of normal patients who reacted to each of the antigens is shown in table 6, along with the mean diameters

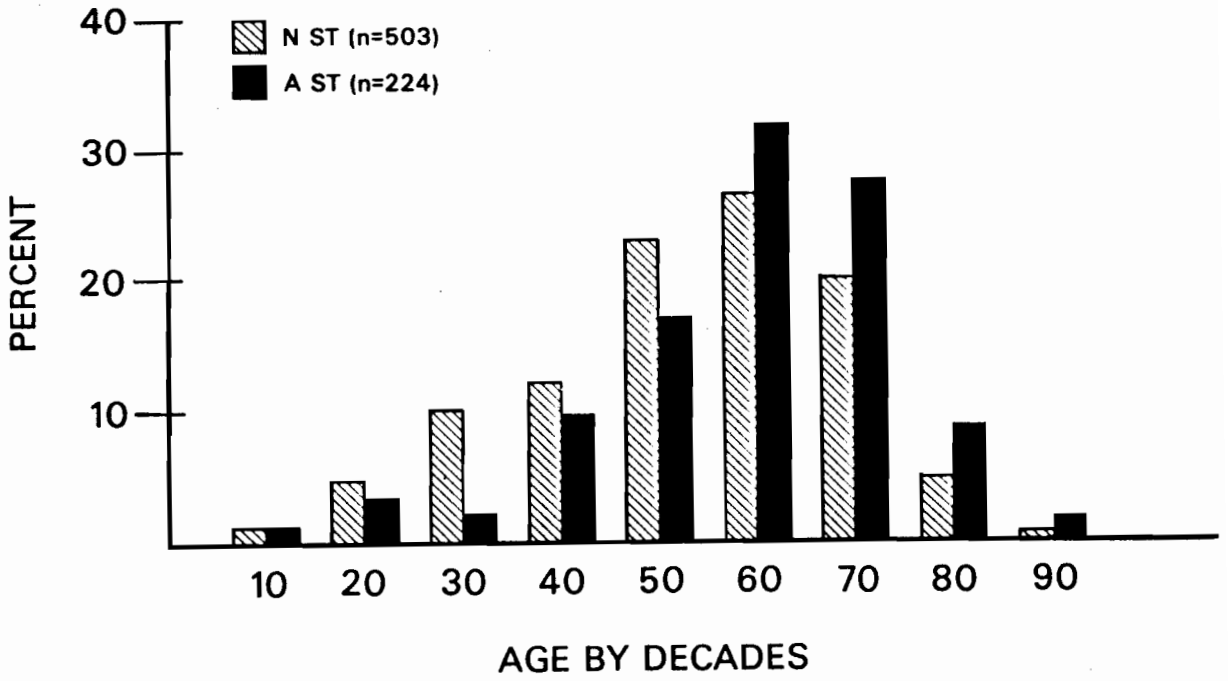


Figure 2. The percentage of total patients of a particular age demonstrating normal or anergic skin test response.

TABLE 6

The percent of patients in this region who responded to each of the injected antigens and the mean diameter ± S.E. of induration of each response (n=499)

ANTIGEN	PATIENTS (%)	MEAN INDURATION
Candida	370 (74.2)	8.58 ± 0.20
Mumps	458 (91.8)	9.46 ± 0.32
PPD	242 (48.2)	14.0 ± 0.47
Trichophytin	206 (41.3)	8.41 ± 0.47
Varidase	477 (95.6)	17.00 ± 0.78

± SEM for each antigen. In this geographic location (Greater St. Lawrence Valley), Varidase, Mumps and Candida are the antigens most frequently eliciting a positive response (95.6%, 91.2% and 74.2% respectively) with PPD (48.5%) and Trichophytin (41.3%) eliciting responses less frequently.

Table 7 lists the primary diagnosis of all patients included in this pre-operative study. From the wide spectrum of surgical disease afflicting these patients, the diagnosis of hemorrhage from the gastrointestinal tract and sepsis showed a significantly higher percentage of patients with failed cutaneous responses. The type of operations performed listed in table 8, demonstrate that other than drainage of the infection, all three groups had similar operative procedures.

The data used to compare age, severity of operation, pre-operative albumin (as a measure of malnutrition) and pre-operative polymorphonuclear neutrophil chemotaxis is shown in table 9, along with hospital stay and clinical outcome. The anergic and relatively anergic groups were older than the normals ($p<0.05$, analysis of variance and Scheffe's test) though there was no difference in age between the RA and A groups. Similar findings were noted with pre-operative PMN chemotaxis. There was a statistically different pre-operative albumin value in all three groups. The severity of operation (classified on a scale of 1 to 5) was not different between any group. The rate of major sepsis was much higher in the RA and A groups, as was the mortality which increased 3.4-fold in the RA group and 6.5-fold in the A

TABLE 7

The percentage of preoperative patients with a particular diagnosis who had normal or depressed skin test response

DIAGNOSIS	NORMAL ST(%) (n=503)	RELATIVE ANERGY (n=58)	ANERGY(%) (n=77)
Cancer	177 (35.2)	25(43.1)	25(32.5)
Cholecystitis ± Cholangitis	45 (8.9)	6(10.3)	4(5.2)
GI Hemorrhage	10 (2.0)	2(3.4)	17(22.1)
Duodenal ulcer (non bleeding)	38(7.6)	-	-
Diverticulitis	30(6.0)	-	2(2.6)
Morbid obesity	25(5.0)	-	-
Bowel obstruction	20(4.0)	-	2(2.6)
Colonic polyps	18(3.6)	-	-
Hernia	17(3.4)	-	1(1.3)
Pancreatitis (acute/chronic)	15(3.0)	3(5.2)	2(2.6)
Abdominal aortic aneurysm	14(2.8)	1(1.7)	-
Gastric ulcer	12(2.4)	-	-
Peripheral vascular disease	12(2.4)	-	-
GI Fistula	12(2.4)	3(5.2)	-
Crohn's Disease	8(1.0)	-	1(1.3)
Obstructive Jaundice	5(1.0)	4(6.9)	3(3.9)
Ulcerative colitis	5(1.0)	1(1.7)	2(2.6)
Perforated viscous	3(0.6)	1(1.7)	1(1.3)
Echniococal cyst	3(0.6)	-	-
Sepsis	1(0.2)	9(15.5)	10(13.0)
Others	33(8.6)	3(5.2)	7(9.1)

TABLE 8

Operations performed upon patients tested preoperatively

OPERATION	NORMAL (%) (n=503)	RELATIVE ANERGY (%) (n=58)	ANERGY (%) (n=77)
Hemicolectomy	57(11.3)	11(19.0)	9(11.7)
Cholecystectomy ± CBDE	64(12.7)	10(17.2)	8(10.4)
Anterior resection	36(7.2)	4(6.9)	5(6.5)
Lapartomy ± biopsy ± bypass etc.	36(7.2)	8(13.8)	13(16.8)
Vagotomy + antrectomy or pyloroplasty	36(7.2)	-	6(7.8)
Colostomy (open,close, revise)	34(7.0)	2(3.4)	5(6.5)
Gastrectomy (total/subtotal)	34(6.8)	1(1.7)	4(5.2)
Abdominoperineal resection	28(5.6)	-	-
Hernioraphy	16(3.2)	-	1(1.3)
Jejunioleal bypass	16(3.2)	-	-
Small bowel resection	15(3.0)	1(1.7)	-
Resection AAA	13(2.6)	-	-
Peripheral vascualr procedure	13(2.6)	-	-
Pulguration of colonic polyp	12(2.4)	-	-
Gastroplasty	7(1.4)	-	-
Esophagogastrectomy	7(1.4)	1(1.7)	4(5.2)
Lysis of adhesions	6(1.3)	-	1(1.3)
Portal systemic shunt	3(0.6)	2(3.4)	2(2.6)
Nissen fundoplication	3(0.6)	-	-
Total proctocolectomy	3(0.6)	1(1.7)	-
Choledochoduodenostomy	4(0.8)	-	-
Whipple's resection	4(0.8)	1(1.7)	-
Radical cystectomy	-	2(3.4)	-
Radical nephrectomy	-	1(1.7)	1(1.3)
Peritoneal-jugular shunt	-	-	2(2.6)
Drain infection	8(1.6)	9(15.5)	10(13.0)

TABLE 9

A comparison of age, severity of operation, preoperative albumin, preoperative polymorphonuclear neutrophil chemotaxis, duration of hospital stay, and clinical outcome in patients with Normal(1), Relatively Anergic(2) and Anergic(3) skin test responses preoperatively.

VARIABLE	NORMAL (%) (n=503)	RELATIVE ANERGY (%) (n=58)	ANERGY (%) (n=77)
Age (1 vs 2,3)	58.1 ± 0.7	64.2 ± 1.8	63.0 ± 1.8
Serum albumin (g/dl) (all)	3.8 ± 0.03	3.54 ± 0.09	3.10 ± 0.06
Severity of operation (none)	2.6 ± 0.1	2.7 ± 0.2	2.6 ± 0.1
Preoperative PMN CTX (1 vs 2,3)	118.5 ± 1.2μ	108.4 ± 2.5μ	103.2 ± 2.6μ
Days in hospital (1 vs 2,3)	22.8 ± 0.8	32.9 ± 5.3	31.3 ± 3.1
Sepsis (1 vs 2,3)	38(7.5)	20(34.0)	22(28.6)
Mortality (1 vs 2,3)	23(4.6)	9(15.5)	23(29.9)
Mortality due to sepsis	2(8.7)	2(22.0)	7(30.0)
Septic → Death	3(9.0)	4(20.0)	13(59.0)

( ) Statistically different groups at $p < 0.05$, Analysis of Variance and Scheffe's test.

group. The deaths caused primarily by sepsis as proven by autopsy as well as the number of septic patients who died were markedly higher in the RA and A groups.

Of interest is the subgroup of 32 patients (6.4% of N responders) who lost their reactivity post operatively for one week or longer. This group is summarized in table 10 and compared to the 471 (93.6%) of N responders who maintained their reactivity post operatively. The mean age was not different but the two groups had a statistically different pre-operative albumin level though the N$\longrightarrow$ A group was still within the normal range. The severity of operation was higher by 1.06 points in the group who dropped their responses. The majority (25) of these patients were tested one week post surgery and followed until recovery of skin test response, discharge or death. Failure to react post-operatively was noted immediately (day 1) in seven patients who were tested the day following their operation. Sepsis and mortality was much higher in the N patients who lost their skin reactivity post-operatively. They also had a much longer hospitalization, 22.8 vs 55.9 days.

The autopsy proven cause of death in the four groups is shown in table 11. There was a lower proportion of N$\longrightarrow$RA/A patients who were autopsied following death. A higher proportion of patients died of overt sepsis or multisystem failure to which sepsis was contributory in the anergic groups (RA, A or N$\longrightarrow$ RA/A) than the normal responders.

TABLE 10

A comparison of normal responders who became nonreactive for longer than one week following an operation.

VARIABLE	N→N (%)		N→RA/A (%)
Number of patients	471 (93.6)		32 (6.4)
Age	58.1 ± 0.7	N.S.	58.2 ± 2.1
Pre-op Albumin (g/dl)	3.82 ± 0.03	*	3.45 ± 0.10
Severity of operation	2.57 ± 0.04	*	3.63 ± 0.17
Days in hospital	22.8 ± 0.80	*	55.9 ± 7.6
Sepsis	25 (5.3)	*	13 (40.6)
Mortality	16 (3.3)	*	7 (21.9)

* statistically significant at $p < 0.001$, Students t-test.

TABLE 11

The autopsy proven cause of death in the normal, relatively anergi anergic preoperative patients, and normal patients becoming anergic for longer than one week following surgery.
(number of deaths in brackets)

CAUSE OF DEATH	N→N (%) (n=16)	RA (%) (n=9)	A (%) (n=23)	N→RA/A(%) (n=7)
Overt Sepsis	1(6.3)	2(22)	7(30)	1(7.7)
Multisystem failure Sepsis contributory	0	3(33)	6(26)	3(23)
Hypovelmic Shock	0	0	2(8.7)	1(7.7)
Multisystem failure Non septic	0	0	2(8.7)	--
Disseminated Cancer	4(16)	1(11.1)	--	2(15.3)
Myocardial Infarct	1(6.3)	2(22)	--	--
Aspiration Pneumonia	1(6.3)	--	--	--
Renal Failure	1(6.3)	--	--	--
Number Autopsied	8(50)	8(89)	17(74)	4(54)

The 89 RA/A patients who were detected during this study but who did not undergo an operation were compared to all the operated normals and operated RA/A (table 12). Again, the mean age was higher in the RA/A groups compared to N as was the serum albumin (non-operated RA/A had a value below the mean normal range of the hospital assay) and PMN chemotaxis. The length of hospital stay was much shorter in the non-operated RA/A group since they were not subjected to an operation. The same held for sepsis, where the non-operated RA/A group was not statistically different from the normal. Mortality was higher in this population compared to normals. The resistance to sepsis once it became established, was not equivalent to the normals, and comparable to the operated RA/A group as shown by the numbers of septic patients who died in the three group. The presenting diagnosis in this non operated RA/A group were: Cancer (35.3%), GI hemorrhage (25.8%), acute pancreatitis/cholecystitis (13.5%), dehydration/malnutrition (5.62%), and others (small bowel obstruction, diverticulitis, bowel fistula, ulcerative colitis, etc. 24.7%). Of the 20 of 27 deaths (74% who were autopsied in this group), 7 (35.0%) died from overt sepsis and three from multiple causes to which sepsis was contributory. Multisystem failure - non septic, aspiration pneumonia (? sepsis), asphyxia secondary to tumour and myocardial infarcts accounted for the remainder of the 7 deaths.

TABLE 12

A comparison of normal(1) and anergic patients(2) undergoing an operative procedure with those non-reactive patients who were not operated upon(3).

VARIABLE	OPERATED N (n=503)	OPERATED RA/A (n=135)	NON-OP RA/A (n=89)
Age (1 vs 2,3)	58.1 ± 0.7	63.5 ± 1.3	64.5 ± 1.4
Serum albumin (g/dl) (1 vs 2,3)	3.8 ± 0.03	3.32 ± 0.06	3.14 ± 0.08
PMN CTX (1 vs 2,3)	118.5 ± 1.2	106.5 ± 1.9	107.6 ± 2.2
Days in hospital (All)	22.8 ± 0.8	32.1 ± 3.2	15.6 ± 1.0
Sepsis (2 vs 1,3)	38 (7.5%)	42 (31.1%)	9 (10.4%)
Mortality (1 vs 2,3)	23 (4.6%)	32 (23.7%)	27 (30.3%)
Septic Death (1 vs 2,3)	3 (7.9%)	17 (40.5%)	6 (66.7%)

( ) groups which differ significantly from each other with $p < 0.05$, Analysis of Variance and Scheffe's test.

12.3 The Effects of Gastrointestinal Hemorrhage

One hundred and twenty two patients with endoscopically or angiographically proven diagnosis of gastrointestinal hemorrhage were analyzed to determine the reason for the higher proportion of anergy in these patients. There were 73 patients with normal responses and 48 patients with RA and A. Seventy five patients were studied pre-operatively (most are included in the pre-operative analysis presented earlier) and 47 post operatively. A greater number (42% vs 13%, $p < 0.05$) of patients bleeding from the esophagus were in the A group, but other sites of bleeding in the two groups were similar. Anergic patients had an increased rate of liver disease, 10/48 (21%) versus none in the normal group, with no other differences in associated diseases. There was no difference in mean age (57.7 ± 1.9 vs 62.2 ± 2.1), admission hemotocrit (31.1 ± 1.0 vs 28.6 ± 1.2), systolic BP (128.8 ± 3.2 vs 117 ± 5.6), or diastolic BP (76.6 ± 2.0 vs 71.1 ± 3.2). The data which differed significantly are listed in table 13. Anergic patients had a significantly higher admission pulse, greater blood transfusion requirements both early (pre ST) and late (post ST) and increased colloid infusions. The sex distribution was also significantly different with a larger number of females in the anergic group. Morbidity in the form of sepsis, despite aggressive use of therapeutic antibiotics, was higher in the anergic group. From a total of 17 deaths in this group, two were due to autopsy proven septicemia and in 9 sepsis was contributory

TABLE 13

A comparison of hemodynamic parameters, transfusion requirements, and clinical outcome in patients with G.I. hemorrhage and normal or anergic skin test response

VARIABLE	NORMAL PATIENTS	ANERGIC PATIENTS
Number	74	48
Male/Female	3.2	1.8 *
Admission Pulse	92 ± 4.4	109 ± 3.9 **
Blood Transfused Pre-ST (Units)	3.4 ± 0.4	8.0 ± 0.8 *
Blood Transfused Post-ST (Units)	0.8 ± 0.2	5.8 ± 0.9 *
Total Blood (Units)	4.1 ± 0.4	13.9 ± 1.3 *
Total Coloid Transfused	0.2 ± 0.5	3.2 ± 0.5 *
Sepsis	1.3%	42% *
Mortality	5.4%	50% *

* $p < 0.001$ ** $p < 0.02$

to the death. The remaining six deaths were due to hypovolemic shock. The patient who died in the N group suffered a myocardial infarction. The evidence for hypovolemia in anergic patients is the significantly higher admission pulse rate and transfusion requirements (blood and colloid).

12.4 The Effect of Cancer

Two hundred and five patients with surgically proven neoplasia were analyzed, in order to assess the influence of cancer and its extent, on host defense as reflected by delayed hypersensitivity responses. There were 140 normal responders (95% studied preoperatively) and 65 in the anergic group (62% studied preoperatively). Mean age and weight, male/female, numbers operated upon, numbers autopsied, and distribution of cancer at each site namely: esophagus, stomach, pancreas, gallbladder, cervix, breast and lymphoid organs were not significantly greater in the anergic group. Serum albumin at first skin test and lowest value during hospitalization, were statistically lower in the anergic cancer patients (table 14). Sixty five percent of the cancer patients studied had normal skin tests and 35% were anergic. The distribution of anergic and normal responders was the same for patients with metastases, carcinomatosis, and Dukes' C and D colon lesions. In patients with Dukes' A and B lesions, 78% were ST normal, a higher percentage, but not statistically different, from all patients with cancer. If malnutrition is defined as 5 kg weight loss and/or serum albumin of 3.2 g/dl or less, 1% of normals and 55% of anergic patients

TABLE 14

Nutritional status, extent of disease and clinical outcome of normal and anergic patients with cancer

I NUTRITIONAL	NORMAL (n=140)	RA/A (n=65)
Albumin(g/dl)	3.95 ± 0.03	3.43 ± 0.08 *
Weight loss (kg)	3.25 ± 0.48	5.23 ± 0.74 *
Worst Albumin	3.3 ± 0.04	2.90 ± 0.09 *
II EXTENT OF DISEASE		
Patients with Cancer	65%	35%
Patients with Metastasis	66%	34%
Patients with Carcinomatosis	67%	33%
Colorectal Dukes' A&B	78%	22%
Colorectal Dukes' C&D	66%	34%
III CLINICAL OUTCOME		
Sepsis	9.2%	55% *
Death	6.4%	61% *
Septic ⟶ Death	23%	50% *

* $p < 0.05$, X^2 or unpaired t-test

were malnourished ($p < 0.005$). Again, the incidence of sepsis and mortality, as well as the number of septic patients who died was much higher in the anergic group compared to normals ($p < 0.005$).

13. LYMPHOCYTE STUDIES

13.1 Total Lymphocyte Counts

Total lymphocyte counts are shown in figure 3. There was a statistically significant decrease in the peripheral lymphocyte count of the anergic group compared to laboratory normal controls. ($p < 0.05$, Analysis of Variance). The small sample size of the RA patients does not allow for valid comparisons. Pooling all normal responders and all anergic responders does not demonstrate a significant difference between the two groups ($p \sim 0.5$, Students t test), though the anergic patients had a lower peripheral circulating lymphocyte count.

13.2 Total and Active E-Rosettes

The percentage of circulating lymphocytes that formed rosettes with sheep erythrocytes is shown in figure 4. Total E-rosettes as well as active E-rosettes are shown. Though there is a lower proportion of circulating lymphocytes that formed rosettes in the relatively anergic and anergic patients, there is no statistical difference between any of the groups compared

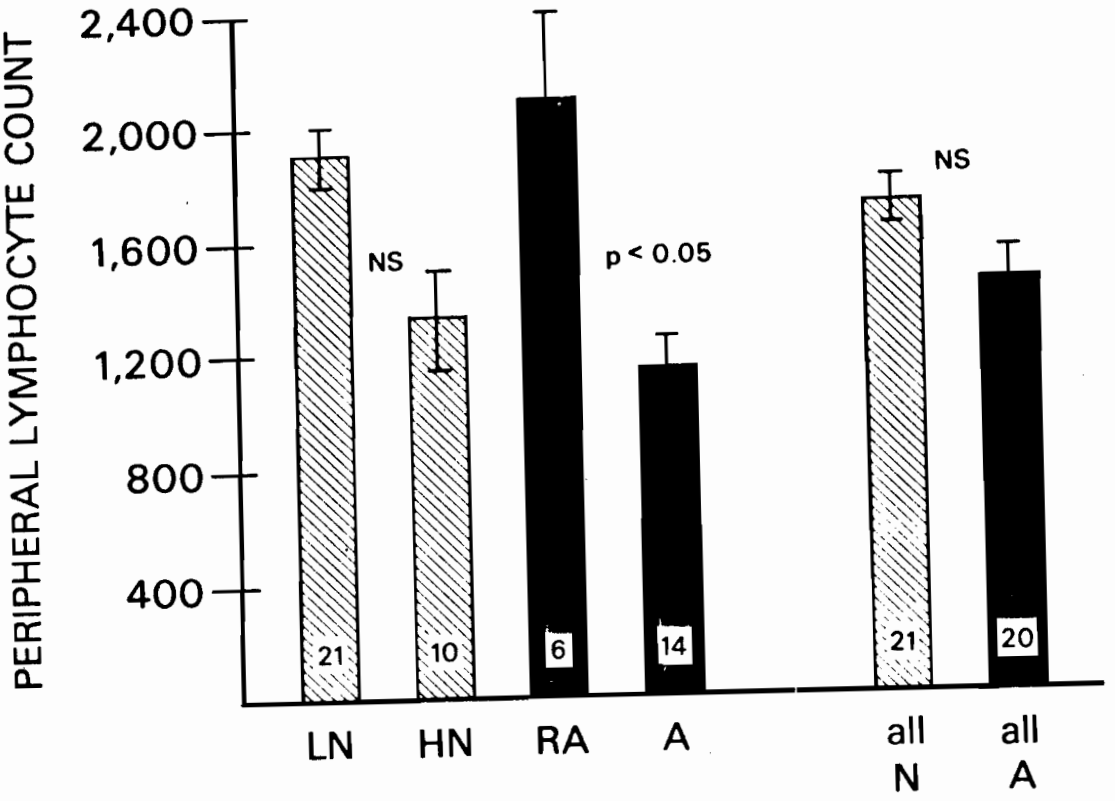


Figure 3. Peripheral lymphocyte counts in laboratory normal controls(LN), hospitalized normals(HN), relatively anergic(RA), and anergic(A) patients. The pooled data are shown on the right.

