HUMORAL IMMUNITY

in

SURGICAL PATIENTS

by

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ABSTRACT

Humoral immune function was studied in surgical patients. The antibody response to vaccination with a protein antigen, tetanus toxoid (TT), was reduced among all patients, especially those with reduced delayed type hypersensitivity (DTH) and increased degree of physiologic derangement. The antibody response to a polysaccharide antigen, pneumococcal polysaccharide (PPS), was normal. In trauma patients, the antibody response to TT was normal. The *in vitro* production of specific and total immunoglobulin (Ig) by blood mononuclear cells was studied. Patients that failed to produce a serum antibody response to TT also failed to produce anti-TT *in vitro*. Anti-PPS production was normal. More total Ig was produced by patients, especially those with reduced DTH responses. Some patients showed a reduction, rather than the normal increase, in Ig synthesis with mitogen stimulation. These data show evidence of humoral immune deficiency to protein antigens, and *in vivo* activation of the B cell system.

RÉSUMÉ

On a étudié la fonction d'immunité humorale chez des patients chirurgicaux. *In vivo*, la réponse d'anticorps à la vaccination avec un antigène protéique, le toxoïde du tétanos (TT), s'est avérée réduite chez tous les patients mais particulièrement chez ceux dont l'hypersensibilité retardée (DTH) était diminuée et qui présentaient des perturbations physiologiques accrues. Toujours *in vivo* mais utilisant cette fois l'antigène polysaccharidique du pneumocoque (PPS); la réponse d'anticorps s'est avérée normale. Chez les polytraumatisés, la réponse d'anticorps au TT fut normale. On a étudié *in vitro* la production totale et d'immunoglobulines (Ig) spécifiques par les cellules mononucléées du sang. Les patients ayant démontré une incapacité à fabriquer des anticorps contre le TT se sont aussi montrés incapables d'en produire *in vitro* malgré que la production d'anti-PPS fut normale. Le compte d'Ig total produit par les patients s'est avéré plus élevé que celui des témoins et ceci plus particulièrement chez ces patients ayant une diminution de la réponse d'hypersensibilité retardée. Chez certains patients il y a eu réduction plutôt que l'accroissement habituel dans la synthèse d'Ig stimulée par un mitogène. Ces observations démontrent un défaut de l'immunité humorale vis-à-vis les antigènes protéiques, ainsi qu'un activation *in vivo* des cellules de souche B.

PREFACE

The experimental work for this thesis was done while the candidate was training in General Surgery at the Royal Victoria Hospital, McGill University, Montreal, Quebec, Canada. The literary style of the "Uniform requirements for manuscripts submitted to journals", as published by the Annals of Surgery, has been followed. All work contained in this thesis is that of the candidate. The mononuclear cell culture methods used were either modified or developed, and largely performed, by the candidate. Under direct supervision, some routine cell cultures were carried out by Mr. G. Mazzei and Ms. L. Chisson after instruction by the candidate. The radioimmunassays used were either modified or developed, and largely performed, by the candidate. Under direct supervision, some routine assays were carried out by Mr. B. Kapadia after instruction by the candidate. For analysis of cell surface phenotypes, cells were prepared by Ms. D. Charrette under the supervision of the candidate, and analyzed in the laboratory of Dr. R. Guttman. Dr. D. Latter participated in the Pneumovax experiments, under the supervision of the candidate. Skin testing and clinical followup of patients was performed under supervision by Ms. M. Broadhead, Ms. B. Penning, and Ms. L. Lapointe. Dr. N.V. Christou provided the serum immunoglobulin data from ongoing data collection facilities. Computer programs for calculation and analysis of radioimmunoassay data were written by the candidate. Additional computer usage for calculations, databases and statistics was done by the candidate within the framework of commercial software. The thesis was prepared by the candidate, with the assistance of Ms. N. Theoret for the tables.

CONTRIBUTIONS TO ORIGINAL KNOWLEDGE

1. Specific humoral immune responses to tetanus toxoid are reduced among all surgical patients, worst in those with reduced delayed type hypersensitivity.

2. Degree of abnormal physiology and depressed delayed type hypersensitivity reactions correlate with depression of the humoral immune responses to tetanus toxoid, and with each other.