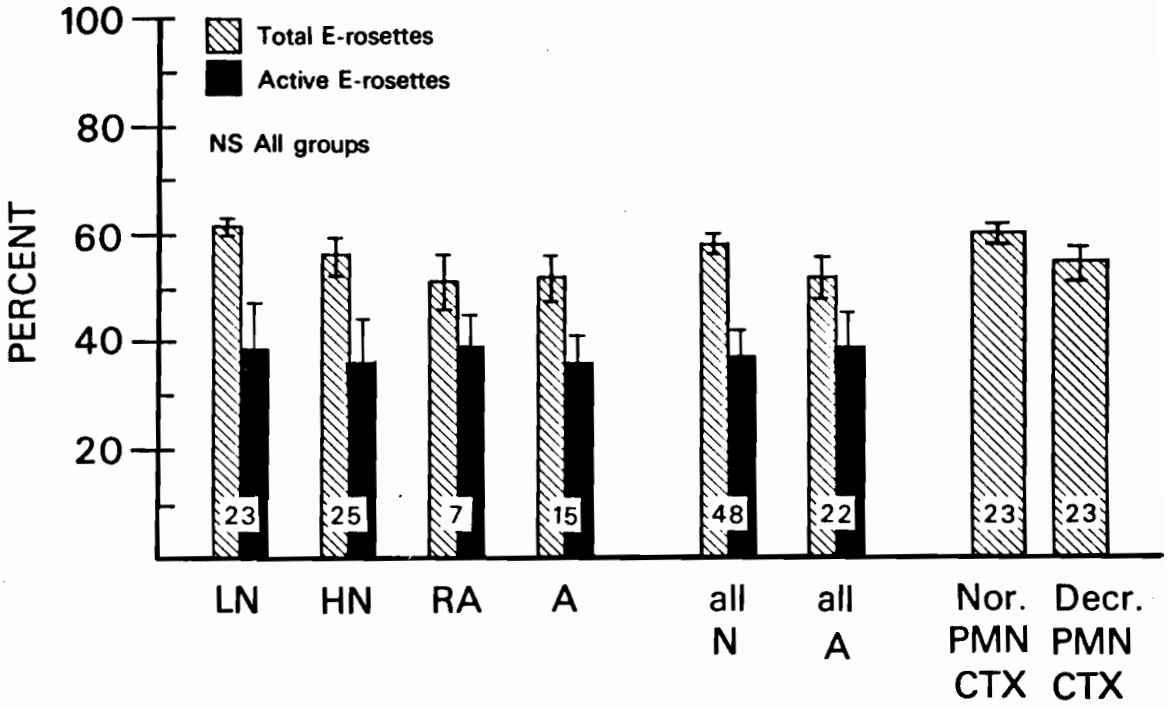


Figure 4. The percent of total and active lymphocyte rosettes in laboratory normal controls(LN), hospitalized normals(HN), relatively anergic(RA), and anergic(A) patients. E-rosettes from all patients with normal or decreased CTX is also shown.

by analysis of variance, nor if all normals and all anergic patients are combined and analyzed ($p = 0.8$, Students t-test). The number of active E-rosetting cells is also not different. Patients with decreased PMN chemotaxis had a lower percentage E-rosetting cells but, again, not statistically different from patients with normal chemotaxis. No correlation could be demonstrated between PMN chemotaxis and E-rosette forming cells in this study ($r = 0.07$, $t = 0.55$, $n = 60$, N.S.)

13.3 PHA Stimulation of Lymphocytes from Normal and Anergic Patients

The results of *in vitro* transformation of lymphocytes from normal laboratory controls and hospitalized surgical patients are shown in figure 5. No significant difference was measured in the stimulation index of any one group compared to the others, in this *in vitro* system which is free of *autolgous serum* and supplemented with 15% AB heterologous serum.

13.4 Lymphocyte Chemotaxis

13.4.1 Development of Method

Various experimental protocols were tried in order to determine the most suitable method for measurement of lymphocyte chemotaxis from normal laboratory controls and hospitalized patients. The main reason for our lymphocyte transformation experiments was to obtain lymphocytes suitable for chemotaxis.

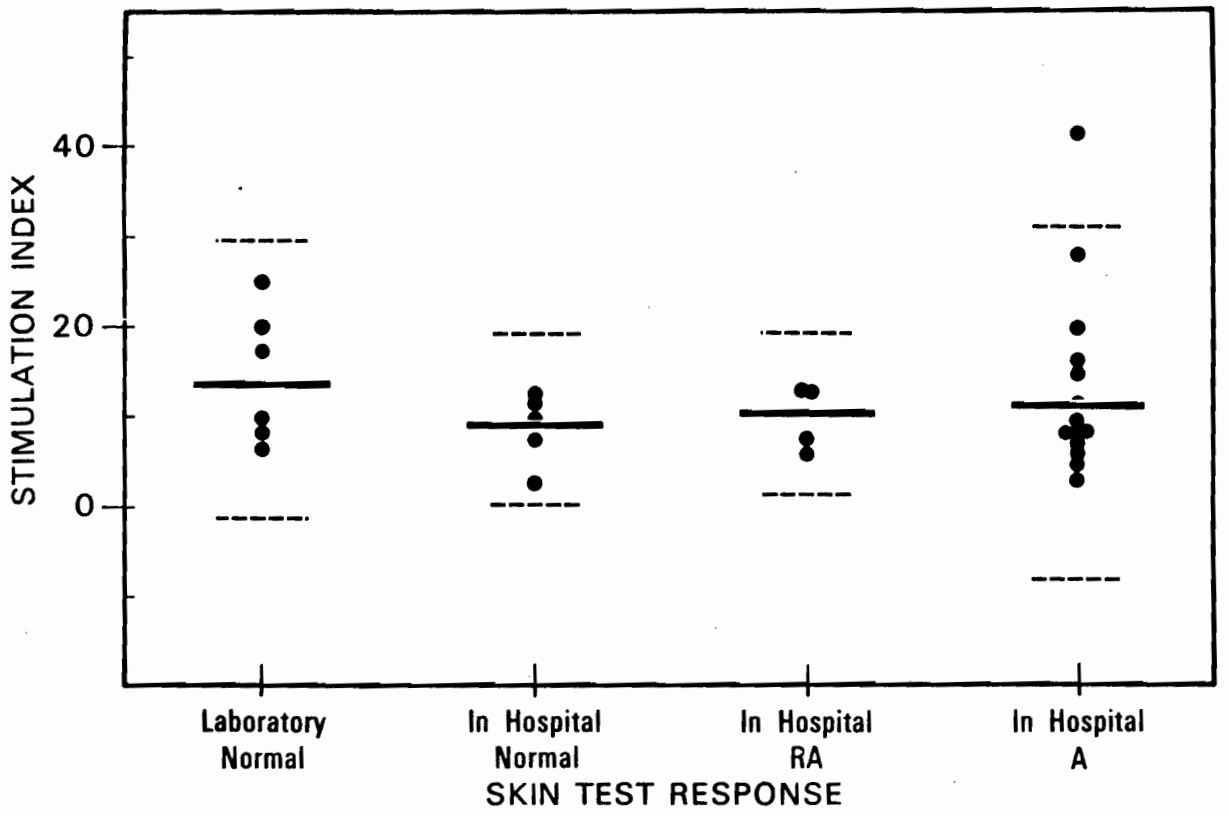


Figure 5. The effect of PHA stimulation of lymphocytes from normal controls and hospitalized surgical patients (mean ± S.D).

Other investigators working in this field indicated that lymphocytes had to be stimulated and transformed before they would migrate into 8 or 12 μ nucleopore or sartorious filters[274,275]. This was possible in this study, however, transformed lymphocytes clumped together making the experiments difficult. Lymphocyte chemotaxis of normal controls or anergic patients was not altered by mitogen stimulation (table 15). The lymphocyte stimulation with PHA was ommitted, and lymphocyte chemotaxis was measured with freshly harvested cells.

The initial experiments were aimed at standardizing chemotaxis system using normal control lymphocytes. Lymphocyte chemotaxis was optimal in filters of 3 μ pore diameter and reduced in 5 μ and 8 μ pore filters (table 15). Random migration was unaffected by pore size. The optimal time to measure maximal normal control lymphocyte chemotaxis was determined to be 5 hours (figure 6) giving enough space within the filter to detect decreased chemotactic values. Normal lymphocytes travelled at 0.36 μ/min.

Inhibition of glycolysis with potassium fluoride 0.1 M and divalent cation chelation with ethylenediaminetetracetic acid 0.1%, was carried out by adding these to the lymphocyte resuspension medium. After 30 minutes incubation at 37°C the cells were placed in chemotaxis chambers and chemotaxis was assessed after 5 hours. Lymphocyte aliquots previously heated at 70° for 30 min

TABLE 15

Effect of Mitogen Stimulation on the chemotaxis of lymphocytes from normal controls and anergic patients (mean ± SD of five experiments)

TREATMENT	LYMPHOCYTE CHEMOTAXIS (μ)	
	Normal Conrols	Anergic Patients
0 day - Fresh cells	110.2 ± 4.8	85.2 ± 2.1
3 day + PHA	108.1 ± 3.2	84.2 ± 4.1
3 day - PHA	105.2 ± 3.6	86.8 ± 3.3

TABLE 16

The effect of varying the filter pore diameter on the random and chemotactic migration of normal control lymphocytes (mean ± SD of 3 experiments)

FILTER PORE DIAMETER	RANDOM MIGRATION (μ)	CHEMOTACTIC MIGRATION (μ)
3 μ	24.8 ± 1.0	105.5 ± 5.0
5 μ	22.1 ± 1.8	49.4 ± 4.9
8 μ	24.8 ± 2.1	37.6 ± 4.0

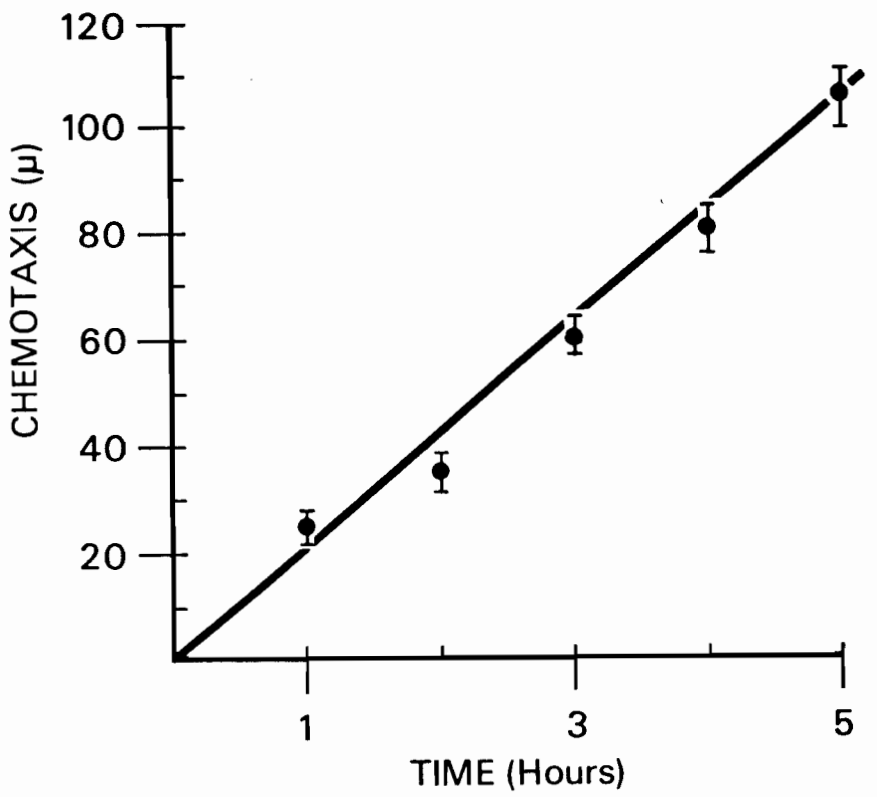


Figure 6. The rate of chemotactic migration of normal control lymphocytes into 3 μ pore diameter filters (means of 3 experiments are shown).

(after which cell viability dropped to about 1%) were also assessed. Inhibition of lymphocyte chemotactic migration was observed in all instances with cells from normal controls (table 17) and three anergic patients (data not shown).

The numbers of myeloperoxidase positive cells were 20.8 ± 9.6% (mean ± SD) in the normal control lymphocyte suspension and 19.0 ± 5.4% in the anergic patient lymphocyte preparations (NS, Student's t-test). The number of small mature lymphocytes were 63% ± 18% in normals versus 66.0 ± 22.6% in the anergic lymphocyte suspensions. The rest of the mononuclear cells were considered to be "large lymphocytes" with diameter > 8μ. Chemotaxis was optimized by 1/3: 2/3 ratio of large to small lymphocytes. Decreasing the myeloperoxidase positive cells to less than 1% by passage of the mononuclear cell suspension through cotton wool columns, gave normal control lymphocyte chemotaxis of 108.2 ± 0.84 μ as long as the "small lymphocyte" to "large lymphocyte" ratio was the same as above. When larger capacity columns were used to obtain 99% small mature lymphocytes, the chemotactic migration was variable and not reproducible, though cell viability remained better than 98%.

Contaminating polymorphonuclear cells were easy to identify and ignore when reading the filters. No lymphocyte sample with greater than 5% PMN contamination was used for chemotaxis. The distinction between lymphocytes and monocytes was not easy however, especially when the lymphocytes were in an extended locomotive form.

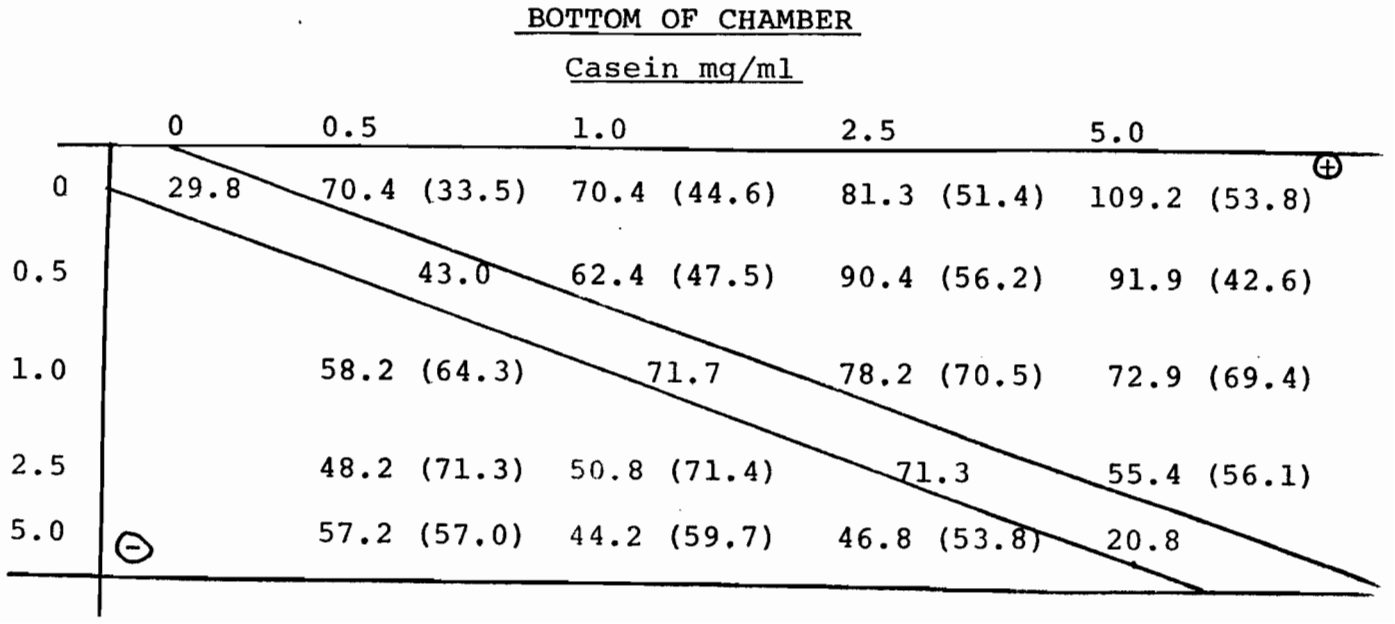
TABLE 17

The effects of glycolytic pathway inhibitors and cell killing by heat on the chemotaxis of normal control lymphocytes (Mean ± SD of 3 experiments)

TREATMENT OF CELLS	CHEMOTAXIS (μ)
0.1 M KF - MEM-FCS	13.0 ± 2.8
0.1% EDTA-MEM-FCS	19.0 ± 3.2
70°C x 30 min-MEM-FCS	18.0 ± 2.7
MEM-FCS	106.2 ± 3.2

TABLE 18

The effect of varying the direction of the chemoattractant gradient on normal control lymphocyte chemotaxis. Numbers represent migration distance in μ.



It was not possible to stain for either myeloperoxidase positive cells or nonspecific esterase containing cells in the filters using conventional wet mount staining methods. Most myeloperoxidase positive activity was seen on the starting side of the filter and only the occasional PMN with myeloperoxidase activity was detected on the opposite side.

13.4.2 Separation of Chemotaxis from Chemokinesis

The effect of varying the direction and degree of the chemotactic gradient on the chemotaxis of normal control lymphocytes is seen in table 18. Numbers within the parallel diagonal lines represent cell migration in the absence of a gradient but increased absolute concentration of the chemoattractant. The numbers outside that area represent the observed cell migration, and the numbers within the brackets represents the calculated expected migration if no gradient existed and cells were moving by chemokinesis only. Cells are in a positive chemotactic gradient above the parallel diagonal line and a negative gradient below. It is apparent that lymphocytes responded with chemotaxis and not simply chemokinesis. It is also noteworthy that high absolute concentrations of casein were inhibitory to all lymphocyte motion and lead to decreased migration whether stimulated or directed.

13.4.3 Normal Laboratory Control and Hospitalized Patient Lymphocyte Chemotaxis

Simultaneous measurements of skin test response, polymorpho-

nuclear neutrophil chemotaxis and lymphocyte chemotaxis were done in 13 laboratory controls and 74 hospitalized patients (figure 7). The mean ± SE laboratory normal control lymphocyte chemotaxis of 106.4 ± 1.1 μ was not different from that of the hospitalized normal skin test response lymphocyte chemotaxis 104.5 ± 1.6 μ ($p = 0.5$). However, the mean hospitalized normal lymphocyte chemotaxis was significantly different from the mean relatively anergic lymphocyte chemotaxis of 89.9 ± 2.8 μ ($p < 0.001$) and the mean anergic lymphocyte chemotaxis 87.5 ± 1.3 μ ($p < 0.001$). Relatively anergic to anergic lymphocyte chemotaxis was not different ($p \sim 0.5$).

The simultaneous measurements of lymphocyte and polymorphonuclear neutrophil chemotaxis in 74 surgical patients with various skin test responses and 13 normal laboratory controls showed excellent correlation (figure 8), $X = 22.4 + 0.67\ Y$, $r = 0.7904$ $p < 0.0005$).

13.4.4 Effect of Patient Serum and Levamisole on Lymphocyte Chemotaxis

Sera were obtained from normal, relatively anergic and anergic patients with decreased PMN chemotaxis, to assess their ability to inhibit normal control lymphocyte chemotaxis in vitro. The decrease of both lymphocyte chemotaxis and PMN chemotactic migration after treatment with inhibitory sera is highly significant

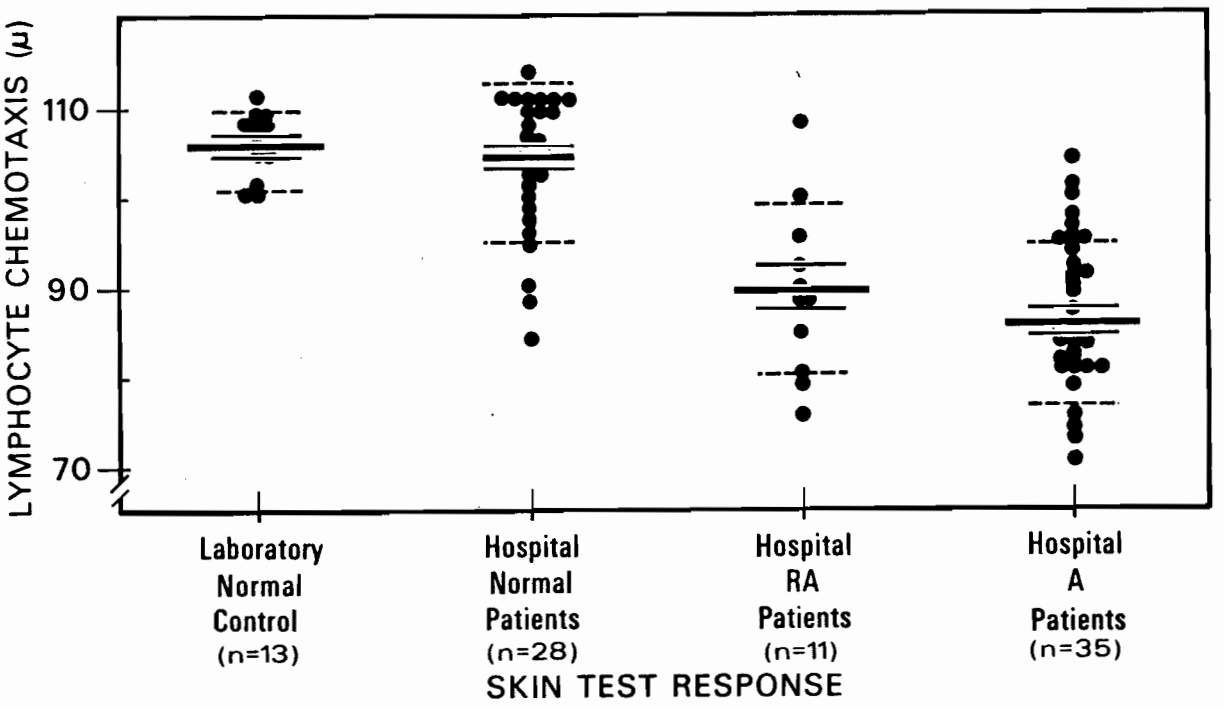


Figure 7. The skin test response and lymphocyte chemotaxis in normal controls and hospitalized surgical patients. Dark solid lines are means, light lines S.E., and broken lines are S.D.

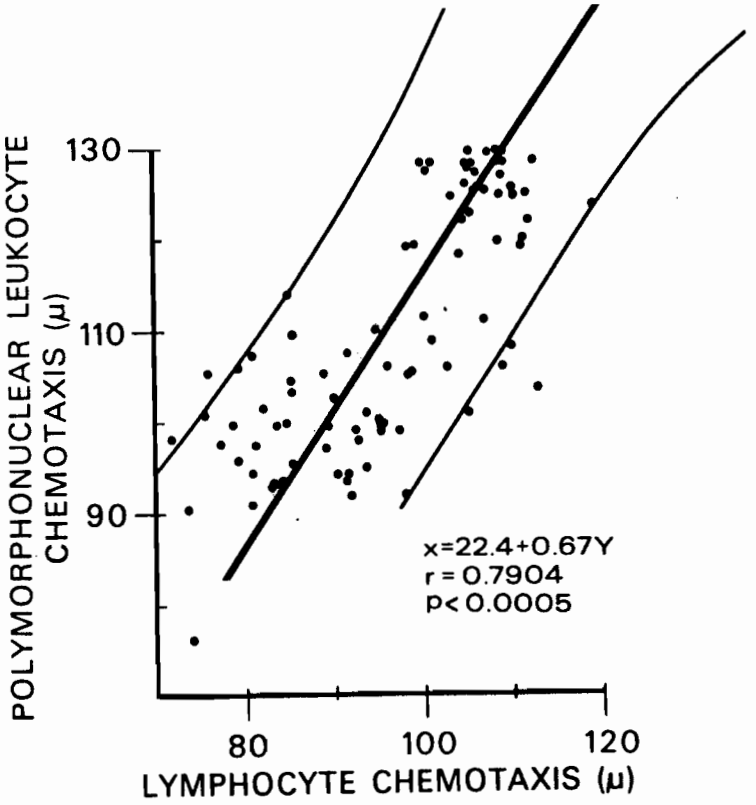


Figure 8. The correlation between simultaneous determination of PMN chemotaxis and lymphocyte chemotaxis in 87 subjects. Dark line is the regression slope and 95% confidence limits are represented by the light lines.

TABLE 19

The effect of patients sera on the chemotaxis of normal control lymphocytes and polymorphonuclear leukocytes (n = number of sera tested; chemotactic values are mean ± SD)

SKIN TEST OF SERUM DONOR	PMN CHEMOTAXIS (μ)	LYMPHOCYTE CHEMOTAXIS (μ)
Normal control (n = 3)	128.1 ± 2.4	112.2 ± 3.1
Relative Anergy (n=3)	95.1 ± 1.7	81.5 ± 2.2
Anergy (n=7)	92.5 ± 1.8	79.0 ± 2.0

TABLE 20

The effect of Levamisole on the chemotactic migration of lymphocytes

MEDIUM	NORMAL CONTROL	ANERGIC PATIENT
MEM-FCS	112.7 ± 1.0 μ	81.3 ± 1.1 μ
MEM-FCS $+10^{-4M}$ Levamisole	113.0 ± 0.4 μ	108.9 ± 1.5 μ

(table 19, $p < 0.005$). The effect of levamisole -HCl on normal laboratory control lymphocyte chemotaxis and on a patient with skin test A was tested by adding levamisole in the medium. As can be seen from table 20, normal lymphocytes migration was not affected, but decreased lymphocyte migration was corrected to normal in this experiment.

14. POLYMORPHONUCLEAR LEUKOCYTE STUDIES AND SKIN TEST RESPONSE

14.1 Adherence

14.1.1 Development of the Method

Existing systems in the literature were modified and adapted in order to measure adherence of neutrophils from normal controls and hospitalized patients, as well as testing for the effect of plasma, serum and drugs. Neutrophil adherence was proportional to the fiber weight in both the whole blood adherence and purified neutrophil adherence systems and exhibited a non-linear relationship (figures 9 & 10). The fiber weight of 5 mg for the purified neutrophil system gave a control neutrophil adherence of 72.7 ± 5.7% (n = 21, mean ± SD). The comparable fiber weight for the whole blood system was found to be 30 mg and gave a control adherence of 71.5 ± 3.8% (n = 21). Variations in the cell concentration from 1×10^6 to 50×10^6 neutrophils/ml in the purified neutrophil system resulted in all adherence values falling within 1 standard deviation of the control (figure 11). Variation of the pH of the resuspending medium

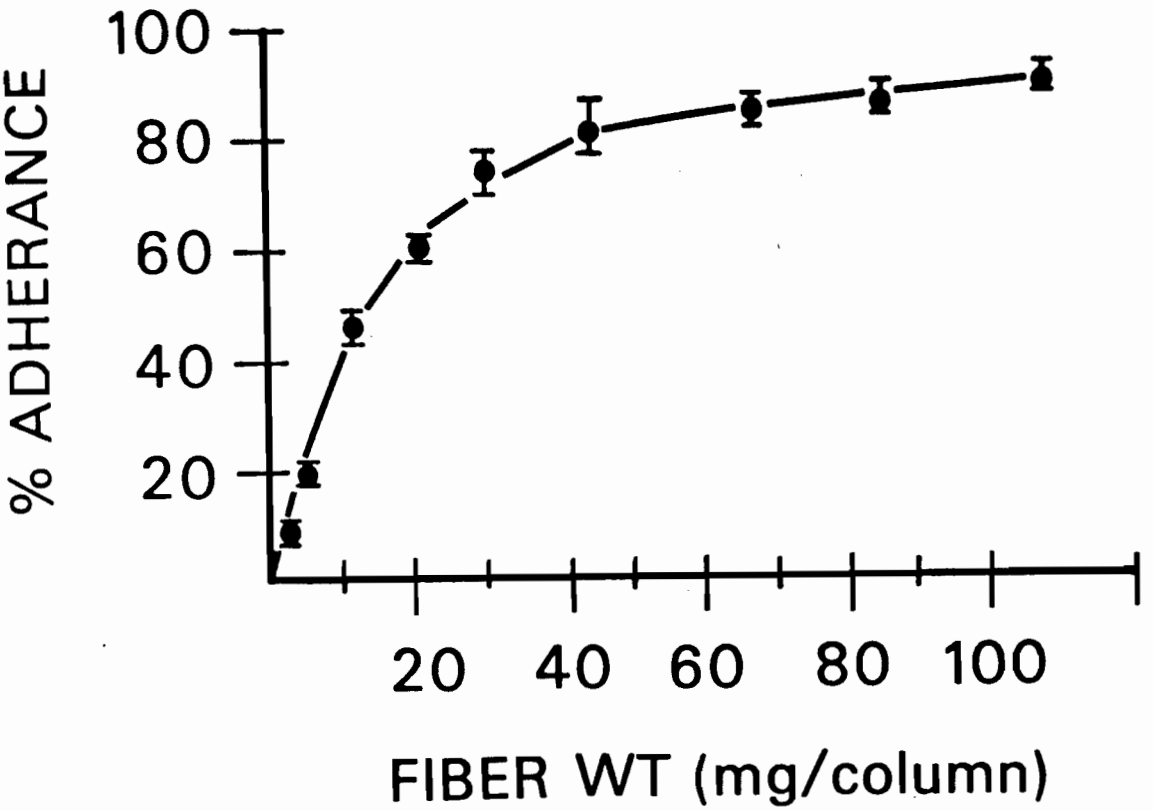


Figure 9. The relationship between fiber weight and neutrophil adherence in the whole blood system.

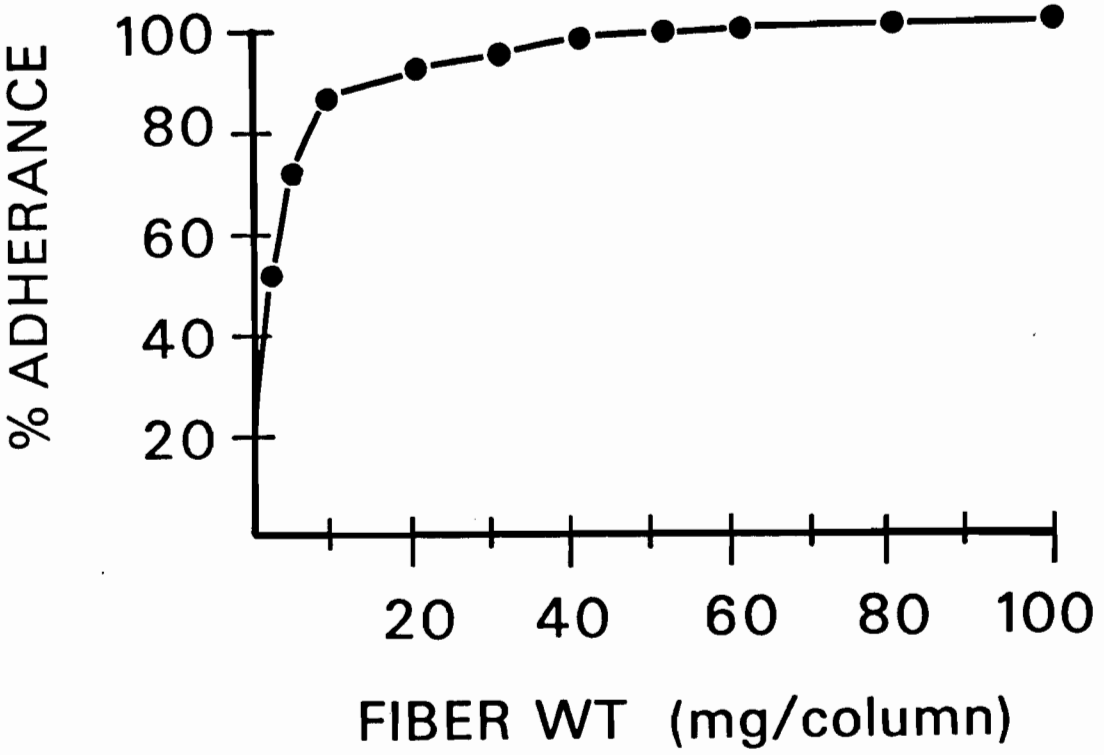


Figure 10. The relationship between fiber weight and adherence in the purified neutrophil system.

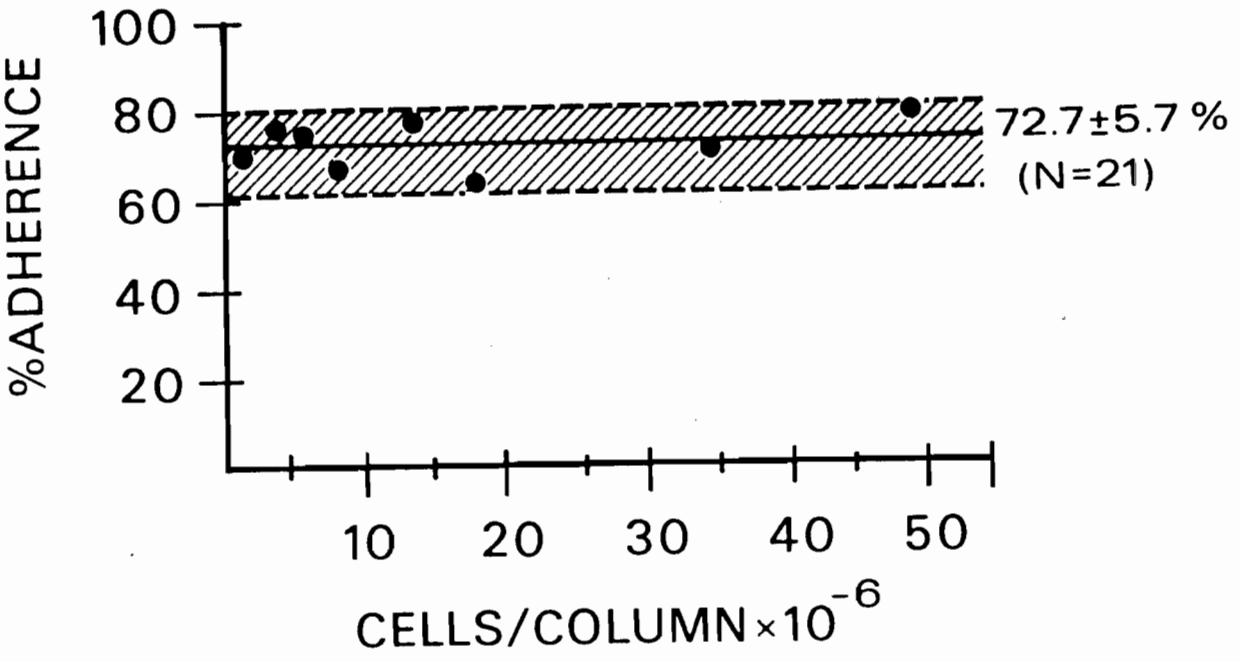


Figure 11. The effect of the variation in the cell concentration on their adherence in the purified neutrophil adherence system.

showed linear constant adherence between pH 6 to 7.8. The adherence became markedly decreased above pH 8.5 (figure 12).

Day to day variation in control subjects was low as shown in table 21 for the purified adherence system. Similar results were obtained with the whole blood system. Glycolytic inhibitors and cation chelators resulted in decreased adherence and chemotaxis of PMNs from controls and patients with skin test anergy (table 22).

In order to ensure that measurements of neutrophil adherence using the two systems was indeed representative of granulocyte adherence, the PMNs were separated from mononuclear cells using ficoll-hypaque gradients in six control subjects and then measurements of the adherence of these PMNs were made using 5 mg columns. The mean adherence was 73.7 ± 5.9% (± SD) which did not differ significantly from the mean laboratory normal control adherence, measured with the whole blood system (72.7 ± 5.7%), or the purified neutrophil system (72.4 ± 4.4%). Differential counts performed manually on five whole blood adherence samples gave a PMN adherence of 72.4 ± 5.1%.

14.1.2 Adherence of Neutrophils from Normal Laboratory Controls and Hospitalized Patients

The cumulative results of adherence measurements with the whole blood system are shown in figure 13. The twenty one laboratory controls had two or more different determinations (n = 54)

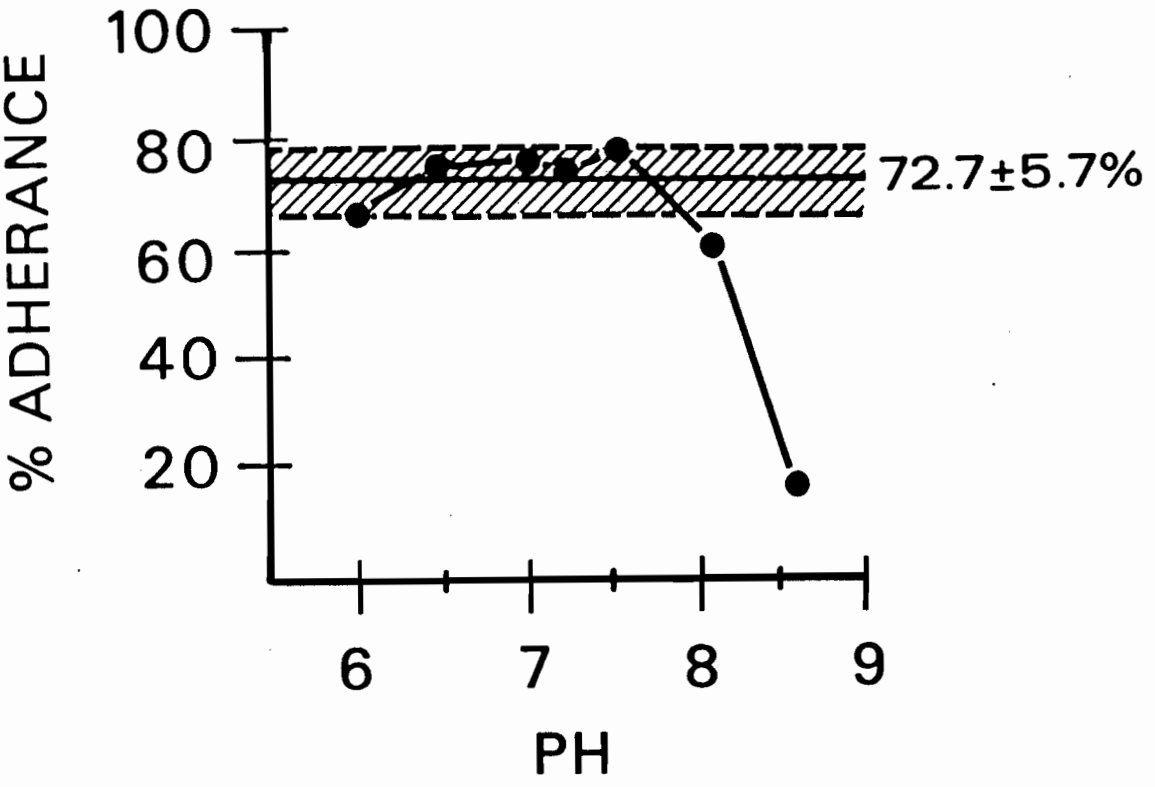


Figure 12. The effect of the hydrogen ion concentration on the adherence of the purified neutrophils.

TABLE 21

The day to day variation in the purified leukocyte adherence of control subjects

SUBJECT	DAY 1	DAY 2	DAY 3	DAY 4	DAY 5
N.C.	76%	74%	71%	77%	72%
I.H.	72%	74%	72%	69%	-
J.M.	70%	73%	-	73%	75%

TABLE 22

The effect of glycolytic pathway inhibitors on leukocyte adherence from control and anergic surgical patients

NEUTROPHIL SOURCE	TREATMENT	CHEMOTAXIS (u)	ADHERENCE
Control	MEM	125.0 ± 0.28	67%
"	KF 0.1M	6.2 ± 0.14	31%
"	EDTA 0.1%	6.1 ± 1.0	18%
Anergic	MEM	91.7 ± 0.42	89%
"	KF 0.1M	7.5 ± 0.42	22%
"	EDTA 0.1%	7.3 ± 1.3	17%

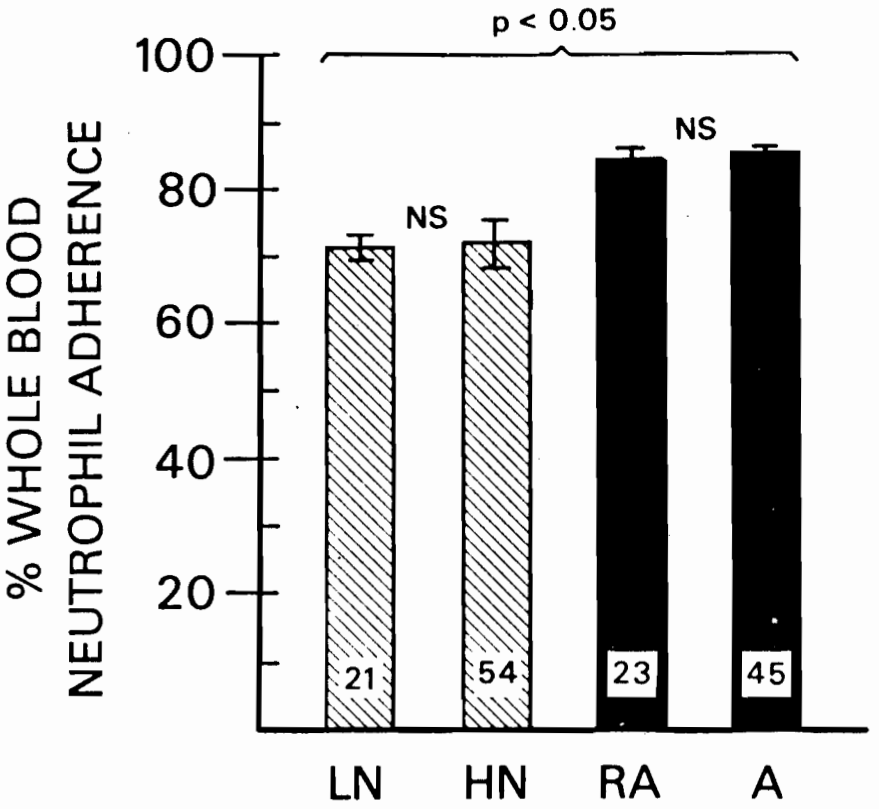


Figure 13. The relationship between whole blood neutrophil adherence and skin test response.

during the course of this study and the mean (± SE) of these readings was used as the laboratory control neutrophil adherence, (71.5 ± 0.51; 95% CL of mean = 70.4 to 72.5 and 95% CL of sample = 64.1 to 78.9%). Fifty four hospitalized patients who demonstrated normal skin test response had a slight reduction in neutrophil chemotaxis. Their adherence was 72.5 ± 1.7%. These patients showed a wide standard deviation because some of them had an evolving hospital course. Some deteriorated after they were studied, and some were recovering both their health and skin test response. Twenty three relatively anergic patients had a significantly increased adherence from the laboratory normal control and the in-hospital normals, and similarly 45 anergic patients had significantly increased adherence compared to the laboratory controls and in-hospital normals ($p < 0.001$) but not the in-hospital relatively anergic population. Eighty four surgical patients who had simultaneous measurements of neutrohil adherence and chemotaxis, are shown in figure 14. The regression equation is $Y = 137.7 - 0.51X$ with a correlation coefficient, $r = 0.81$, $p < 0.001$.