3. Antibody responses to pneumococcal polysaccharide are normal, even in severely ill surgical patients.

4. After traumatic injury, antibody responses to tetanus toxoid are normal, although delayed type hypersensitivity is reduced in some patients, worst in those with blunt compared to penetrating injury. Nonetheless, there are correlations between antibody responses, and physiologic status and delayed type hypersensitivity results obtained immediately after injury.

5. The kinetics and magnitude of spontaneous *in vitro* production of specific antibody by blood mononuclear cells harvested after tetanus toxoid or pneumococcal polysaccharide immunization *in vivo* parallel, and correlate with, changes in serum antibody levels. This extends previous observations in this area. Stimulation with pokeweed mitogen reduces the synthesis of IgG and IgA, but not IgM, antibody by cells harvested early after vaccination.

6. Blood mononuclear cells from surgical patients who did not produce a serum antibody response to tetanus toxoid also failed to produce antibody *in vitro*. This likely indicates a failure of specific sensitization *in vivo*, especially in patients with reduced delayed type hypersensitivity.

7. The *in vitro* synthesis of anti-tetanus toxoid antibody after vaccination *in vivo* of trauma patients is abnormal, and indicates an inability to generate antigen-specific memory cells after traumatic injury.

8. *In vitro*, blood mononuclear cells from surgical patients with reduced delayed type hypersensitivity responses spontaneously produce larger amounts of total IgG and IgA, but not IgM, than normal cells. The pattern is similar to that seen in normal subjects after vaccination with a bacterial polysaccharide antigen. This suggests that the increased *in vitro* synthesis of IgG and IgA by mononuclear cells from surgical patients may be due to *in vivo* anti-bacterial antibody responses. As an *in vivo* corollary observation, elevated serum IgG and IgA levels, but not IgM, were observed in septic surgical patients.

9. Pokeweed mitogen stimulation of blood mononuclear cells from patients with reduced delayed type hypersensitivity produces variable effects on total immunoglobulin synthesis; cells from some subjects produce more immunoglobulin *in vitro*, a normal effect, while cells from others produce the same or less than in unstimulated cultures. Abnormal responses were more common in ill patients with reduced delayed type hypersensitivity responses. This pattern was present for IgG and IgA, but not IgM.

10. Hydrocortisone *in vitro* alters the production of total immunoglobulin in a manner similar to that seen in ill surgical patients, suggesting that increased *in vivo* corticosteroid levels may be partly responsible for the increase in *in vitro* immunoglobulin synthesis.

11. Percentage of B cells in the blood of surgical patients is modestly reduced. Circulating monocytes are increased. There are only minor abnormalities in blood T cell phenotypic analysis.

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I would like to acknowledge the influence and contribution of the following individuals: Dr. J.L. Meakins, whose achievements initially attracted me to research, and who is continually guiding my development with great patience and skill: Dr. N.V. Christou, for unending enthusiasm and continuous assistance in many ways; Dr. L.D. MacLean, who created the environment of academic excellence at the Royal Victoria Hospital that set the stage for this work to take place; Dr. J. Gordon, whose knowledge was a necessary part of the development of the research program in host defence mechanisms; Dr. H. Rode, for his generous teaching; and Dr. J. Bohnen and Dr. A. Forse for encouragement.

I would like to acknowledge the contribution to my formation of all the surgeons that have trained me, for they taught me the significance of disease, and how to place basic knowledge in the context of human illness.

I would like to thank the technicians, Ms. D. Charrette, Ms. L. Chiasson, Mr. B. Kapadia, and Mr. G. Mazzei, and the research nurses, Ms. B. Penning, Ms. M. Broadhead, and Ms. L. Lapointe, for the mutual instruction and always pleasant interactions. I would also like to thank the ward nurses of the Royal Victoria Hospital, for their cheerful cooperation.

Finally, the strength of my gratitude and appreciation for my wonderful wife, Karen, and our children, Eva, Petrea and Lene, cannot be adequately expressed; suffice it to say that they provide the very reason to exist.