14.1.3 The Effects of Plasma and Serum on Neutrophil Adherence

The effect of serum and plasma on the adherence of purified normal control neutrophils was assessed using this system. In aliquots of 15×10^6 cells, MEM was removed by centrifugation and the cells were resuspended in 3.5 ml of normal control serum, normal control plasma, anergic serum, or

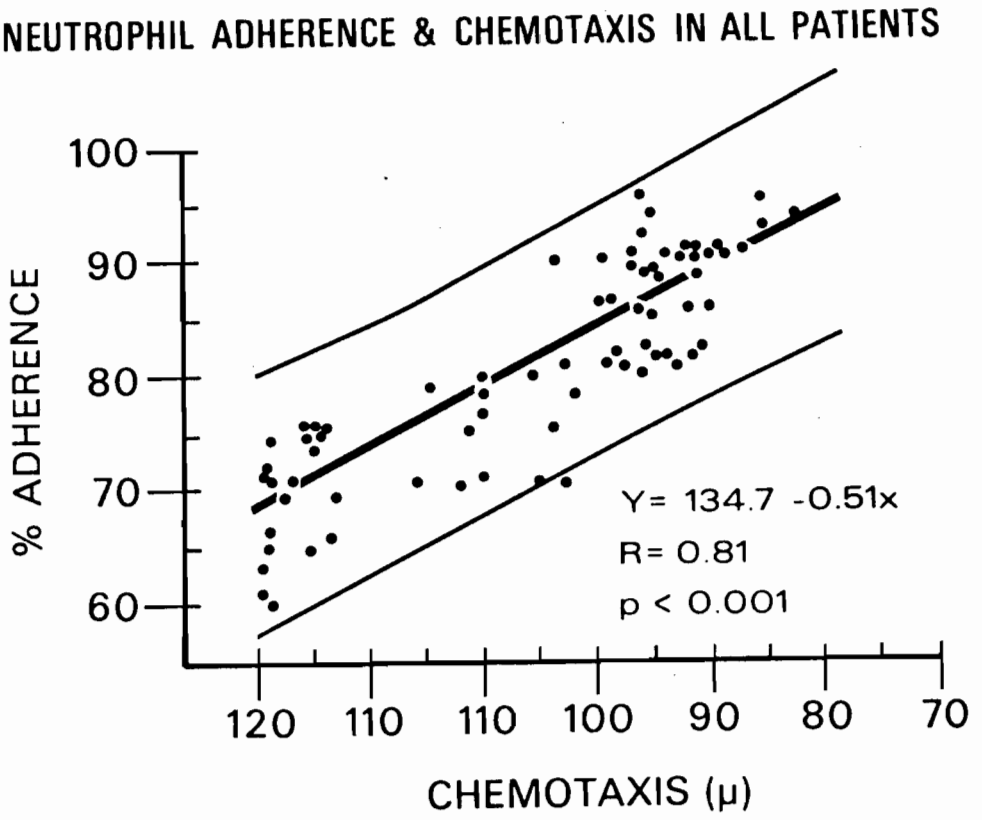


Figure 14. The correlation between increased neutrophil adherence and decreased neutrophil chemotaxis in anergy. The regresion line and 95% confidence limits are shown as dark and solid lines respectively.

anergic plasma. Serum and plasma from four different normal and four anergic patients were used. One aliquot was resuspended in MEM only, as a control. After a 10 min incubation the adherence was measured with 5 mg columns. Neutrophil chemotaxis was assessed simultaneously. The anergic serum donors (n = 4) demonstrated a mean autologous neutrophil adherence of 90.3 ± 1.3 (± SD) and chemotaxis 93.7 ± 4.5 μ (± SD), both significantly abnormal. Table 23 shows that normal neutrophils resuspended in MEM showed the expected adherence of 72.3 ± 4.4% as well as normal chemotaxis. Resuspension in normal serum or serum from patients with skin test anergy demonstrated no significant changes in the adherence, though the chemotaxis of the normal cells dropped to 94.7 ± 4.5 μ (± SD) after treatment with anergic serum. Resuspension of normal neutrophils in normal plasma gave an adherence that was different from cells without treatment with plasma, as measured with the purified neutrophil system. However, resuspension of normal cells in plasma from the four anergic patients demonstrated a significantly increased adherence of 74.5 ± 11.3% (± SD) over the normal plasma treated cells and decreased chemotaxis of 93.3 ± 2.7 μ (± SD).

14.1.4 The Effects of Drugs on Neutrophil Adherence

A number of drugs were investigated for their ability to alter neutrophil function <u>in vitro</u>. Adherence and chemotaxis was measured simultaneously on the same sample. Hydrocortisone sodium succinate (figure 15) had no effect at concentrations from 0 to 5 mg% on either measurements from normal or anergic patients.

TABLE 23

The effect of treating normal control neutrophils with serum or plasma from normal controls and anergic surgical patients (n = 4)

TREATMENT OF CELLS	CHEMOTAXIS (μ)	ADHERENCE (%)
MEM	128.6 ± 2.6	72.3 ± 4.4
Normal Serum	127.1 ± 2.4	68.7 ± 2.5 *
Normal Plasma	127.7 ± 1.8	24.0 ± 6.0 **
Anergic Serum	94.7 ± 4.5	68.8 ± 4.5 *
Anergic Plasma	93.3 ± 2.7	74.5 ± 11.3 **

* N.S.; ** $p < 0,001$ Students t-test

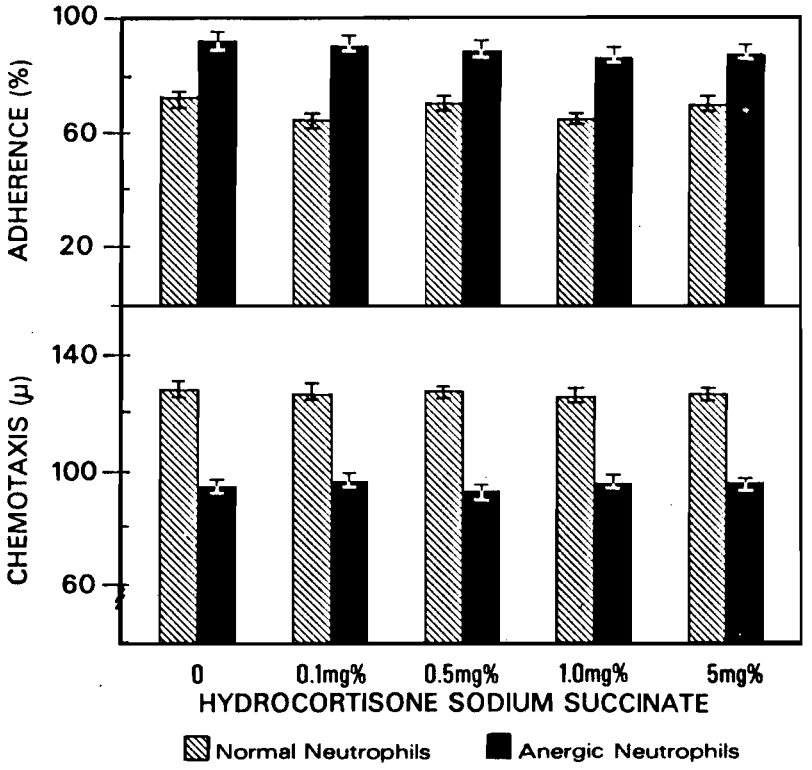


Figure 15. The effect of hydrocortisone on the adherence and chemotaxis of neutrophils from normal and anergic patients.

Ascorbic acid (figure 16) at 0 to 5.0 mg% also had no effect. Ethanol (figure 17) at levels 0-1 g% was found to markedly affect both the chemotactic behaviour and adherence of control and anergic neutrophils. Two other drugs were tested in vitro. Acetylsalycilic acid (ASA) at 0 to 12 x 10^{-5}M,was found not to alter neutrophil adherence measured with the whole blood system. The mean adherence of 11 patients (3N, 2RA, 6A) was 79.8 ± 8.5% (±SD) before ASA and 76.4 ± 8.6% after treatment (p = 0.5). Levamisole-HCl was added directly at $10^{-7M}$to whole blood. The mean whole blood adherence of 9 patients (1N, 4RA, 4A) was 80.2 ± 4.7% before levamisole treatment and 81.0 ± 6.5% after (p$\sim$ 0.5). Individual patients also showed no significant differences.

14.2 Chemotaxis

14.2.1 Comparison Between the Leading Front Technique and the Cells per High Power Field Method for Assessing PMN Chemotaxis

The chemotactic system used in these studies has been standarized previously and was used without modifications. Parallel chemotactic chambers were set up and incubated for 90, 120 and 180 minutes. Reading of the filters was carried out by the standard leading front technique or by counting the cells per high power field (CPF) on the far side of the filter. Table 24 shows the data. Both techniques will detect a statistical

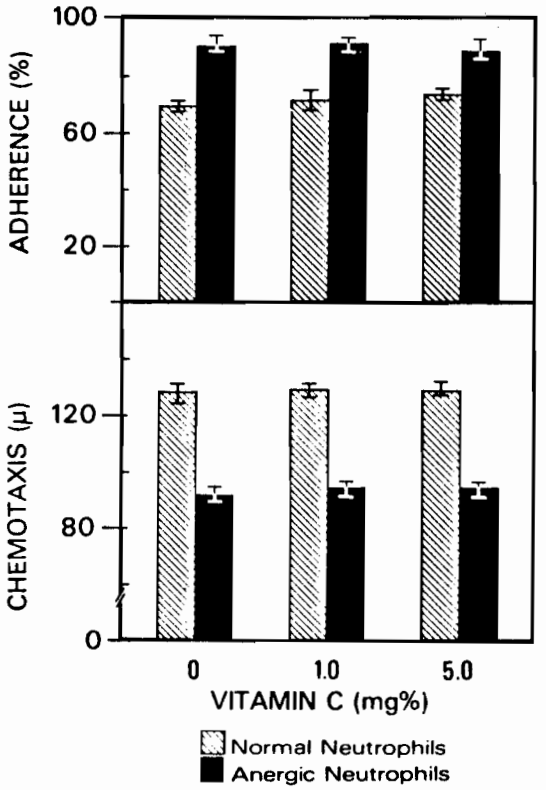


Figure 16. The effect of ascorbic acid on the adherence and chemotaxis of neutrophils from normal and anergic patients.

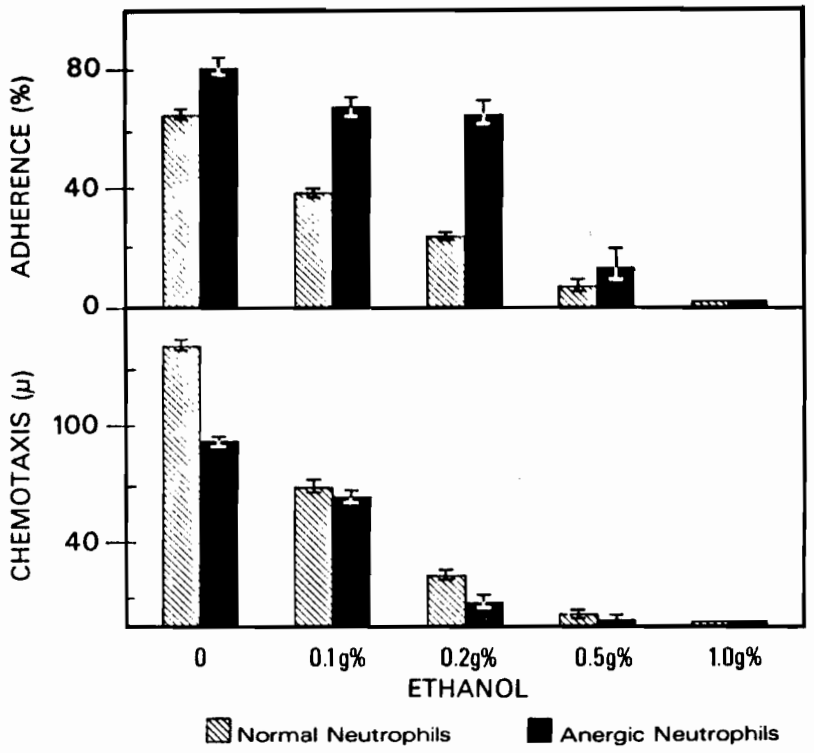


Figure 17. The effect of ethanol on the adherence and chemotaxis of neutrophils from normal and anergic patients.

TABLE 24

A comparison of the Leading Front Technique (LFT) and the Cell per High Power Field Method (CPF) for measurement of PMN chemotaxis (numbers in brackets are 95% confidence limits of the sample)

METHOD	NORMAL PMN	INHIBITED PMN
$LFT_{90\ min}$	125.7 ± 0.5 μ (121-130)	97.2 ± 1.6 μ * (84-109)
$CPF_{120\ min}$	8.3 ± 0.6 CPF (4-13)	18.4 ± 1.3 CPF * (8-29)
$CPF_{180\ min}$	20.2 ± 1.2 CPF (10-30)	32.9 ± 0.9 CPF * (25-39)

* $p < 0.05$, Students t-test

difference in PMN chemotaxis when normal control and maximally inhibited PMN are compared. However,due to considerable overlap in the 95% confidence limits of the sample with the CPF, this method is not ideal for measuring differences in PMN CTX. Pooling the data of normal PMN and inhibited PMN migration distance μ and CPF at 120 or 180 yeilded a significant correlation ($r = 0.54 p < 0.05$ Again, the 95%-CL of the sample about the mean of the CPF were too wide (7.5 to 28.4) to allow for detection of changes in chemotactic migration with the CPF method.

14.2.2 Chemotactic Reponses of PMN from Normal Controls and Hospitalized Patients

Data on the chemotactic migration of PMN from pre-operative surgical patients have already been presented in tables 9 and 12. Table 25 shows all the data collected on 40 laboratory normal controls and 712 hospitalized patients who had skin tests and chemotaxis performed within three days of each other, most on the same day. No separation of patients is made as to pre- or post-operative, trauma, burn, sepsis, etc. The laboratory control PMN chemotaxis based on 95 determinations,(26M, 14F, age range 22-56 years) is 127.4 ± 0.2 μ with 95% confidence limits of the sample equal to 123.8 to 131.8 μ. Hospitalized normal skin test responders had significantly lower PMN chemotaxis than control normals as did RA and A patients, with all groups being statistically different from one another at $p < 0.001$ (analysis of variance). The 95% confidence limits of the samples of the

TABLE 25

PMN chemotactic migration and skin test response. PMN CTX and skin tests performed within 3 days of each other. (CL = Confidence Limits)

GROUP	n	PMN CTX	95% CL-MEAN	95%-CL SAMPLE
Laboratory N Controls	95	127 ± 0.2 *	127.4 - 128.1	123.8 - 131.8
Hospital Normal	329	112.3 ± 0.7 *	111.0 - 113.6	88.5 - 136.0
RA	139	105.9 ± 1.0 *	103.8 - 107.8	82.0 - 129.2
A	244	101.9 ± 0.8 *	100.3 - 103.5	77.4 - 126.5

* $p < 0.001$, Analysis of variance and Scheffe's test

four groups were found to overlap. It was noted clinically that PMN chemotaxis tended to vary depending on the clinical course of the patient.

14.2.3 Rates of Chemotactic Migration of Normal Control PMN and Maximally Inhibited PMN From Anergic Patients

Sixty samples of PMN from normal laboratory controls and 39 samples of inhibited PMN from hospital anergic patients were studied in order to determine the rates of PMN chemotactic migration. Normal PMN demonstrated isolated chemotactic migration at 90 min within the 95% CL of the normal laboratory control sample 123.8 to 131.8. Maximally inhibited PMN demonstrated CTX at 90 min less than 100 μ (lower 95%-CL of anergic PMN CTX = 100.3 μ). To assess the rates of migration, chemotaxis was allowed to occur for 15, 30, 45, 60, 75 and 90 min in each case. Figure 18 shows the slopes and 95%-CL of the sample for the normal control PMN and the maximally inhibited PMN. Normal PMN migrated 1.32 μ/min and maximally inhibited PMN migrated 1.11 μ/min. These two rates of migration, then, are the two extremes expected in these patients using these systems.

14.2.4 The Clinical Significance of Decreased PMN Chemotaxis

Two hundred and fifty four patients, (some included in the analysis of skin test response presented earlier) who had sequential chemotactic measurements were used in this analysis.

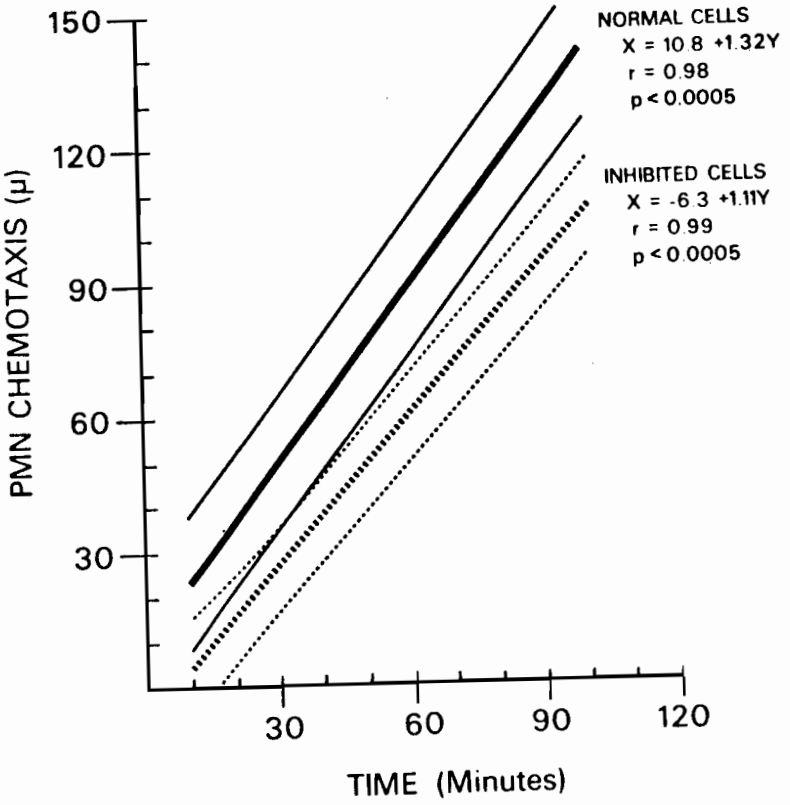


Figure 18. The rates of chemotactic migration of normal and inhibited PMN . Regression slopes are shown as dark lines, and 95% confidence limits by the light lines.

The purpose was to demonstrate any association between depressed neutrophil chemotaxis and clinical outcome. All patients had sequential skin tests and chemotaxis measurements. PMN chemotaxis showed a wide spectrum of values between the normal and anergic range depending upon the clinical state of the patient. The "trend" of PMN chemotactic migration, eg. improvement towards normal level, signalled an improvement in the patient's clinical course. The worst chemotactic measurement achieved by a patient during his hospitalization is recorded in table 26, along with the skin test response at that time, and the eventual clinical outcome. As a patient demonstrated progressively slower PMN chemotaxis particularly values less than 99 μ, the incidence of septic complications increased as did the mortality rate. The worst prognosis was seen in patients who demonstrated cutaneous anergy and PMN chemotaxis less than 99 μ, at least once in their clinical course. No patient demonstrating both normal skin tests and normal PMN CTX throughout the hospital course had sepsis or died. Normal PMN chemotaxis was defined greater than 123.8 μ since 95% confidence limits of the normal laboratory sample was found to be 123.8 to 131.8 μ. The non septic patients had decreased chemotaxis for a period of 1.78 + 0.08 weeks (n = 165, mean ± SE) and the septic patients had PMN CTX decreased for 2.14 ± 0.12 weeks (N = 89, mean ± SE, $p < 0.001$). In 44 septic patients, PMN chemotaxis was clearly depressed <u>before</u> the septic episode <u>was detected</u>.

TABLE 26

The correlation between clinical outcome, skin test response and neutrophil chemotaxis in 254 surgical patients

CHEMOTAXIS	NUMBER	SKIN TEST	SEPSIS	DEATH
> 123.8 μ	80	70 N	0	0
		3 RA	1	0
		7 A	0	0
			1 (I.3 %)	0
100-123.8 μ	66	6 N	0	0
		28 RA	10	3
		32 A	18	11
			28 (42%)	14 (21%)
< 99 μ	108	8 N	0	1
		21 RA	8	3
		79 A	52	29
			60 (56%)	33 (31%)

14.3 Polymorphonuclear Neutrophil Phagocytosis

14.3.1 Characterization of the Method

Two laboratory controls were used to evaluate the technique. Table 27 shows the comparison of the phagocytic capacity of PMN from a normal male, compared to a normal female. Tests were done on different days over 12 weeks. Maximal phagocytosis was seen at 10 min and this represented only ~ 50% of the available bacteria. This uptake includes all bacteria detected in the PMN pellet after thorough washing. To assess the proportion of bacteria that were sticking to the cell membranes but not internalized, heat killed PMN ($70^{o}C$ x 20 min) were mixed with the bacteria in separate tubes and the same procedure carried out as with live cells. Normal control PMN, demonstrated 14.9 ± 5.6% (±SE, 95%-CL of mean 9.3-19.5) of the bacteria to be PMN associated but not internalized. Similarly, to determine the proportion of bacterial contamination in the PMN pellet by free bacterial cosedimentation, tubes with bacteria only were carried through the assay. This was found to be 7.7 ± 1.0%. PMN: Bacteria ratio varied from 1:2 to 1:20 demonstrated similar overall uptake, however the numbers of assays with each ratio ($<$ 10) did not permit for reliable statistical analysis.

14.3.2 Phagocytic Capacity Between Laboratory Controls and Hospitalized Patients

The phagocytic capacity of PMN from 21 healthy laboratory

TABLE 27

The phagocytic capacity of PMN from two healthy laboratory normal controls and the variability of the method. (Mean ± SE of bacterial uptake is shown. In brackets are the 95%-CL of the mean.)

TIME	CONTROL #1 (n=7)	CONTROL #2 (n=5)	p
5 min	45.1 ± 3.9% (34.4-53.8)	46.6 ± 5.2% (32.0-61.2)	N.S.[1]
10 min	46.3 ± 5.3% (31.6 - 58.3)	51.2 ± 7.3% (30.9 - 71.5)	N.S.
20 min	43.8 ± 4.6% (30.9 - 54.3)	41.0 ± 5.8% (25.0 - 50.0%)	N.S.

1 - Analysis of Variance and Scheffe's test

controls against staphylococcus 502A was measured and compared to that from 18 hospitalized patients with normal skin test response and 37 patients with relative anergy or anergy (9RA, 28A). Analysis of variance was used to compare all nine groups simultaneously and significance was determined using Scheffe's test and the Studentst distribution. Table 28 shows that PMN from anergic patients had a statistically higher percentage of phagocytosed bacteria detected at all times sampled, as compared to laboratory control PMN. No other statistically significant difference was detected using Scheffe's test. Using the t distribution to assess significance, differences were noted between laboratory controls and normal patients PMN at 10 and 20 min, as well as between patient normal and anergic PMN at 5 and 10 minutes.

Simultaneous measurements of PMN chemotaxis in the three groups showed that there were patients with depressed chemotaxis in the normal hospitalized patient group and normally migrating PMN in the anergic group. Normal laboratory control PMN chemotaxis was 127.1 ± 0.5 μ, hospitalized normal PMN CTX was 121.0 ± 2.2 μ and anergic patients PMN CTX was 116.6 ± 1.6 μ. Figure 19 shows a graphic representation of phagocytosis of PMN with normal chemotaxis equal to 125.6 ± 0.4 μ compared to those with decreased chemotaxis of 109.0 ± 1.5 μ. There was a significantly higher percentage of bacteria associated with the cells which demonstrated decreased chemotaxis. Furthermore, there was a significant correlation between depressed chemotaxis and phagocytosed bacteria

TABLE 28

Phagocytic capacity of PMN from laboratory controls, hospitalized normal, and anergic skin test responders

TIME (min)	LAB N (n = 21)		HOSP N (n=18)		ANERGIC (n=37)
		N.S.		N.S.	
5	* 40.8 ± 3.5		52.2 ± 4.8		65.6 ± 3.1 *
		N.S.		N.S.	
10	** 48.6 ± 4.3		62.6 ± 5.3		76.1 ± 2.8 **
		N.S.		N.S.	
20	*** 40.9 ± 3.3		62.2 ± 5.6		64.0 ± 2.6 **

* vs * $p < 0.05$

** vs ** $p < 0.05$

*** vs *** $p < 0.05$

* vs ** vs *** N.S.

Analysis of Variance and Scheffe's test

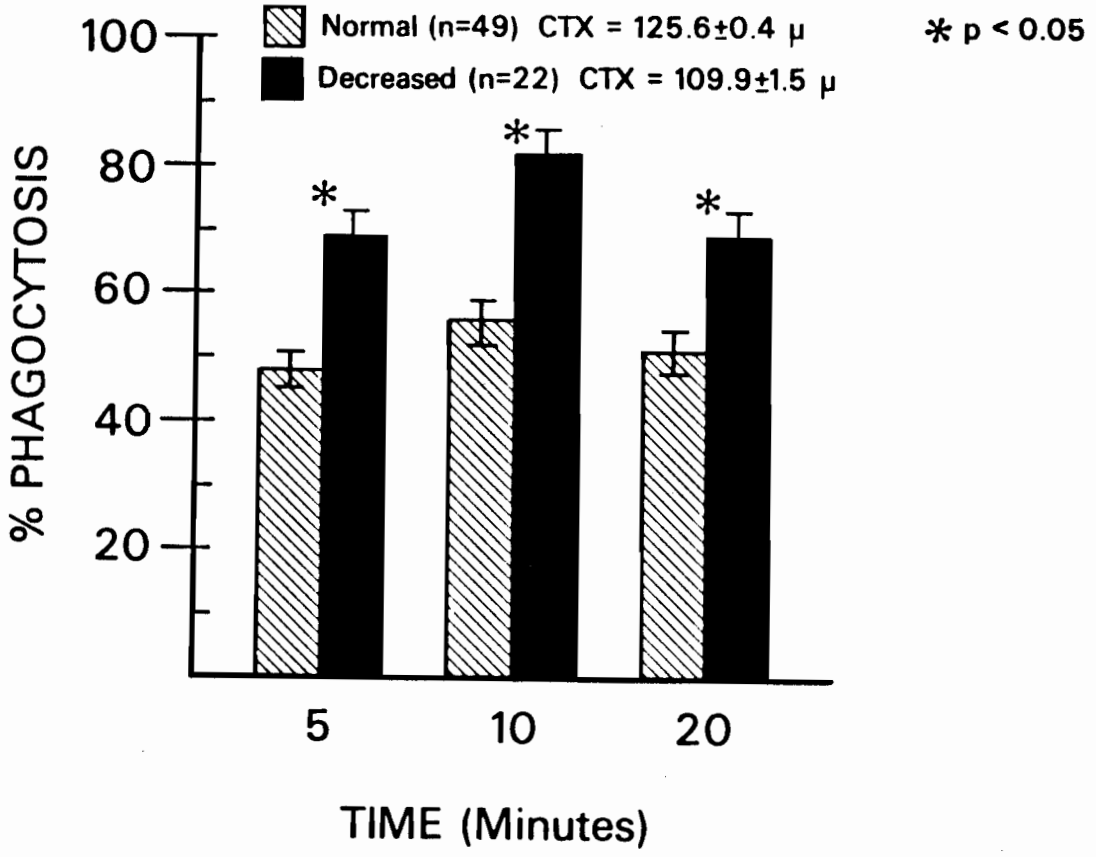


Figure 19. A comparison of the phagocytic capacity between PMN with normal and decreased chemotaxis.

at all times tested (5 min, $r = 0.47$, $p < 0.001$, 10 min, $r = 0.54$ $p < 0.001$, 20 min, $r = 0.26$ $p < 0.05$). The numbers of bacteria attached to PMN in the anergic patients but not internalized (determined using killed PMN) was 19.8 ± 1.6% (mean ± SE, 95% CL of mean 16.6 - 22.8), which was not significant from the membrane associated bacteria of normal control PMN.

14.4 Polymorphonuclear Neutrophil Bactericidal Function

14.4.1 Characterization of Method

As for PMN pahgocytosis, the same two laboratory controls were used to characterize the technique used to measure PMN bactericidal function. Table 29 shows the data. There was 85.5% kill of the initial inoculum by the control PMN, reducing the inoculum by $\log_{10} \sim 0.67$ or from $\sim 3 \times 10^8$ cfu to $\sim 5 \times 10^7$ cfu after 240 min. In the first 10 min 50 to 60% of the bacterial inoculum was killed. The 95% confidence limits were very wide especially at the early sampling times. No difference in killing was noted in the two controls. Bacteria incubated without PMN in medium lost no viability up to 240 min, and actually increased in number.

14.4.2 Polymorphonuclear Neutrophil Bactericidal Function Between Laboratory Controls and Hospitalized Patients

Table 30 shows the data on the bactericidal capacity of PMN from 19 laboratory normal controls, 6 hospitalized normal

TABLE 29

Bactericidal capacity of PMN from two different laboratory controls (% of total bacteria killed ± SEM; 95%-CL of the mean is shown in brackets)

TIME (min)	CONTROL #1 (n=7)	CONTROL #2 (n=5)	p
10	60.3 ± 3.7 (44.3 - 69.4)	52.0 ± 7.0 (21.9 - 82.1)	N.S.[1]
20	65.2 ± 4.1 (57.0 - 70)	64.0 ± 5.4 (40.9 - 78.9)	N.S.
40	69.3 ± 3.2 (60.4 - 77.1)	74.8 ± 4.7 (61.9 - 87.7)	N.S.
80	75.3 ± 3.5 (65.7 - 83.8)	78.0 ± 4.5 (65.6 - 90.4)	N.S.
160	83.4 ± 2.9 (75.4 - 90.5)	82.2 ± 4.8 (68.9 - 95.5)	N.S.
240	85.5 ± 3.2 (78.1 - 92.1)	-	-

1 Students t-test

TABLE 30

Bactericidal capacity of PMN from Laboratory controls, hospitalized normal and anergic skin test responders (% kill of initial inoculum ± SEM)

TIME (min)	LAB N (n=19)		HOSP N (n=6)		ANERGIC (n=15)
10	* 63.5 ± 2.6		*45. ± 3.2	N.S.	50.0 ± 2.9 *
20	64.1 ± 2.6	N.S.	60.0 ± 4.3	N.S.	60.8 ± 2.3
40	73.3 ± 2.2	N.S.	69.8 ± 4.5	N.S.	73.2 ± 2.3
80	78.6 ± 2.1	N.S.	77.3 ± 3.2	N.S.	83.0 ± 1.7
160	83.9 ± 2.0	N.S.	80.9 ± 2.6	N.S.	86.5 ± 1.6

* $p < 0.05$ Analysis of Variance and t distribution

* N.S. Analysis of Variance and Scheffe's test

skin test responders and 15 anergic skin test responders. Analysis of variance using Scheffe's test for differences of means showed no significant differences in any of these groups, though the t distribution indicated that hospital normal and anergic patients had a significant decrease in bactericidal function after 10 min. No difference could be detected when PMN with normal chemotaxis were compared to those with decreased chemotaxis (figure 20). Also, there was no correlation between PMN chemotaxis and bactericidal capacity. Paired t-test analysis of normal controls and anergic patients studied the same day showed no difference in bactericidal function though the anergic PMN demonstrated increased phagocytosis ($p < 0.05$).

15. CHARACTERIZATION OF THE PMN CHEMOTACTIC INHIBITING ACTIVITY IN SERUM

15.1 Chemotactic Inhibitors in Serum of Patients with Decreased Autologous PMN Chemotaxis

Sequential determinations of PMN chemotaxis in trauma patients demonstrated that decreased autologous PMN chemotaxis appeared rapidly and was detectable within 2 hrs of the traumatic insult in most patients. To account for this, a search was carried out for circulating serum inhibitors of PMN chemotaxis in the serum of patients with decreased autologous PMN chemotaxis. Of 170 patients with A or RA tested, 160 (94%) demonstrated

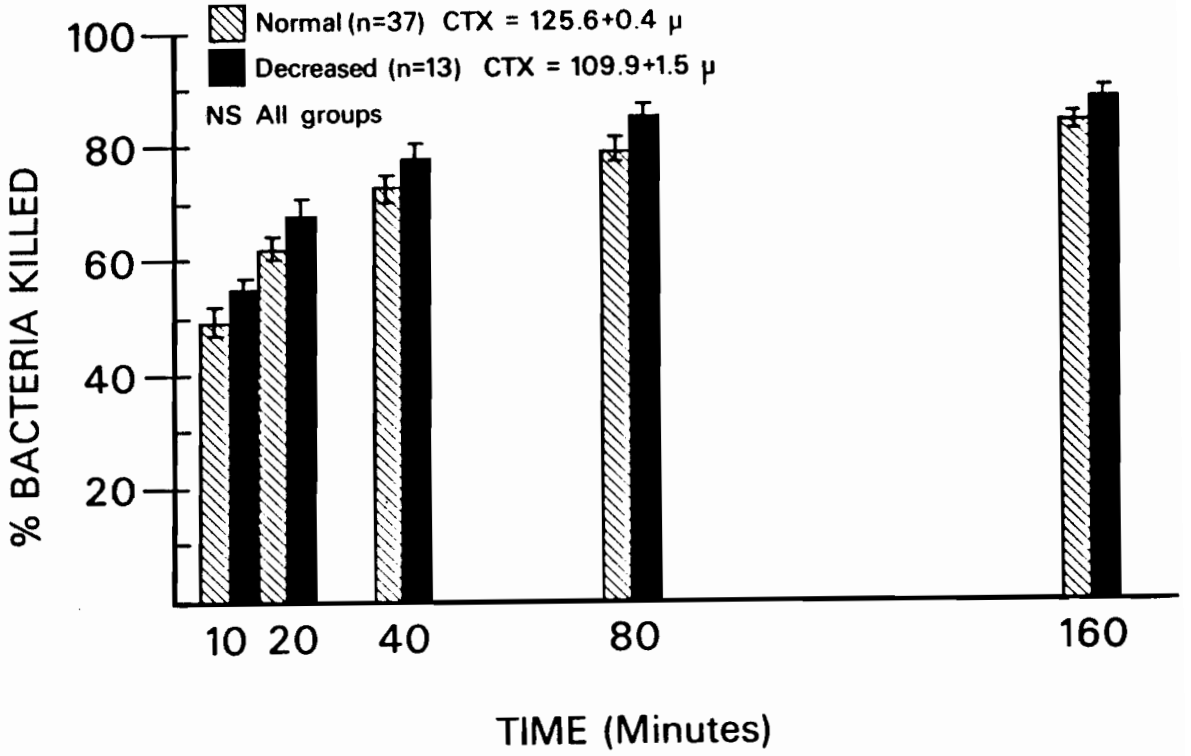


Figure 20. A comparison of the bactericidal capacity between PMN with normal and decreased chemotaxis.

decreased autologous PMN chemotaxis. One hundred and eleven sera from this pool were tested and in every instance, autologous serum decreased the migration of heterologous neutrophils to the same distance as the autologous neutrophils of the serum donor. A typical experiment is presented in table 31.

This serum factor was found to bind avidly to the neutrophil membrane. Washings of anergic neutrophils up to eight times with MEM failed to improve their altered chemotaxis (figure 21). Successive resuspensions of fresh 5 x 10^6 cell aliquots of normal neutrophils in 2 cc of inhibitory serum removed a small quantity of inhibitor sequentially up to 12 resuspensions, at which time, there still remained some chemotactic inhibition in the serum (figure 22). Washing these "converted" neutrophils exhaustively with MEM failed to correct their chemotaxis to normal.

The dose response curve of the effect of various dilutions of anergic serum on the chemotaxis of normal neutrophils is shown in figure 23. Inhibitory serum from a patient with autologous PMN chemotaxis of 92.0 ± 1.2 μ was diluted with MEM to yield sera concentrations from 0-100%. One ml aliquots were used to preincubate normal neutrophils before chemotaxis was assessed. These results have been consistently obtained with 5 different inhibitory sera indicating a heterogeneous system of chemotactic inhibitors.

TABLE 31

Inhibition of normal neutrophil chemotaxis by treatment with anergic serum

NEUTROPHILS	SERUM	CHEMOTAXIS (μ)
Normal	Normal	123.0 ± 1.1
Anergic	Anergic	90.0 ± 1.9
Normal	Anergic	90.0 ± 1.1

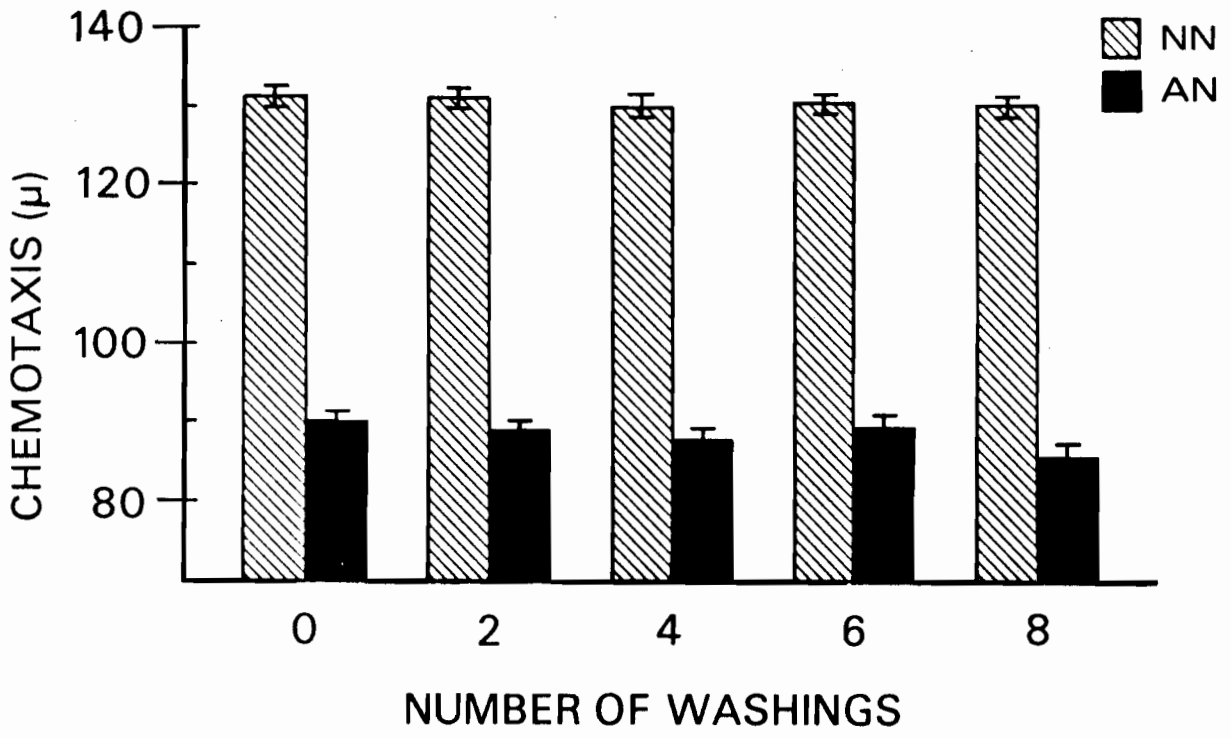


Figure 21. The effect on neutrophil chemotaxis of repeated washings of cells from normal controls or in-hospital anergic patients. NN=normal control, AN=anergic cells.

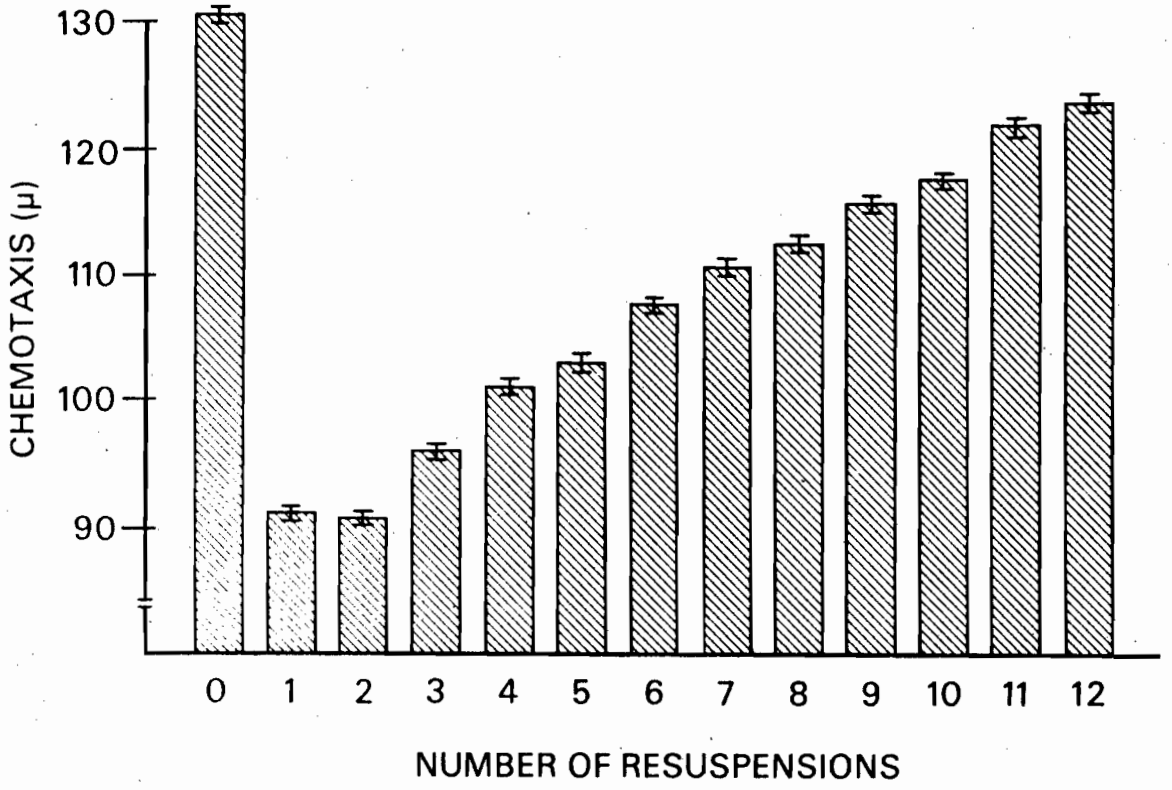


Figure 22. The removal of chemotactic inhibitor from a constant volume of anergic serum by repeated resuspension and removal of fresh batches of 5×10^6 normal neutrophils.

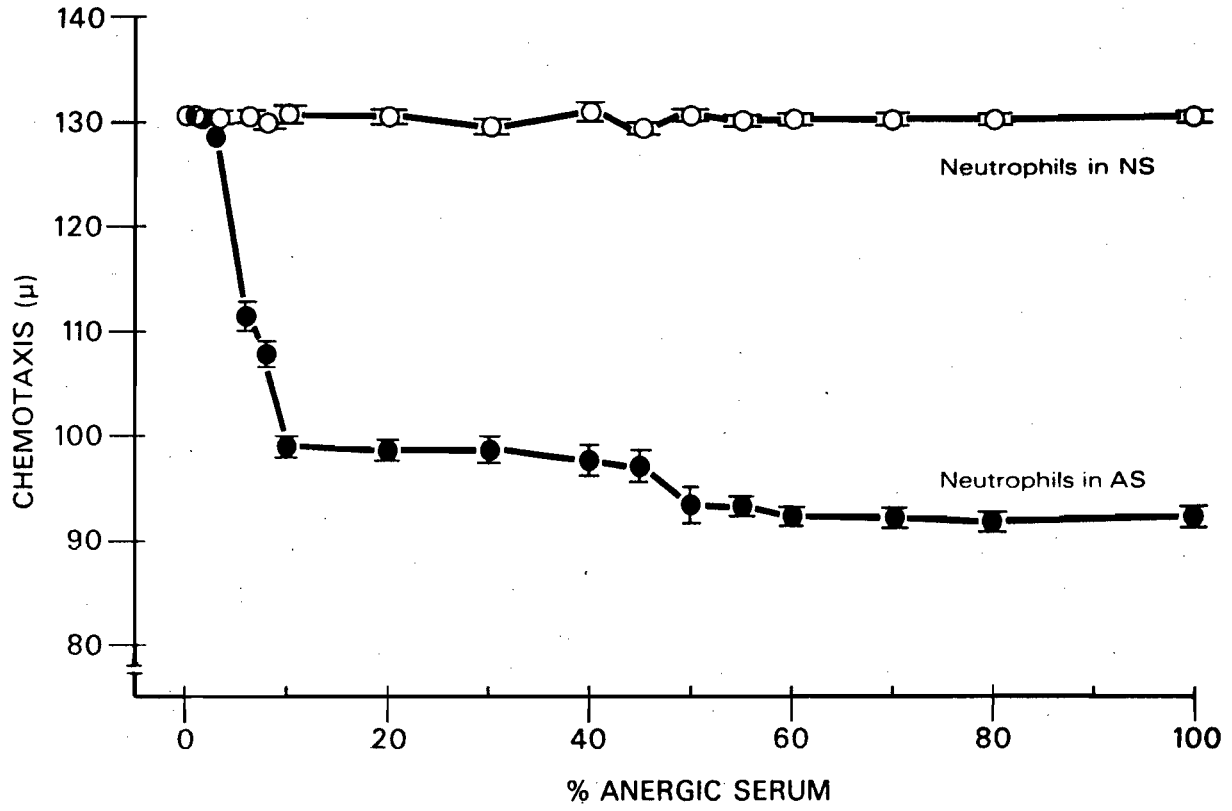


Figure 23. The dose response curve of the ability of various dilutions of anergic serum to inhibit the chemotaxis of normal control PMN. Normal serum dilutions have no effect.