ABBREVIATIONS

ADH ..... adherence (cell isolation procedures)
AET ..... 2-amino-ethyl-isothiourunium bromide
APACHE II ..... Acute Physiology Score and Chronic Health Evaluation
BSA ..... bovine serum albumin
CPM ..... counts per minute
E ..... sheep red blood cell
FACS ..... fluorescence activated cell sorter
FBS ..... fetal bovine serum
FITC ..... fluorescein isothiocyanate
g ..... gram
HBSS ..... Hank's balanced salt solution
HBSS+H ..... Hank's balanced salt solution with heparin
HC ..... hydrocortisone
hr ..... hour
ICU ..... Intensive Care Unit
Ig ..... immunoglobulin
IL ..... interleukin
ITS ..... insulin-transferrin-selenium medium supplement
kg ..... kilogram
l ..... liter
L ..... latex particle
Lf ..... Limes flocculation units
M ..... molar
mcg ..... microgram
mCi ..... milliCurrie
mcl ..... microliter
mcU ..... micro-unit
ml ..... milliliter
MNC ..... mononuclear cell
mU ..... milli-unit
mg ..... milligram
min ..... minutes
MISS ..... modified injury severity score
MNC ..... mononuclear cells
ng ..... nanogram
NS ..... normal saline
PBS ..... phosphate buffered saline
PBS-AZ ..... phosphate buffered saline with sodium azide
PC ..... packed red blood cells

PMN.....polymorphonuclear neutrophil
PPS.....pneumococcal polysaccharide
PVC.....polyvinyl chloride
RIA .....radioimmunoassay
r_s.....Spearman rank correlation coefficient
SD.....standard deviation
SEM.....standard error of the mean
sIg.....surface immunoglobulin
SPCIRA.....solid phase competitive immunoradioassay
SPRIA.....solid phase radioimmunoassay
TT.....tetanus toxoid
U.....unit
WBC .....white blood cell
WSRT.....Wilcoxon signed rank test
WRST.....Wilcoxon rank sum test

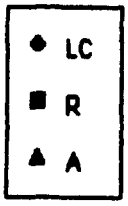
PATIENT GROUP ABBREVIATIONS

LC.....lab control
WR.....walk-in DTH reactive patient
HR.....hospital DTH reactive patient
WA.....walk-in DTH anergic patient
HA.....hospital DTH anergic patient
IA.....intensive care unit DTH anergic patient

FIGURE SYMBOLS

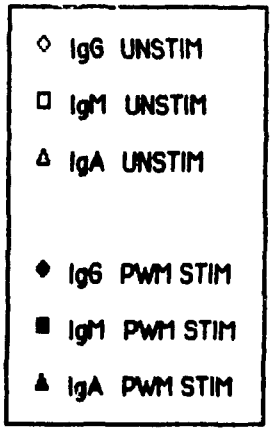
The graph symbols were used consistently as follows:

to indicate DTH classes:



LC = lab control
R = DTH reactive
A = DTH non-reactive (anergic)

to indicate Ig isotypes:



UNSTIM = unstimulated (spontaneous)
open markers

PWM STIM = pokeweed mitogen stimulated
solid markers

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INTRODUCTION

Sepsis is a major problem in surgery. Infections are usually caused by common Gram-negative and -positive bacteria. Estimates of incidence in hospitalized populations range from less than 1 to 10% [1,2,3]. The overall case fatality rate is approximately 30%. There are three determinants of infection: the bacteria, the environment, and the host. The systemic host defence mechanisms most important in dealing with invasive bacterial sepsis are the humoral immune and phagocytic systems [4,5].

Immunosuppression consists of detrimental alterations in those aspects of host anatomy and physiology normally dedicated to defense against infections. Because of the invasive nature of surgery, and because defects in systemic immunity have been described in most major surgical illnesses, it is reasonable to state that the immune system of all, or nearly all, surgical patients is altered in some manner to some degree. The overall acquired immunodeficiency in any given individual is the sum of several disease-related or iatrogenic changes in the patient.

In surgical patients, defects in phagocytic systems, including reduced polymorphonuclear neutrophil (PMN) chemotaxis [6,7], serum inhibitors of chemotaxis [8], decreased *in vivo* inflammation [9,10], and altered phagocytosis and killing [11], have been demonstrated. Some aspects of lymphocyte function have also been examined. There are reduced T cell percentages in peripheral blood, and a serum inhibitor of sheep red blood cell (E) rosette formation by T cells [12]. *In vitro* T lymphocyte function, measured by mixed lymphocyte culture, cell mediated lympholysis and antigen stimulation, seems normal [13]. In these studies, patients have been stratified according to delayed type hypersensitivity (DTH) skin testing, because decreased DTH responses have been found to be associated with an increased risk of sepsis and mortality in diverse groups of surgical patients [12,14,15,16,17,18,19]. In this context, altered DTH is viewed as an indicator of a multi-faceted immunodeficiency, rather than as a test of specific immunity [14].

The objective of the present studies was to determine the functional state of the humoral immune system in surgical patients.

REVIEW OF THE LITERATURE

1. HISTORY OF THE CONCEPTS OF HUMORAL IMMUNITY

The latin word *immunis* is related to the concept of exemption [20], originally used in Rome in a legal context to indicate freedom from duty. Although the concept of resistance to infection by prior contact has similarly existed since BC, when Greek historians described the non-lethality of a second exposure to plague, the use of the word immunity in this context is of more recent origin. This usage can be traced to the 14th century, when the phrase *E quibus Dei gratia ego immunis evasi* (From these things by the grace of God I have escaped.) described the escape of the author from a plague epidemic [20]. Although the 16th century saw speculation about the contagious nature of disease, and the possibility of deliberately producing a state of immunity against pestilence in the host, the word *immunity* was not extensively linked to the concept of acquired resistance to disease until the 19th century, in relation to smallpox vaccination by Edward Jenner. The role of the host in this interaction was not yet understood, but the publication in 1858 of the cell theory of Virchow laid the groundwork for the modern understanding of the host-infectious agent interaction in immune processes [21]. The further formulation of modern theories of acquired immunity awaited the germ theory of Pasteur [22]. The addition of the work of Koch in 1878 completed the basics of microbiology [23]. Thus, close to the end of the 19th century, the conceptual elements needed for the development of the modern theories of acquired immunity had been assembled; an infectious etiologic agent, and a host defense system that could respond to infections with an acquired resistance to repeated infections by the same agent.

Many significant events in the development of humoral immunity occurred at the end of the 19th century. Pasteur demonstrated protection against chicken cholera by vaccination with attentuated organisms [24]. On December 4, 1890, a landmark paper was published by Behring and Kitasato [25]. They described active vaccination against tetanus in rabbits, and showed passive protection by the inoculation of immune serum into un-vaccinated mice before toxin challenge. The antitoxic mechanism of action of the serum was shown by incubation of immune serum and tetanus toxin before test injection. In a footnote, the term *antitoxisch* was used, but not as a noun. The word *antitossina* was first used as a noun the following year in Italy by Tizzino and Cattani, and is the origin of the word *antibody* [26].

Subsequent studies lead to a rapid expansion of knowledge about antibodies. Ehrlich [27] produced antitoxins to plant products, and demonstrated a key feature of antibodies, namely antigen specificity. The few years left before the 20th century produced several more key discoveries. Agglutinating antibodies and precipitins were described. Complement, which had been a confounding factor in some early studies on antibody, because of a lack of knowledge, was also described by Bordet before the turn of the century.

The great debate between the proponents of the cellular and humoral theories of host defense occurred in the late 19th century [28,29]. Metchnikoff's observations of phagocytosis in 1882 [30] were the basis of the theory that cells were the most important defense mechanism against infection. This cellular approach to host defense was integrated with humoral immune concepts by the description and naming of opsonins [31]. The vital importance of the contributions of both cellular and humoral schools was further recognized by the joint award of the 1908 Nobel prize in Physiology to Metchnikoff and Ehrlich, the respective champions of the cellular and humoral approaches to immunity. Other Nobel prize winners from this era were Bordet in 1919, Koch in 1905 and Behring in 1901.

Thus, most of the important concepts of the role of humoral immunity in protection of the host against infection were developed in the late 19th century. In the early 20th century, plasma cells were shown to be the producers of antibodies [32]. From then to the present, the major developments have been in the fields of genetics and cellular processes involved in humoral immunity.