Storage of the inhibitor in the form of anergic serum up to one year at -70°C yielded a 25% loss in activity. The chemotactic inhibiting activity of this serum also survived at 22°C for up to 24 hrs. Heating experiments demonstrated the total loss of chemotactic inhibition at 60°C for 30 mins., the loss being progressive from 50°, 56° to 60°C. Exhaustive dialysis of anergic serum against 0.02 M sodium phosphate buffer pH 7.4 or 1% glycine solution failed to remove the chemotactic inhibitory activity towards normal neutrophils. Storage at -20°C leads to complete progressive loss of activity within one year.

15.2 Does the Serum Inhibitor of PMN Chemotaxis Prevent the Cells from Detecting the Chemotactic Gradient?

It was evident early in this investigation that autologous inhibited PMN rarely migrated less than 85 μ towards casein in the assay used. It is conceivable that such a distance could be achieved by chemokinesis alone, that is, stimulated random cell migration due to the presence of casein in the chambers. To separate chemotaxis from chemokinesis and assess whether the serum inhibitor "blinded" the cells, normal control PMN before or after pretreatment with inhibitory serum were prepared. They were resuspended in various concentrations of casein and run against similarly varying concentrations so as to have different chemotactic gradients to which the cells could respond. The data is shown in tables 32 and 33. The migration distance within the diagonal lines represents migration in the absence

TABLE 32

The effect of varying the concentration gradient of the chemoattractant on the chemotaxis of normal control PMN. Mean migration distance of two experiments are shown

TOP COMPARTMENT Casein mg/ml	BOTTOM COMPARTMENT Casein mg/ml				
	0	0.5	1.0	2.5	5.0
0	25.5	20.0(47.2)	95.8(51.0)	102.85(84.2)	127.4(69.5)
0.5	x	86.5	99.9(93.5)	117.2(100.5)	120.2(102.3)
1.0	x	100.1(103.7)	110.0	126.7(113.8)	124.6(119.5)
2.5	x	106.1(114.4)	98.2(116.5)	119.2	100.1(124.6)
5.0	x	106.4(115.2)	97.9(115.8)	120.0(116.0)	114.6

TABLE 33

The effect of varying the concentration gradient of the chemoattractant on the chemotaxis of normal control neutrophil pre-treated with inhibitory serum (means of two experiments are shown)

TOP COMPARTMENT Casein mg/ml	BOTTOM COMPARTMENT Casein mg/ml				
	0	0.5	1.0	2.5	5.0
0	24.4	100.5 (27.9)	93.5 (56.5)	105.3 (64.5)	101.2 (56.4) ⊕
0.5	x	85.1	96.4 (91.0)	93.6 (89.2)	101.4 (89.1)
1.0	x	87.1 (94.2)	100.9	96.9 (91.6)	99.5 (95.4)
2.5	x	88.7 (96.5)	73.0 (94.1)	91.3	100.3 (103.2
5.0	x	51.6 (95.6)	99.9 (94.5)	96.1 (93.1)	99.9
⊖					

of a chemoattractant gradient but increasing absolute concentration of the chemoattractant. High concentrations of casein are inhibitory to all cell locomotion. The numbers within the brackets represent the calculated distances based on the migration observed within the diagonal lines. The numbers outside the lines are the actual observed migration distances. It can be seen that cells "read" and respond to the gradient despite treatment with inhibitory serum. The decreased migration observed, is not due to total lack of chemotactic migration, and thus chemokinesis, but due to a decrease in the rate of chemotaxis.

15.3 Isoelectric Focusing of Inhibitory Serum from a Surgical Patient

Serum collected on two separate occasions from the same patient was used. The autologous patient neutrophils migrated to 89.5 ± 1.2 μ and 90.0 ± 2.1 μ on the days serum was collected, and the pooled serum inhibited heterologus control and neutrophils to 92.1 ± 1.3 μ. Isoelectric focusing in a broad pH gradient from 3 to 10 separated the inhibitory activity of this serum into two peaks as shown in figure 24. One has a pI = 4.3 and the other pI = 6.3. Both should be basic at physiologic pH and have a β mobility on electrophoresis. To confirm the observations with this experiment the same serum sample was analyzed as described below.

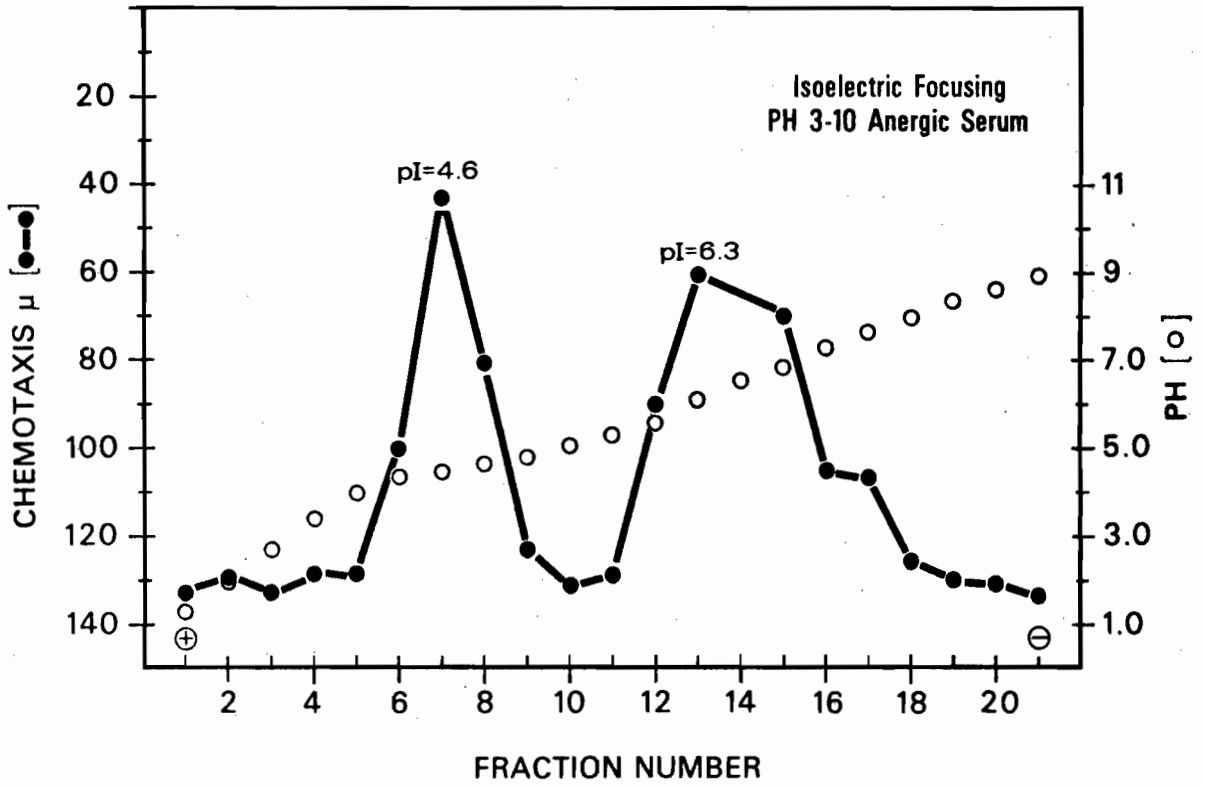


Figure 24. Isoelectric focusing of inhibitory serum in a 0-50% anticonvection sucrose gradient with 1% ampholine in a pH range 3-10. PMN chemotaxis is from top to bottom of figure.

15.4 Sucrose Density Gradient Centrifugation of Inhibitory Serum From a Surgical Patient

The same serum was analyzed on 10-30% sucrose density gradients with cytochrome C, Bovine Serum Albumin and Ferritin as protein markers. Data is shown in figure 25. Two peaks of inhibitory activity were demonstrated. The peaks from the isoelectric focusing experiment were analyzed on similar gradients and demonstrated that the peak with pI = 4.6 is the larger inhibitor with $S_{20,W} = 9.4$, whereas the less basic inhibitor at pI = 6.3 sedimented in the region of the smaller inhibitor with $S_{20,W} = 5.3$.

15.5 Molecular Sieve Chromatography of Inhibitory Serum from Surgical and Trauma Patients

Sephadex G-200 chromatography was utilized. To allow for comparison between columns the partition coefficient K_{av} defined as:

$$K_{av} = \frac{V_e - V_o}{V_t - V_o}$$

where V_e = elution volume

V_o = void volume

V_t = total bed volume

was used. A calibration curve of the K16x85cm column used is shown in figure 26. A chromatogram of the same serum sample used in the

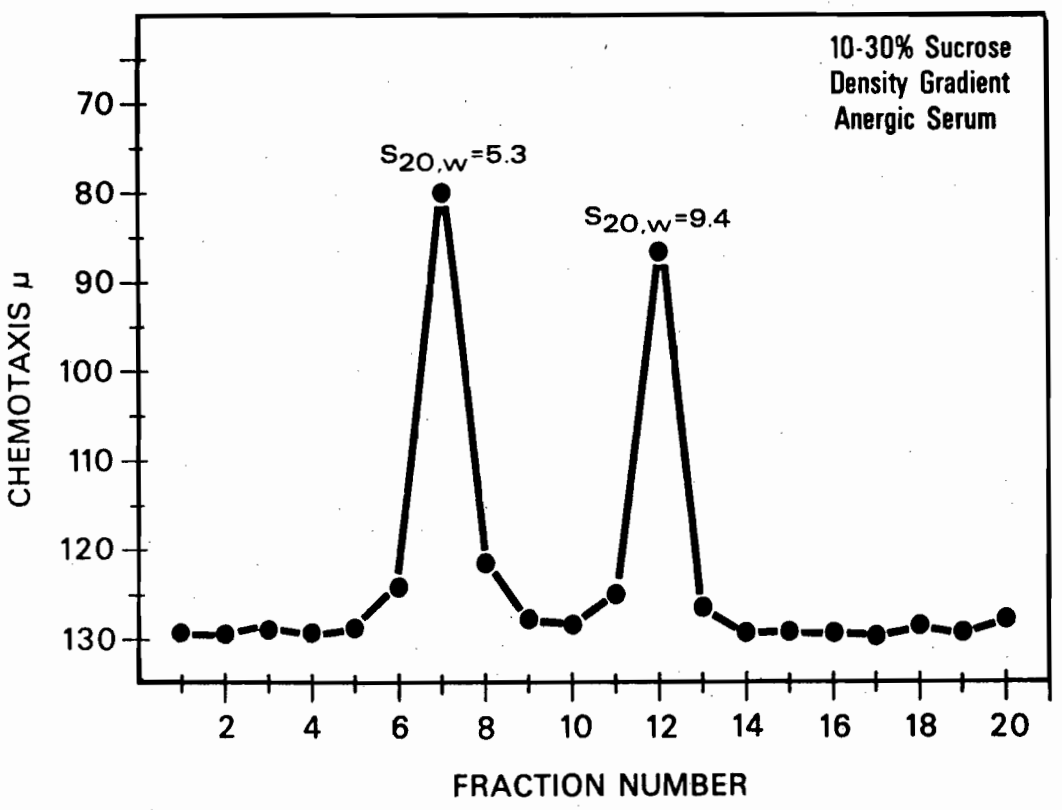


Figure 25. Ultracentrifugation of inhibitory serum in a 10-30% sucrose density gradient at 180,000 x g at 4 °C for 18 h. $S_{20,w}$ values are estimated from the internal reference markers cytochrome c, albumin, and ferritin. PMN chemotaxis is from top to bottom.

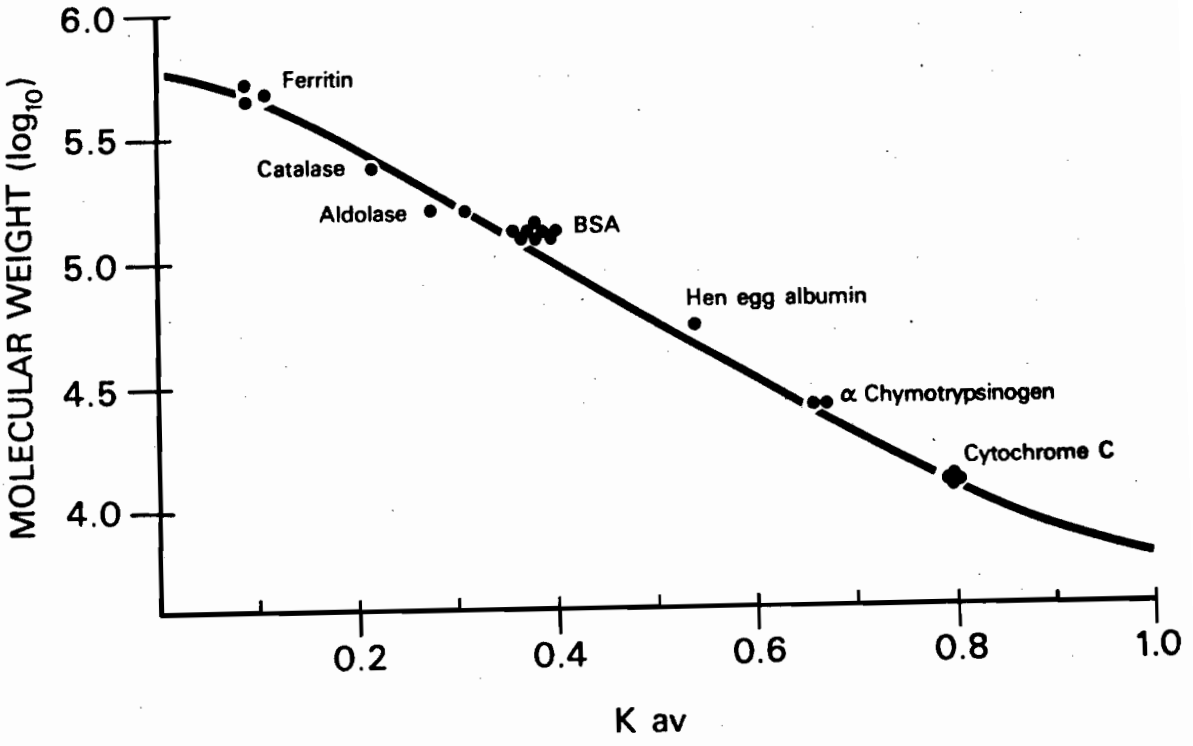


Figure 26. Calibration curve of the K16x85 cm Sephadex G-200 column used in these experiments.

electrofocusing and sucrose density gradient experiments described above is shown in figure 27. The same column on which the serum was developed was calibrated separately and the MW estimation shown was computed using elution volumes alone. When K_{av} is used and an adjustment is made for the K_{av} of the serum albumin (eg. K_{av} albumin = 0.33 for this column, whereas several separate calibration chromatograms showed this to have $K_{av} = 0.38$. Thus 0.05 is added to both the albumin and the inhibitory peaks to standardize this for comparison between columns) the estimated molecular weights become 380,000 and 136,000. Table 34 shows a number of inhibitory sera analyzed by this means. In all cases but two, there were always two peaks of inhibition detected, one of MW $\sim$380,000 to 457,000, and the other ranging between 115,000 to 158,000. Two sera showed one inhibitor of MW $\sim$220,000. The error in molecular weight estimation with a shift of the peak inhibition by one column fraction or 6.3 ml is 15% for this column.

A different pattern of inhibitory peaks was obtained when inhibitory sera from traumatized patients were analyzed soon after their injury (table 35). Up to five peaks of inhibition were detected, the smallest being of MW $<$20,000 and the largest in the range of the large inhibitor detected in non-traumatized inhibitory patient sera.

TABLE 34

The molecular weights of chemotactic inhibitors in the serum of surgical patients with decreased autologous PMN chemotaxis, determined with Sephadex G-200 Column Chromatography

SERUM #	PMN CTX	PEAK I	PEAK II	PEAK III
68	101.2 ± 2.3	-	275,000	126,000
94-1	92.1 ± 1.4	457,000	-	138,000
94-2	92.3 ± 2.3	457,000	-	138,000
94-3	90.1 ± 1.7	437,000	-	158,000
98-2	93.1 ± 2.7	416,000	-	120,000
144	95.2 ± 2.1	-	240,000	-
150	95.2 ± 3.1	-	208,000	-
181	95.0 ± 2.1	380,000	-	138,000
228	99.7 ± 1.0	436,000	218,000	-
239	92.1 ± 1.0	436,000	218,000	-
251	90.1 ± 1.2	-	254,000	126,000
280	93.1	-	280,000	115,000

TABLE 35

The molecular weights of chemotactic inhibitors in the serum of trauma patients determined with Sephadex G-200 column chromatography. (Hours post trauma are shown in brackets)

TRAUMA #	PMN CTX	PEAK I	PEAK II	PEAK III	PEAK IV	PEAK V
9 (12h)	100.2 ± 1.1	398,000	-	132,000	-	-
11(96h)	91.8 ± 2.1	-	214,000	-	52,000	-
38(30h)	115.8 ± 1.2	436,000	-	-	69,000	< 20,000
39 (5h)	102.0 ± 2.1	400,000	-	120,000	33,000	< 20,000
TD3(18h)	105.0 ± 2.1	416,000	-	-	45,000	< 20,000

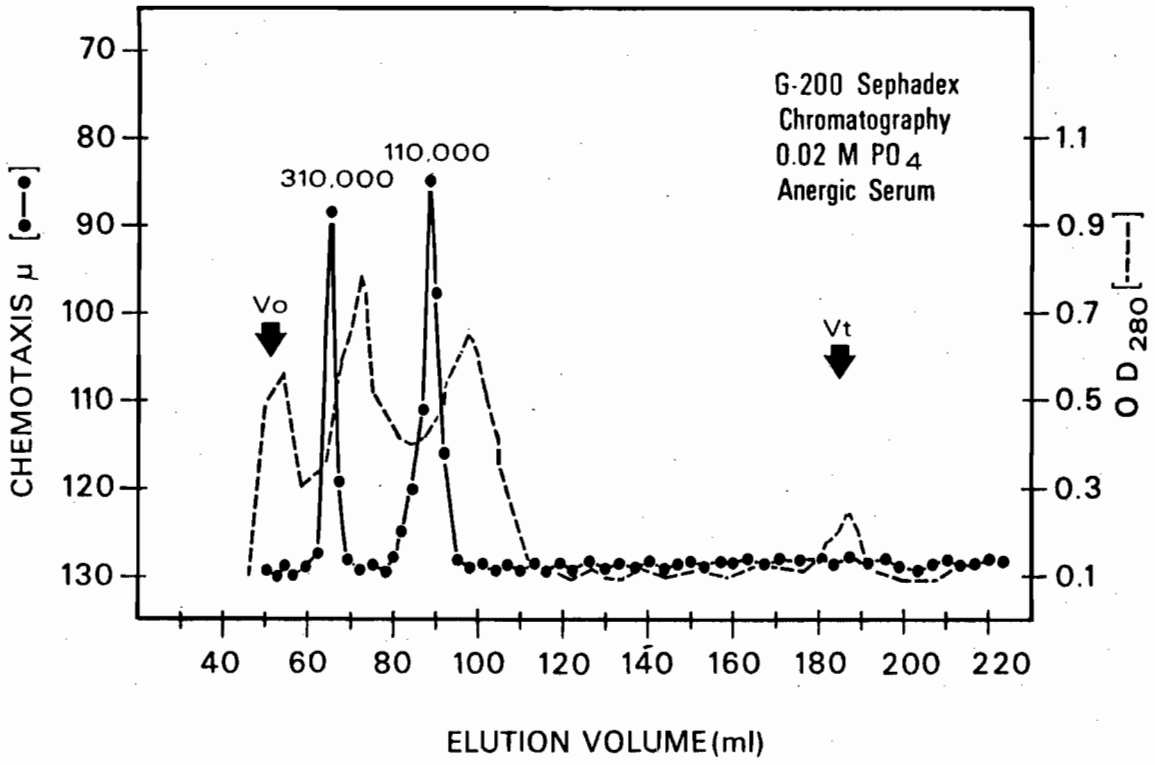


Figure 27. Sephadex G-200 molecular sieve chromatography of inhibitory serum in 0.02 M sodium phosphate buffer pH 7.4. Chemotaxis is from top to bottom of figure.

15.6 PMN Chemotactic Inhibitory Activity in Normal Human Serum

Concentration of normal serum through a semipermeable membrane that retained proteins of molecular weight greater than 30,000 showed that chemotactic inhibition of normal neutrophils could be detected in normal serum after a 3-fold concentration (table 36). This inhibition was destroyed by heating at 60^{o} x 30 and reversed by 10^{-4}M levamisole, indicating that the effect was not due to a mere concentration effect of all the serum proteins, resulting in a change of the osmolarity of the serum.

Serum density gradient analysis of these same normal sera pre and post concentration demonstrated one peak of chemotactic inhibition corresponding to the smaller MW inhibitor detected in inhibitory sera. A comparison of these sera with inhibitory serum from a patient with relative anergy and one with anergy is shown in figure 28. Sephadex G-200 chromatography of 5 normal control sera showed MW = 126,000, 127,000, 140,000, 158,000 and 132,000 respectively. The larger or smaller inhibitors were not detected in these experiments, using normal control serum.

15.7 Correction of PMN Inhibition by Normal Serum, Aspirin, and Levamisole

Pooled serum from laboratory controls was kept at -70^{o}C before use. Parallel experiments were set up where PMN from

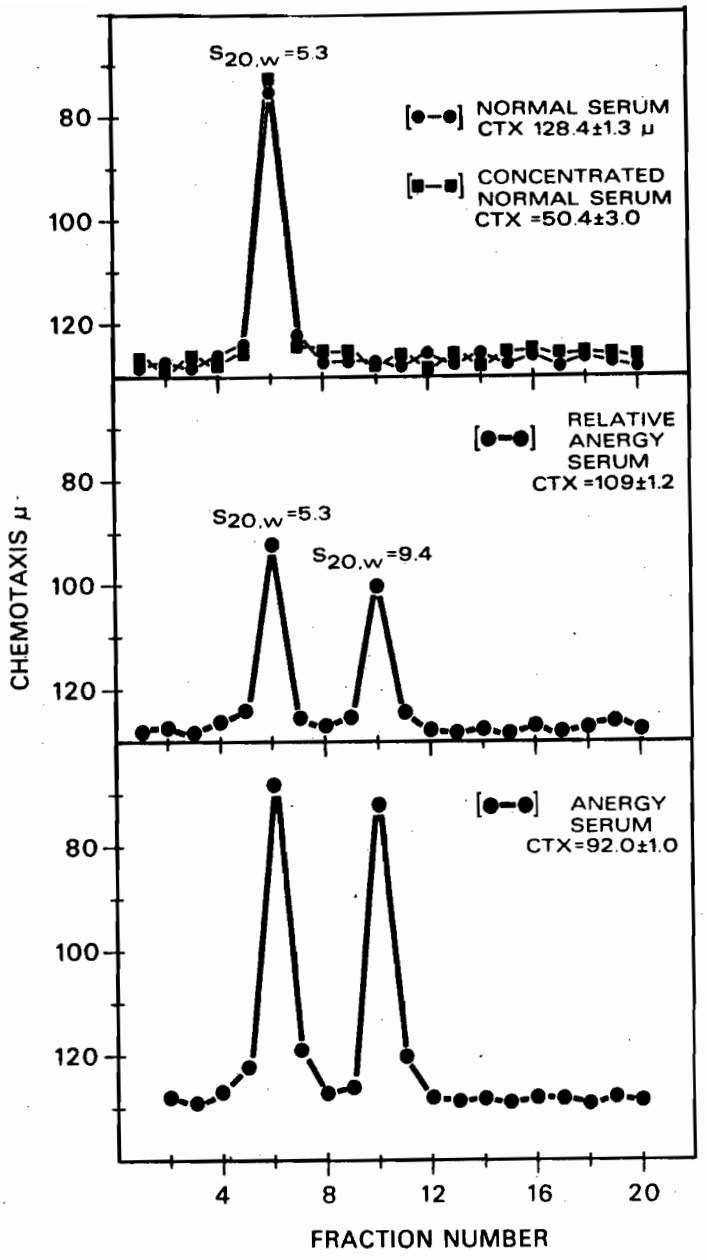


Figure 28. Sucrose density gradient ultracentrifugation analysis of normal control serum, normal control concentrated serum, relative anergy serum, and anergy serum, using 10-30% gradients, 180,000 x g at 4° C for 18 h. Chemotaxis is from top to bottom of figure.