2. THE PROCESS OF A HUMORAL IMMUNE RESPONSE

An understanding of the process of a humoral immune response is important to address antibody deficiencies. In surgical patients generally, intact primary development of humoral immune systems is demonstrated by survival to an adult age. The process of this development in an individual occurs in two phases [33]. The first is an antigen independent, continous production of mature lymphocytes in either the thymus or the bone marrow to populate peripheral lymphoid organs with mature T and B cells, respectively [34]. The second is dependent on antigenic stimulation, and involves antigen encounter and processing, proliferation and differentiation of B cells, lymphocyte traffic within the body, immunoglobulin (Ig) synthesis, and distribution of antibody.

During embryogenesis, hemopoietic stem cells initially migrate from the yolk sac to the liver [35], and subsequently to the bone marrow [36]. Throughout adult life, there is a continuous heavy production of primary B lymphocytes in the bone marrow [37], approximately 10^9 per day [38]. While the presence of cytoplasmic IgM is characteristic of pre-B cells [39], the mature, immunologically virgin B cell is characterized by the presence of surface IgM and IgD [33,40]. Antigenic diversity in this population of cells is generated by rearrangement of the immunoglobulin-variable region genes during the pre-B cell phase [41,42]. These cells form one part of a continuous flow of lymphocyte traffic, passing via the blood to populate the peripheral lymphoid organs, including the follicles and marginal zone of the spleen, and the cortical follicles of systemic and gut-associated lymph nodes [43]. The microanatomic basis for this phenomenon is an interaction between lymphocytes and a variety of endothelial cells found in systemic and gut-associated lymph nodes [44]. Without clonal specific antigenic or polyclonal stimulation, the half-life of these lymphocytes is only 3 to 5 days [45].

The second phase in the development of a humoral immune response is an antigen-driven proliferation and differentiation of B lymphocytes into Ig secreting cells and memory cells [46]. Antigen entry and initial contact with the host immune system may occur at one of three points. Antigens may cross cutaneous or mesothelial barriers, and enter the lymph draining into systemic lymph nodes. Alternatively, they may cross gut barriers and encounter the host immune system at the level of the gut-associated lymphoid tissue. Finally, antigens may initially bypass the lymphatic system, enter the blood stream directly, and secondarily contact lymphocytes in the spleen. There may thus be differences in the initial immune encounter between the host and bacteria originating in the abdomen from perforation, in the biliary tract, in the urine, from the gut lumen, or from subcutaneous infections and implanted vascular devices.

Complex antigens may be processed by macrophages lining the lymph sinuses [47]. These cells, together with phagocytic and non-phagocytic accessory cells such as lymph node follicular dendritic cells [48], interact with antigen, pre-exisiting antibody, and lymphocytes. Passage of an antigen to a specific B cell, together with appropriate inter-cellular soluble signals from T cells and monocytes [49,50], results in binding to surface Ig receptors. After temporary internalization [51], the antigen resurfaces in association with class II molecules [52]. Recognition by helper T cells results in the production of lymphokines that cause the clonal proliferation of the antigen-specific B cells [51,53,54], forming a germinal center over a period of 4 to 5 days [55,56]. These proliferating cells have a transient loss of migratory capacity,

probably due to a loss of receptors necessary for homing to certain lymphoid tissues. Within the germinal center, heavy chain isotype switching occurs, predominantly to IgA in mucosal lymphoid tissue, and IgG in non-mucosal sites [34,57]. This heavy chain selectivity may be related to local T cell and soluble mediator differences between gut-associated and systemic lymph nodes [58].

Antibody synthesis begins in 3 to 4 days, by large B cells described as lymphoblasts. These cells are migratory, and at least in the case of T-dependant antigens, move from the lymphoid tissue to the bone marrow to continue antibody production [59]. Lymphoblasts producing antibody against gut-derived antigens, e.g. bacteria, return to the gut-associated lymphoid tissue [60]. Thus, although antigenic sensitization occurs in the gut-associated lymph nodes, antibody synthesis and subsequent deployment of secretory IgA occurs after the sensitized cells leave the follicles, travel via the thoracic duct and blood stream back to various mucosal sites [61].