TABLE 36

The result of concentrating normal serum through a semipermeable membrance and subsequently assessing chemotactic inhibitory acitvity against normal PMN

SUBJECT	CONCENTRATION	CHEMOTAXIS (μ) PRE-CONC.	POST.CONC.
N.C.	3-fold	128.4 ± 2.4	51.5 ± 0.7
C.H.	3.3-fold	124.1 ± 0.56	60.8 ± 1.4
F.G.	3.1-fold	126.1 ± 1.9	60.1 ± 1.5
J.M.	3.4-fold	128.2 ± 1.1	50.2 ± 1.1

TABLE 37

The effect of 10^{-4M} Levamisole in anergic serum (L-AS) on the chemotaxis of normal neutrophils

PREINCUBATION	CHEMOTAXIS (μ)
MEM	127.3 ± 1.0
10^{-4M} L-MEM	128.3 ± 2.1
AS	91.3 ± 1.2
10^{-4M} L-AS	125.1 ± 1.1

hospitalized patients were assayed resuspended in MEM or in the presence of 100% pooled Normal human serum. The data are shown infigure 29. There is partial correction of the chemotactic defect in every patient tested (n = 24, 9 hospitalized N, 3 RA, 12 A patients). There was no enhancement of PMN chemotaxis if this was near the normal laboratory control range. A number of drugs were also tested for their ability to modulate PMN function. The data on ascorbic acid, Vitamin C, Hydrocortisone and ethanol have already been presented under the neutrophil adherence section. The data on acetylsalicylic acid is shown in figure 30. One experiment is shown demonstrating partial correction of the PMN chemotactic defect at 3×10^{-5M} ASA without further improvement at higher doses. The chemotaxis of normal control PMNs was not affected. The experiment has been repeated in separate patients. Crystalline levamisole -HCl ((S) - (-) - 2,3,5,6,-tetrahydro-6-phenylimidazo (2,1-6) thiazole hydrochloride) was dissolved directly in the resuspending MEM at various concentrations and the chemotaxis of both maximally inhibited patient PMN (figure 31), and normal control PMN inhibited by treatment with inhibitory serum (figure 32) was assessed. There was correction of the chemotactic defect in both cases, whether autologous inhibited PMN were used, or heterologous PMN, whose chemotactic migration was reduced by pretreatment with inhibitory serum. The reversal of the PMN chemotactic inhibition was constant down to 10^{-9}M, a clinically achievable concentration, and then became reduced in a dose response manner. Figure 33

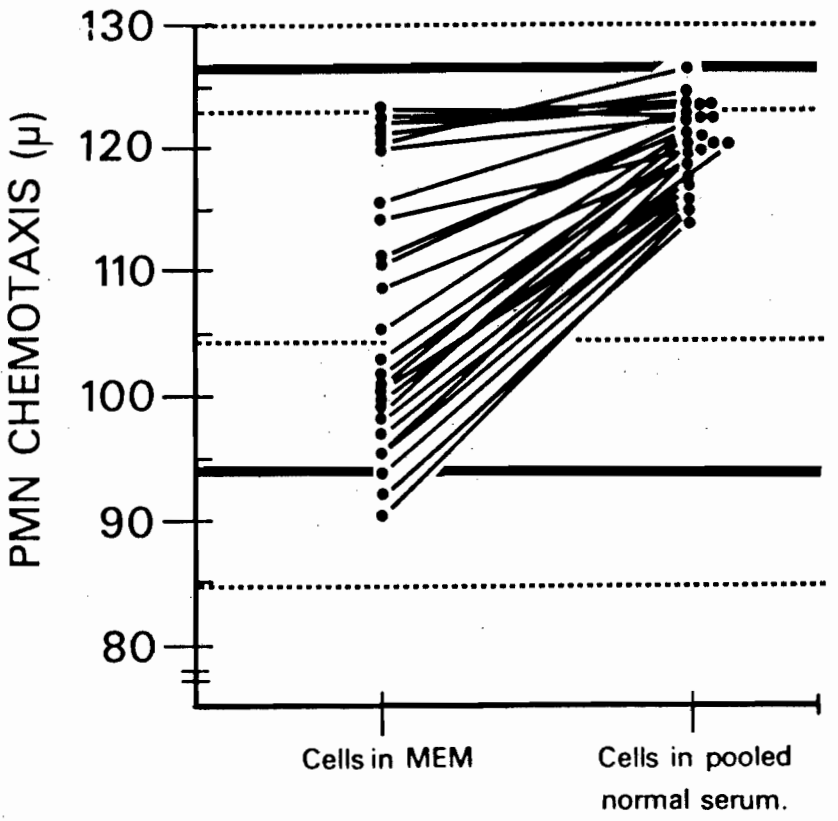


Figure 29. The partial corection of the PMN chemotactic defect by pooled normal human serum obtained from normal controls. Means ± 95% CL the sample normal control PMN CTX (n=95) and maximally inhibited PMN CTX (n=34) are shown by heavy and light lines respectively.

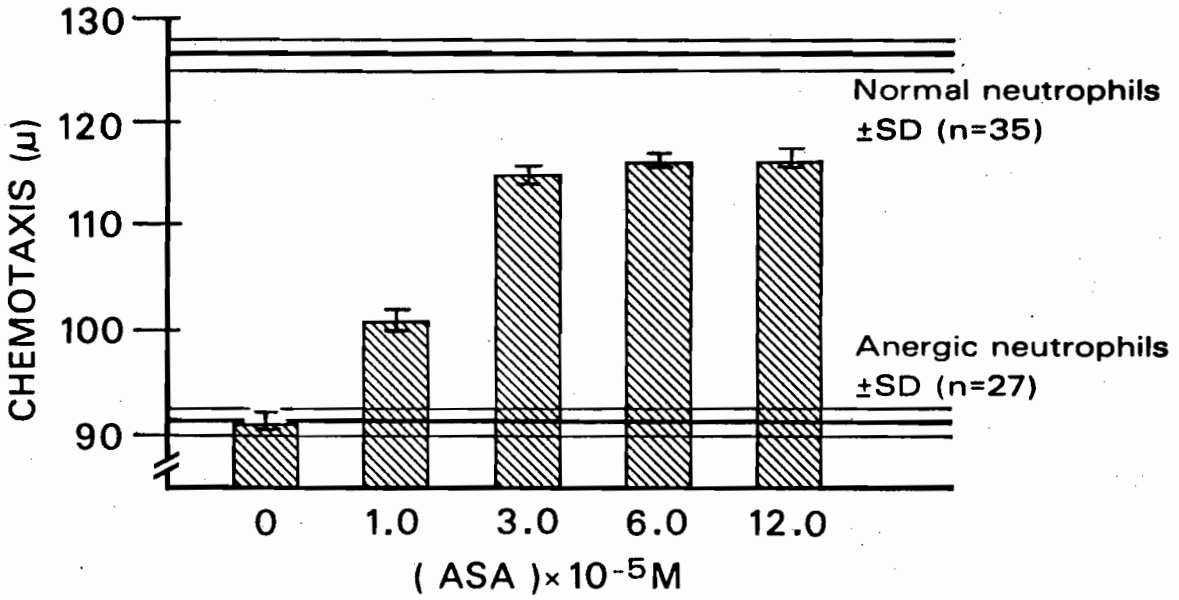


Figure 30. The effect of acetyl salicylic acid on the chemotaxis of neutrophils from an anergic patient. Normal PMN chemotaxis was not altered. The mean±SD of the normal laboratory control PMN CTX and anergic PMN CTX based on an earlier set of measurements is shown by the solid lines and light lines respectively.

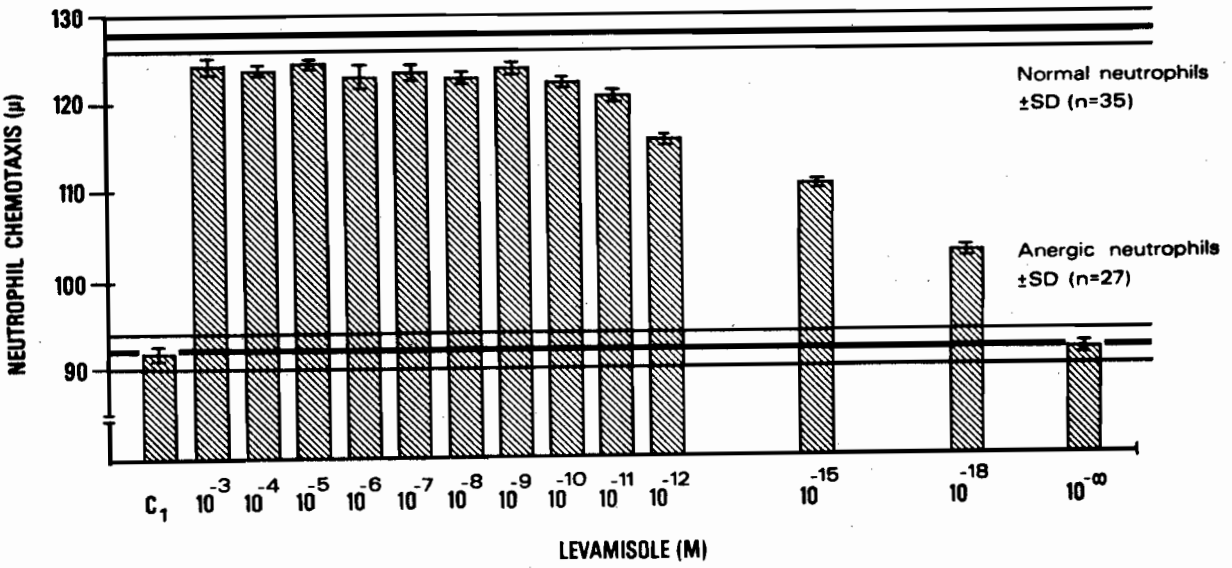


Figure 31. The correction of the decreased anergic PMN chemotaxis by different concentrations of levamisole in vitro. C_1 = PMN from the anergic patient resuspended in MEM only. Normal and anergic PMN CTX ranges as per figure 30.

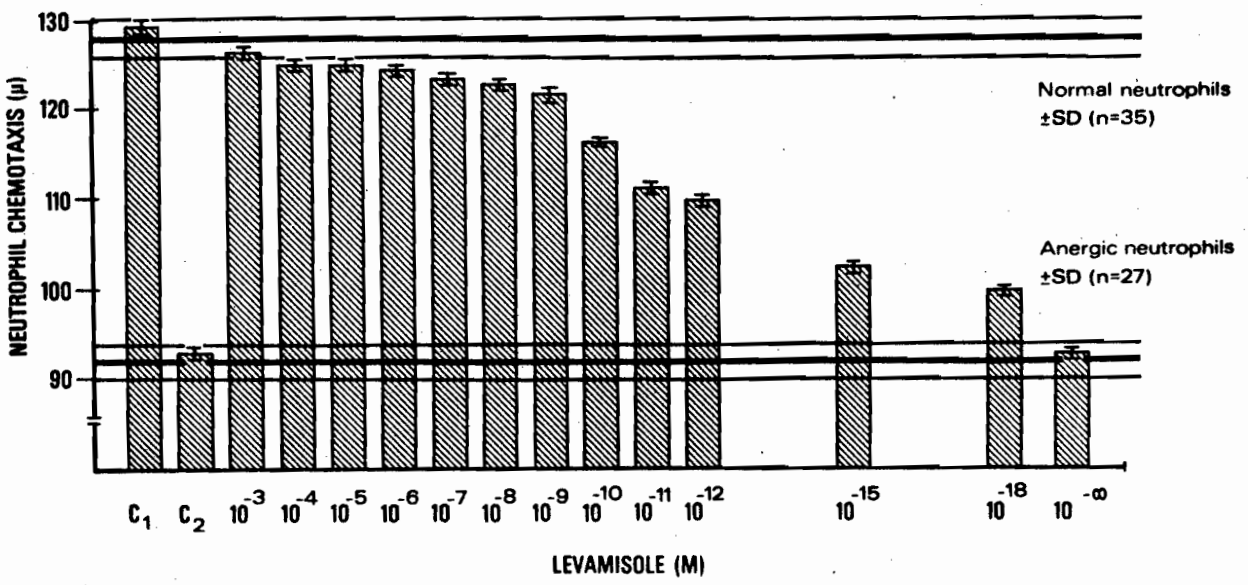


Figure 32. The correction of anergic serum produced decreased normal control PMN chemotaxis by levamisole in vitro. C_1= Normal control PMN resuspended in MEM. C_2= Normal control PMN pretreated with inhibitory serum and resuspended in MEM. Normal and anergic PMN CTX ranges as per figure 30.

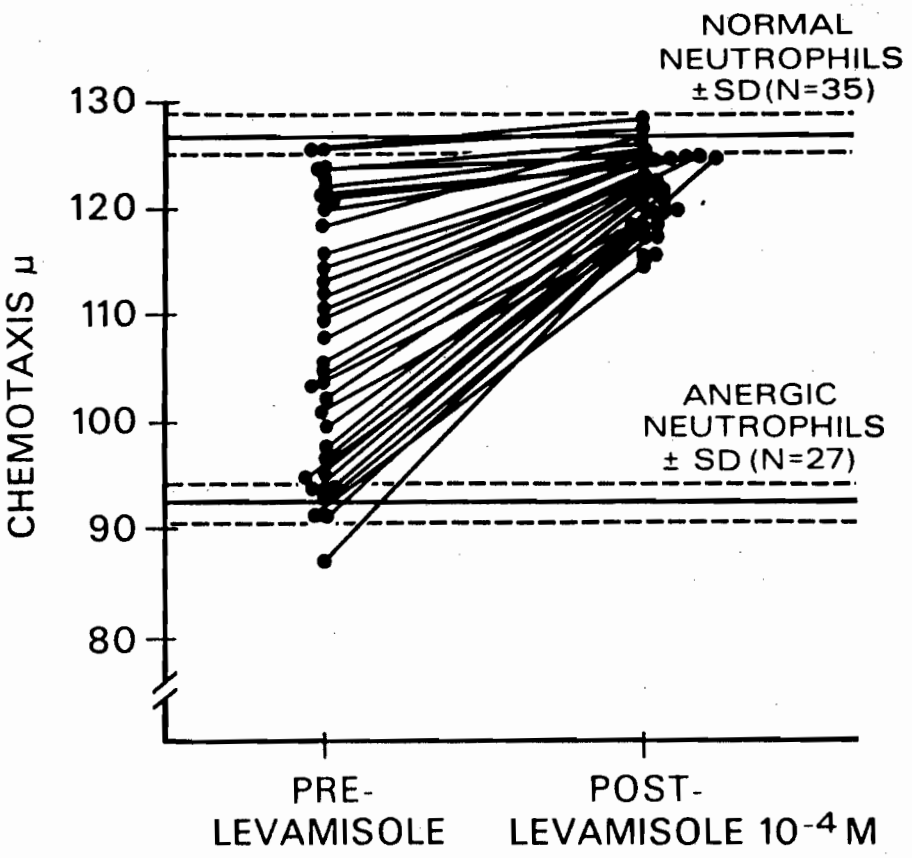


Figure 33. The partial correction of the PMN chemotactic defect by levamisole at 10^{-4} M in vitro.

shows data on PMN CTX of 35 surgical patients in the presence or absence of levamisole at 10^{-4}M. There was significant improvement of the decreased chemotaxis of these cells in every instance, whereas normally migrating PMN were not affected. Simultaneous presentation of levamisole and anergic serum to normal neutrophils (table 37), prevented the inhibitory effect of the serum upon those cells. Pretreatment of normal neutrophils with levamisole at 10^{-4}M, washing and then resuspension in anergic serum, gave partial blockade of the effects of anergic serum despite the presence of excess chemotactic inhibitor in the form of anergic serum (table 38).

In an effort to stimulate the <u>in vivo</u> situation when a patient is given levamisole by mouth, levamisole was added at 10^{-7}M to normal whole blood and anergic whole blood. After a 30 minute incubation at 37° C the neutrophils were isolated and their chemotaxis measured (table 39). Neutrophils in normal whole blood were not affected by levamisole treatment. However, anergic neutrophils which migrated to 92.6 ± 1.0 μ with no treatment were caused to migrate to 119.1 ± 1.0 μ, a significant correction of their abnormal chemotaxis by levamisole ($p < 0.001$, Student's t-test).

15.8 Partial Purification of Serum Chemotactic Inhibitors With Ammonium Sulfate Precipitation

The pooled serum used for the isoelectric focusing, ultracentrifugation and chromatography experiments was used to partially

TABLE 38

The effect of preincubation with 10^{-4M} Levamisole in MEM (10^{-4M} L-MEM) on the chemotaxis of normal neutrophils resuspended in anergic serum (AS)

PREINCUBATION	RESUSPENSION	CHEMOTAXIS (μ)
MEM	NS	123.5 ± 1.0
10^{-4M} L-MEM	NS	123.4 ± 1.0
MEM	NS	92.1 ± 1.4
10^{-4M} L-MEM	NS	109 ± 1.0

TABLE 39

The effect of Levamisole 10^{-7M} (10^{-7M}-L) added to normal whole blood (NWB) or anergic whole blood (AWB) on the chemotaxis of neutrophils isolated from such blood

NEUTROPHIL SOURCE	ADDITIVE	CHEMOTAXIS (μ)
NWB	MEM	130.2 ± 1
NWB	10^{-7M} L-MEM	130.6 ± 1
AWB	MEM	92.6 ± 1
AWB	10^{-7M} L-MEM	119.1 ± 1

purify the PMN chemotactic inhibitors using sequential ammonium sulfate precipitation. In order to allow for calculation of specific activity and monitor the purification, a unit of chemotactic inhibition was defined as follows. "One unit of PMN chemotactic inhibition is that amount of inhibitor which will decrease the chemotactic migration of 2.5×10^6 normal control neutrophils in 0.5 ml of MEM by 3 μ after 90 min migration at 37°C, using casein 5 mg/ml as attractant, a modified Boyden chamber and the leading front technique". Table 40 shows the results of sequential fractionation of pooled inhibitory serum from the same patient, collected on two separate occasions. The symbol S_x denotes the supernatents after the sequential cuts at 20%, 40%, ...100% ammonium sulfate saturation and P_x denotes the precipitates. Two of the precipitate fractions P_{40} and P_{100} contained all the inhibitory activity of the precipitates. None was detected in various dilutions of P_{20}, P_{60} and P_{80}. Considerable chemotactic inhibition was detected in S_{100} after overnight dialysis against PBS. An aliquot of a saturated ammonium sulfate solution in distilled water, similarly dialyzed, failed to show any inhibition. If the dialysis was carried out for 3 days using 5 ml of S_{100} and three changes of PBS, 4 l per change, the inhibitory activity was lost completely. Recovery of inhibitory units in the P_{40} and P_{100} was 80% from that of S_o, but overall recovery including the activity in S_{100} was 209%. Chromatography of S_o, P_{40} and P_{100} is shown in figure 34.

TABLE 40

The partial purification of the PMN chemotactic inhibition from serum using ammonium sulfate precipitation (I.U. = inhibitory units)

FRACTION	VOLUME	PROTEIN (mg/ml)	PROTEIN (total mg)	CHEMOTAXIS (I.U./ml)	CHEMOTAXIS (total I.U.)	SA (I.U. / mg protein)	PURIFICATION
S_0	35 ml	50.0	1700	37.1	1300	0.76	-
S_{40}	36 ml	-	-	0	0	-	-
S_{60}	33 ml	-	-	0	0	-	-
S_{80}	30 ml	-	-	37.0	1110	-	-
S_{100}	32 ml	1.6	51.2	51.2	1638	32.0	42X
P_{20}	5.3 ml	1.1	5.8	0	0	-	-
P_{40}	8.3 ml	32.3	268.0	82.0	681	2.5	3.4X
P_{60}	6.5 ml	78.4	510.0	0	0	-	-
P_{80}	11.3	70.2	793.0	0	0	-	-
P_{100}	5.2	1.3	6.8	78.3	407	59.9	81X

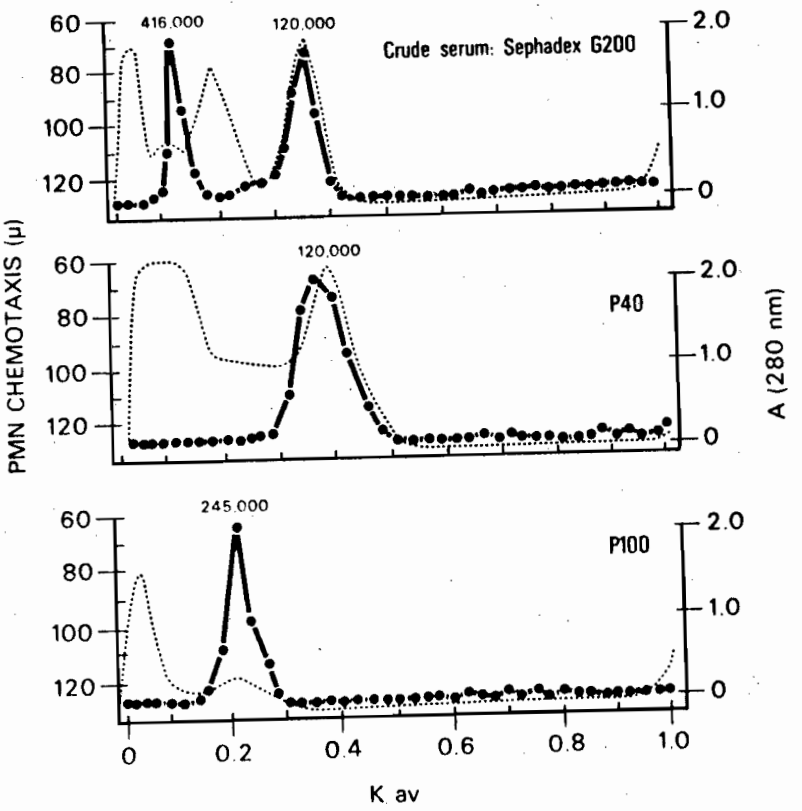


Figure 34. Sephadex G-200 chromatography of crude inhibitory serum and the activity detected in the precipitates at 40% and 100% saturation with ammonium sulfate.

15.9 Evidence for Counter Inhibiting Activity in the Serum

Sucrose density gradient centrifugation and Sephadex chromatography, both techniques which lead to dilution of original samples, demonstrated inhibitory activity in separated fractions of normal serum where no activity was detected in the starting serum. The correction of decreased autologous PMN chemotaxis to near normal levels by pooled normal serum has been presented in figure 29. The ammonium sulfate fractionation gave 209% recovery of starting chemotactic activity once the separated fractions were analyzed. This was taken as evidence that counter inhibitory moities were present in serum. Mixing two inhibitory sera together and testing for their ability to decrease the CTX of normal control neutrophils ha no effect. Inhibition was still present. However, mixing three or more inhibitory sera together led to partial loss of inhibitory activity. Similarly, mixing any inhibitory serum with normal serum led to loss of inhibiting potential. Mixing of the inhibitory and non-inhibitory ammonium sulfate precipitates demonstrated partial neutralization of their PMN chemotactic inhibition (table 41).

TABLE 41

Demonstration of Counter chemotactic inhibitory activity in serum (I.S. = inhibitory serum; N.S. = normal control serum)

SAMPLE	PMN CHEMOTAXIS (μ)
IS_1	96.3 ± 0.42
IS_2	94.1 ± 3.1
IS_3	93.6 ± 4.1
NS	125.6 ± 3.4
MEM	124.6 ± 2.7
IS_1 + IS_2 (0.5 ml each)	91.6 ± 2.7
IS_1 + IS_2 + IS_3 (0.5 ml each)	118.4 ± 0.56
IS_1 + NS (0.5 ml each)	113.8 ± 3.2
IS_2 + NS "	114.0 ± 2.6
IS_3 + NS "	116.1 ± 1.8
P_{40} (100μl) + MEM (400 μl)	105.4 ± 2.1
" + P_{60} (400 μl)	119.4 ± 1.5
" + P_{80} (400 μl)	117.4 ± 1.2
P_{100} (100μl) + MEM (400μl)	91.0 ± 1.2
" + P_{60} (400μl)	118.0 ± 1.2
" + P_{80} (400μl)	117.1 ± 0.6

DISCUSSION

The skin test data on 1776 surgical patients clearly indicate the association between failed skin test reactivity, morbidity due to sepsis, and mortality. The worse the skin test response, the worse the prognosis. Sequential skin testing was found to be a very useful method to follow the patient's clinical course and assess prognosis. Grave outcome was signalled by a depression of skin reactivity from normal. Thirty one of 43 patients (74%) who lost skin reactivity died, the majority from overwhelming sepsis. Similarly, if patients did not recover skin reactivity from anergy, mortality from sepsis was 66%. In contrast, 155 patients who recovered their skin reactivity to normal had a mortality equal to 2% despite a rate of sepsis of 40%, which occurred when these patients were at risk with depressed host defenses. Similar data has been reported by Pietsch et al[196], who found that all 6 patients who lost their skin reactivity died, whereas 135 patients who recovered their skin test response or did not become worse, the mortality was 2.1%. The findings of Johnston et al[126] and Hiebert et al[112] also support this conclusion.

These data raise the age old question of what comes first, the "cart or the horse". Does anergy appear first followed by sepsis and poor clinical outcome, or does a poor clinical outcome and/or established sepsis lead to anergy? Is anergy just another signal that a patient is grieviously ill and is therefore of

no predictive use? The analysis of 727 preoperative patients undergoing major surgery, help to put this question in perspective. A normal preoperative skin test response was associated with 7.5% sepsis and 4.6% mortality compared with 30% sepsis and 23% mortality in patients with altered cutaneous reactivity. The age difference between these two populations was not enough to explain their susceptibility to infection and the high proportion of patients who died as a result of sepsis in the anergic group. The spectrum of surgical disease was similar in these preoperative patients as was the degree of surgery performed. It would appear then that in a preoperative group of patients skin test anergy identifies a population at risk for morbidity from sepsis.

The preoperative patients who had depressed skin reactivity and were not subjected to an operative procedure had a low rate of sepsis, but mortality comparable to the operated pre-operative anergic patients. This interesting result indicates that patients at risk as indicated by skin test anergy, develop sepsis only when challenged by breakdown of other defense barriers such as the skin and gastrointestinal tract. Once such patients do develop an infectious process they do as poorly in containing their infection as the operated anergic patients. Sepsis leads to death in 66.7% of the cases. An interesting finding was the high proportion of patients with gastrointestinal hemorrhage who were anergic on admission. Hypovolemia with increased transfusion requirements was correlated with depressed skin reactivity. Sepsis and mortality was much higher in the anergic group. Cancer has always been thought as causing anergy but

this assumption was not substantiated here. No difference of the "cancer load" on skin test response could be demonstrated. Though it is difficult to accurately measure tumor load, i.e. the proportion of tumor to body weight, the lack of correlation between skin test response and extent of disease is quite apparent in this study.

The severity of operation in 32 pre-operative normal patients was found to depress their host resistance. This resulted in a rate of sepsis equal to 40.6% and mortality of 22% compared to those who remained normal post operatively, with sepsis 5.3% and mortality 3.3%. It would appear that another of the many etiologies of anergy would be controlled operative trauma. The severity of blunt trauma has a similar effect on host defense mechanisms[169]. Greater degrees of trauma produce more severe defects which last longer than simple injuries.

Sepsis as the primary admitting diagnosis was much higher in the patients with failed responses. These patients presented with intracavitary abscesses secondary to previous surgery or to a perforated viscus. It is not clear in this group, whether sepsis itself, was responsible for the observed anergy. Meakins et al[167] have shown that drainage of intracavitary abscesses in some patients resulted in recovery of skin test response within 7 days. This would support a causative role of sepsis and anergy. There are 119 RA/A patients remaining after exclusion of the 19 patients with sepsis as the admitting diagnosis. These remaining patients developed post operative septicemias and

and intracavitary abscesses at a rate of 20% compared to 6% for the preoperative normal patients. Only 9% of the septic patients died in the normal group compared to 20% in the relatively anergic and 59% in the anergic group. This is the strongest evidence that failed cutaneous reactivity indicates a host's inability to contain and resolve bacterial pathogens resulting in death. It therefore appears that anergy predisposes to sepsis and that sepsis may produce anergy. However, the failure to resolve the anergic state synonymous to correcting depressed host defense in both instances, results in significant morbidity due to sepsis and a higher proportion of mortality due to sepsis.

The delayed hypersensitivity response reflects cell mediated immunity and the ability to mount a response indicates this component is intact. The nature of the cells evaluated are the lymphocytes. The exact population of lymphocytes assessed is not clear. The development of induration in delayed hypersensitivity responses following the injection into the dermis of recall antigens, is a result of a predetermined sequence of events. These are reviewed in the Review of Literature, Section 2.2.2. This complex process was simplified in figure 35, to test the hypothesis that decreased accumulation of effector cells at the injection site due to a block as shown by the arrow, alters a finely balanced system. The non-recruitement of large lymphocytes at the site of antigen deposition along

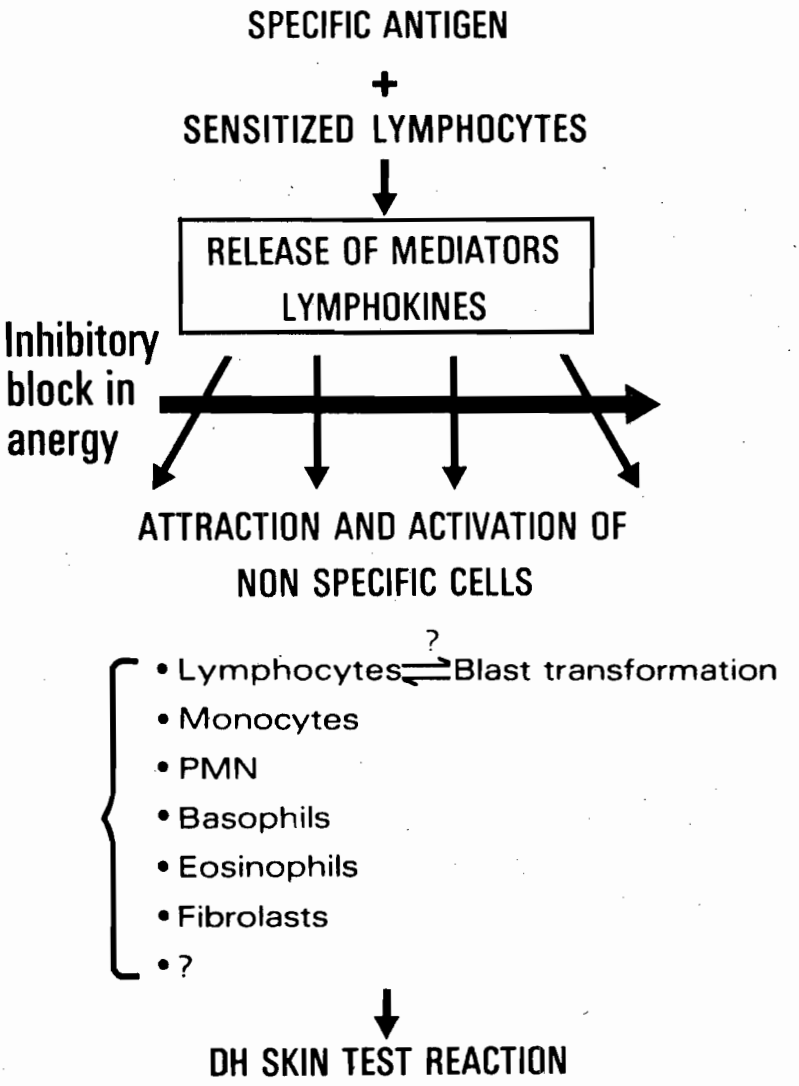


Figure 35. Schematic representation of the delayed hypersensitivity response and the postulated defects that could be created by the chemotactic inhibitors described herein.

with lack of PMNs leads to failed skin test response.

Previous investigations have demonstrated decreased total peripheral lymphocyte counts, as well as decreased levels of T-lymphocytes in patients with anergy. Data presented here were not statistically different, though patients with anergy had lower total lymphocyte counts than controls. The E-rosetting assay for measuring T-lymphocytes demonstrated a wide sample variation, thus differences between normal controls and the three classes of hospitalized patients N, RA and A were not significant. The lack of enough effector cells may explain the anergy observed in severely malnourished patients as well as experimental animals. McLuskey has clearly shown that $>$ 90% of the lymphocytes observed in the skin test response of guinea pigs consisted of large lymphocytes that had been synthesized within the last 3 days. A malnourished host may be unable to synthesize enough of these cells (along with other defects discussed later) to mount a delayed hypersensitivity response. A similar explanation may be used for the anergy observed following severe blood loss depleting this cell population.

The data presented here correlating decreased large lymphocyte chemotaxis and skin test anergy would support the non-recruitment of effector cell hypothesis. To the author's knowledge this is the first demonstration that lymphocyte chemotaxis is abnormal in surgical patients with skin test anergy. Furthermore, the demonstration that lymphocyte chemotaxis closely paralleled PMN chemotaxis and both cell types migrated towards

the same chemoattractant, suggest that the phenomenon of leukocyte chemotaxis is uniform. Serum inhibitors from patients with skin test anergy equally inhibited the chemotactic migration of both normal control lymphocytes and normal control PMN adding suport to this concept. The high correlation between decreased lymphocyte chemotaxis and cutaneous anergy would offer an explanation for the absent delayed hypersensitivity response observed in surgical and trauma patients.

It is of interest that a 3 micron millipore filter is optimal for chemotactic migration of human lymphocytes, in which they travel at the rate of 0.36 μ/min towards casein. It may be that the fibrilar lattice network of a 3 micron filter most closely resembles the space in the ground substance through which the lymphocytes migrate *in vivo*. There is no doubt that lymphocytes show random migration, stimulated migration or chemokinesis, as well as directed migration or chemotaxis. Previous stimulation and transformation of lymphocytes with PHA as suggested by Wilkinson *et al*[257,258] was not found essential. As long as enough large lymphocytes were present in the suspension, reliable chemotaxis was observed. Small mature lymphocytes showed no reliable migration in these studies.

Lymphocyte function studies of this nature, including delayed hypersensitivity, reflect the adequacy of the cell mediated component of host defense. Yet the types of infection observed in these patients were of bacterial nature with only

the exceptional patient developing fungal infection. It is difficult to explain why skin testing alone is predictive of bacterial sepsis. However,when the phagocytic component of host defense is studied in these patients an explanation becomes apparent. Preoperative surgical patients with normal ST responses had PMN chemotaxis very close to the laboratory controls, whereas RA and A patients had depressed PMN chemotaxis. The same was observed when 712 measurements were carried out stimultaneous to evaluation of the skin test response. As a group, there was excellent correlation between depressed skin test response and decreased PMN chemotaxis. On an individual determination, due to the wide 95% confidence limits of the sample, decreased PMN chemotaxis did not always specifically indicate anergy or relative anergy. It is apparent that the probability of a normal skin test responder having depressed PMN chemotaxis is much less compared to the patient with skin test anergy. Skin testing becomes a means of identifying patients at risk for developing sepsis. The etiology is not due to the failed cell mediated immunity,but to coexisting defects in phagocytic cell function.

Neutrophil adherence whether measured in whole blood or with purified cell preparations was decreased in these studies. The method used for measuring adherence was found to be reliable and reproducible. The correlation of decreased PMN chemotaxis and increased adherence was excellent. It would appear that factors modulating each neutrophil function are distinct. The

factor which augments neutrophil adherence was found in the plasma, whereas the inhibitor of PMN chemotaxis was found in the serum. These data support the findings of McGreggor[152] in patients with inflammatory conditions and those of Bryant and Sutcliffe[39].

Phagocytosis as indicated by the numbers of intact bacteria detected within neutrophils was increased in patients with skin test anergy, and the increase was highly correlated to decreased PMN chemotaxis. The assay as performed here identifies cell associated bacteria on the basis of relatively intact DNA. Free thymidine of thymidine incorporated into small chain polynucleotides ($<$ 1,000 nucleotides) would not be retained by the glass fiber filters and counted. One explanation for the higher proportion of radioactivity associated with PMN would be that the disposal of bacteria is different. The numbers of intracellular bacteria were less at 20 min in both normal control PMN and inhibited PMN compared to the 10 min value. Assay of bactericidal activity was not different between control and inhibited PMN. At least 85% kill of the inoculum was observed with normal control cells, yet the maximum phagocytosis detected was only 50%. Also, $>$ 50% of the bacterial inoculum was killed within 10 min. This indicates that once internalized, bacteria are killed and disposed of very rapidly. The thesis then, that the higher proportion of bacteria associated with PMN demonstrating decreased chemotaxis represents decreased disposal, and not uptake, is attractive.

There was no difference in phagocytosis or killing by normal control or patient neutrophils when PMN: bacteria ratios were varied from 1:2 to 1:20. Stressing inhibited PMN did not bring out a difference in bacterial killing potential. A major bactericidal defect does not exist in the anergic patients studies. If a subtle defect in killing is indicated by the phagocytosis data, then more sensitive bactericidal functions must be used to detect it. The first 10-15 min of the assay may be important and initial killing rates at 2 min intervals may be more appropriate. The burn patients studied by Alexander et al[7,8] were severely traumatized patients with marked inhibition of phagocytic cell function. The phagocytosis of heat killed staphylococci was found to be increased by Balch[23]. These patients demonstrated decreased killing, even after 4 hrs according to Alexander. Balch was able to demonstrate decreased delivery of PMN to skin abrasions which today can be accounted for by their decreased chemotaxis. If a phagocytic cell cannot reach the site of bacterial invasion, it is of no use, even if "armed to the teeth" and capable of devastating phagocytic and bactericidal capacity.

The PMN chemotactic defects described here may appear rather insignificant. This is comparable to congenital defects of leukocyte locomotion. It is well accepted that in the pediatric age group, these defects predispose to recurrent infection. The major difference between the acquired defects

described herein and the congenital defects is the "time at risk". A primary defect is present constantly, thus the greater the probability of infection. An acquired defect is transient and will result in sepsis only if the affected patient is challenged.

The decreased chemotaxis of PMN in these patients as well as increased adherence are abnormalities extrinsic to the cell; both are mediated by circulating serum factors. This allows for a much finer regulation of PMN fucntion, as opposed to one where the control is at the cell differentiation level, as occurs with most congenital defects. The nature of the PMN chemotactic inhibitors is interesting. There exists a natural inhibitor whose action in native control serum cannot be measured, simply because it allows for PMN chemotaxis of 127 microns which is considered normal. Only when this inhibitor is separated from other serum components or concentrated, is its presence detected. The same inhibitor is detected in most sera from anergic patients. The inhibitory activity detected in native serum from patients with decreased autologous PMN chemotaxis, occurs with the appearance of a separate inhibitory component. In traumatized patients studied very early post injury, separate inhibitors with smaller MW were detected along with the large $>$ 400,000 MW component. It is possible that these inhibitors are distinct proteins or they represent a small MW inhibitor carried on larger proteins in the serum. The results of ammonium sulfate fractionation where the inhibitor at 416,000 MW in the

native serum appeared at 254,000 MW in the P_{100} fraction, indicates that the small inhibitor hypothesis may be correct. The ammonium sulfate salt would break up low energy bond protein-protein interactions (eg. low energy ionic bonds, hydrogen bonding), leading to disruption of a small inhibitor-large protein complex. With the precipitation of the larger proteins at lower ammonium sulfate saturations, the small inhibitor would attach onto and coprecipitate, with smaller, more soluble proteins. The correction of decreased PMN chemotaxis following treatment of inhibited cells with normal serum, as well as the neutralization of the inhibiting action of the precipitate at P_{40} and P_{100} by mixtures of these, plus the precipitates at P_{60} and P_{80}, indicates that a complex system of PMN regulation exists in the serum.

The data demonstrating the correction of PMN chemotaxis by levamisole, allows for the design of an experiment to test the thesis that decreased PMN chemotaxis predisposes to sepsis. It would be difficult to set up appropriate controls to do this in the usual manner. Most patients who are stressed by blunt trauma, operative trauma or some disease states have decreased chemotaxis. Severely traumatized patients with depressed PMN chemotaxis, and severely traumatized patients with normal PMN chemotaxis do not exist. One would have to identify all severely traumatized patients with depressed PMN chemotaxis and randomize them to receive levamisole or placebo in a double blind fashion. Their chemotaxis and clinical course can be

monitored. The theory would predict that those receiving levamisole (treatment group) would correct their PMN chemotaxis and develop less sepsis, than those receiving placebo (treatment group), who would maintain depressed chemotaxis and increased sepsis due to their depressed host defense.

The same inhibition observed with lymphocytic cells may explain part of the failure of skin test response in the anergic patients. Other factors such as adequate effector lymphocytes may be important. The coexistance of depressed cell mediated immunity and phagocytic cell immunity makes skin testing a very simple but powerful tool in assessing for the adequacy of host defense. Once the anergic patients are identified, detailed study of their phagocytic immunity is required to better correlate those at risk with those who develop sepsis. The thesis predicts that despite the presence of anergy, if phagocytic function is normal, patients should not become septic unless catastrophies such as infarcted bowel or undiagnosed perforated viscus occurs.

Anergy then, an indicator of depressed cell mediated immunity indicates a group of patients who are at risk to develop bacterial sepsis. Acute bacterial sepsis is defended against by the phagocytic component of host defense in conjunction with complement and the humeral component in some instances. In the long run all four components interact to provide for

adequate host defense. Host defense is regulated by a complex system of serum modulators of cell function in the acute state, such as severe trauma and at the cell proliferation state with chronic states, such as malnutrition producing diseases. The relative concentrations of serum inhibitor/counterinhibitor components in the blood regulates cell function following acute stress, be it, controlled or uncontrolled trauma, burns, hemorraghe, or sepsis. Teleologically, this may be a protective mechanism against an overwhelming inflammatory response to self antigens released during trauma or disease states. However, this depression of phagocytic cell function is associated with the "side effect" of increased susceptibility to sepsis. The gradual return to normal of the chemotactic migration of PMN or the evaluation of skin reactivity towards normal passing through a stage of relative anergy[169], following resolution of trauma, indicates return to homeostasis and adequate host defense mechanisms.

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CONTRIBUTIONS TO ORIGINAL KNOWLEDGE

1) Skin test anergy identifies patients at risk to develop sepsis and mortality from sepsis. This is confirmation of some of the data of Pietsch et al[196].
2) Sepsis itself may contribute to the anergic state.
3) The severity of operation is related to the development of depressed host defense in some surgical patients.
4) Severe hemodynamic instability due to gastrointestinal hemorrhage depresses host defense mechanisms.
5) Lymphocyte chemotaxis is depressed in patients with skin test anergy, and the depression varies directly with depressed PMN chemotaxis.
6) The depressed lymphocyte chemotaxis is mediated by serum inhibitors and reversed by levamisole.
7) Leukocyte adherence is increased in patients with skin test anergy and this increase is inversely correlated with PMN chemotaxis.
8) The increased adherence is mediated by factors in the plasma of anergic patients.
9) Several serum inhibitors of PMN chemotaxis exist in anergic patients. Their presence along with counterinhibitory moieties, seem to regulate in a precise manner, PMN chemotactic response.

10) The PMN chemotactic inhibitors can be separated with ammonium sulfate.

11) Major bactericidal defects to Staphylococcus do not exist in patients with skin test anergy and depressed PMN chemotaxis, though there is increased phagocytosis in these PMN.

12) Depressed PMN chemotaxis has a very high correlation with development of sepsis and mortality from sepsis.