Another product of the antigen-induced clonal proliferation is the generation of a long-lived, recirculating population of memory cells [62]. Helped by a process of somatic mutations during proliferation [63], such cells produce high affinity antibody. Their recirculation occurs in two streams depending on the expression of surface receptors capable of distinguishing endothelial cells located in systemic versus gut-associated lymph nodes [64,65]. One stream thus circulates mainly through the peripheral lymph nodes and another through the gut-associated lymphoid tissue. This property of memory cells is in contrast to the virgin lymphocyte, which is capable of interacting with endothelial cells in multiple sites in the body [66].

At any given time, therefore, a variety of B lymphocytes may be found in the lymph [67] and bloodstream [68,69]. This includes virgin cells, long-lived memory cells, and cells that are spontaneously secreting antibody in response to recent antigenic encounters, which are on their way to either the bone marrow or systemic and gut-associated lymphoid tissue.

Once synthesized, the deployment of antibodies to intra-vascular sites requires little more than secretion from the lymphoblastoid or plasma cell. However, many initial contacts between microbe and host occur on the mucous membranes of secretory tissues. To accomplish the distribution of antibodies within and on these surfaces, another synthetic machinery is in place. The predominant isotype is IgA, although there is a secretory form of IgM. Both secretory IgM and IgA are associated with two polypeptides, the J chain, and

secretory component. The latter is produced by mucosal epithelial cells, and aids in transport across the mucosa.

Although there are numerous bacterial protein antigens, most of the surface antigens on the gram-negative bacteria that are responsible for the bulk of surgical sepsis are complex polysaccharides. There are several differences between the antibody responses to protein compared to polysaccharide antigens. Classically, the antibody response to protein antigens was thought to be dependant on the presence of functional T cells, while the response to polysaccharides was independant. However, T cells have more recently been shown to play an immunoregulatory role in humoral immune responses to polysaccharides [70,71], so that this distinction is more one of degree, than absolute. Responses to protein antigens are almost entirely IgG, while IgG, IgM and IgA antibody are produced to polysaccharide antigens. There is also a restriction in the IgG subclasses involved. IgG antibodies to protein antigens are of the IgG_1 subclass [72], while those to polysaccharides are IgG_2 [73,74].

3. THE ROLE OF ANTIBODY IN SEPSIS

The antimicrobial effector functions of antibodies are as follows [75]: inhibition of bacterial adherence, opsonization, complement-mediated cell lysis, neutralization of toxins, agglutination, and effects on bacterial metabolism and motility. The relative importance of these mechanisms varies according to bacteria, body site, and Ig isotype. In any given infectious process, antibody acting in various mechanisms may play a role at several levels in bacterial containment and elimination. The secondary effector mechanisms involved in humoral immune defense are phagocytic cells and complement. Complement provides cytolytic factors, opsonins and chemoattractants.

Inhibition of bacterial adherence to host epithelial surfaces is an important early mechanism of local host defense. Several bacterial species exhibit specialized structures to promote adherence to epithelium [76,77]. Secretory IgA contains antibodies that block these structures [78], limiting the potential for invasive sepsis across mucosal and skin barriers [79,80].

The deposition of antibody on the surface of bacteria may significantly accelerate phagocytosis, and is extremely important in severely ill patients [81]. The opsonically active fragment of complement is C3. To be effective, opsonization depends on intact complement mechanisms [82,83], and an adequate number of functional phagocytes at the site of infection.

Several bacterial antigens may directly activate complement [84,85,86]. In this setting, antibody plays a role by either initiating complement activation, and/or by providing additional ligands for the phagocyte-bacterial interaction. Such opsonizing antibodies may be directed against capsular polysaccharides [87,88], or other surface antigens [89]. The sensitivity to opsonization of some bacterial species, notably gram-negative organisms, is variable, and may be related to the relative efficacy of binding of antibodies against different bacterial determinants [90]. The important opsonizing Ig isotypes are IgM, IgG_1 and IgG_2. In addition to activating the classical complement pathway, the Fc portion of the antibodies also acts as a binding ligand for phagocyte receptors. There are no receptors for the IgA Fc portion, and this isotype is therefore not effective as an opsonin [91]. Whether IgG or IgM is more effective is not known [92,93].

Bacterial cell lysis is another complement-mediated mechanism of action of antibodies. IgM and IgG may initiate complete complement cascades, resulting in cell lysis, especially of gram-negative organisms.

Neutralization of toxin is a classic antibody function in certain diseases, for example diptheria and tetanus. The role of toxins in gram-negative sepsis in surgical patients is less clear. There are many similarities between the presentation of sepsis syndromes, and experimental endotoxin injection, suggesting that one pathophysiologic mechanism in septic man is endotoxemia [94]. Antibody against endotoxin, *anti-toxin*, could thus be protective. Support for this concept comes from data showing increased survival in septic patients with higher anti-endotoxin antibodies [95], and the preliminary findings of therapeutic trials of anti-endotoxin antibodies in the treatment of sepsis [96].

Agglutinins were among the first functionally defined groups of antibodies. These antibodies may reduce numbers of infectious units, thereby limiting the progression of infection. The potential mechanisms of motility inhibition and metabolic alterations are of uncertain significance.

4. ASSESSMENT OF HUMORAL IMMUNITY

It is a caveat that no biological process occurs *in vivo* in isolation. The process of a given host defense mechanism is subject to a vast array of anatomic, cellular and physiologic influences. Conceptually, a thorough evaluation of a single aspect of immunity would

therefore entail a detailed determination of the function of every host organ system, tissue and cell. While acknowledging the influence of global and microenvironmental host conditions on an immune process, the following description of assessment, and the review of known abnormalities of humoral immunity, is focussed on the testing of certain B cell functions.

The status of the humoral immune system in a given individual can be determined by tests in the following categories [97,98,99]: serum Ig levels, measurement of antibody levels to common antigens, specific antibody responses to vaccination, *in vitro* B cell function studies, lymphocyte phenotype enumeration, and histologic examination of lymphoid tissue.

Serum Ig measurements are of little value in the evaluation of acquired immunodeficiency. There is a wide range of normal values, without a clear definition of a lower limit associated with increased susceptibility to sepsis. The normal half life of IgG, 23 days, would also require long-term reductions in Ig synthesis to produce low levels, even without changes in catabolism [100]. Finally, serum Ig levels in surgical patients are subject to influence by fluid and protein shifts. Nonetheless, measurement of serum Ig levels is a standard clinical test in the initial evaluation of humoral immune function [101,102], and if interpreted in the light of these considerations, can provide some indication of the level of activity of the B cell system in surgical patients.

The next level of clinical investigation involves the measurement of natural or commonly acquired antibodies. The presence of serum isohemagglutinins, and antibodies to common antigens, for example diptheria and tetanus toxoid (TT), can provide evidence of past integrity of humoral immunity, but does not provide information about the present state of B cell function.

Further laboratory evaluation of humoral immunity involves the determination of B cell numbers, most easily performed on blood cells. B cells are counted by labelling for surface Ig (sIg) using fluorescent probes or other techniques. The normal percentage of sIg^+ circulating mononuclear cells (MNCs) is from 5 to 15. Some other B cell surface markers also appear on other cells. These include HLA-DR, C3 receptors and Fc receptors [103]. Tests are usually done on cells isolated from blood, but because of lymphocyte traffic considerations, abnormalities do not necessarily imply functional deficiencies [34].

The *sine qua non* of normal *in vivo* B cell function is a specific antibody response of the host to recent antigen encounter. Therefore humoral immunity is best assessed by the study of responsiveness to vaccination [104]. Because of the differences between the responses to protein and polysaccharide antigens, it is useful to test both. Several protein antigens have been used experimentally in the past, most commonly diptheria toxoid, TT, or keyhole limpet hemocyanin [105]. To study immunity to polysaccharide antigens, pneumococcal polysaccharide (PPS) and meningococcal vaccines are available. The usual method of determining responsiveness in these test systems is the measurement of serum antibody levels by one of several methods after vaccination. Hemagglutinin techniques measure predominantly IgM. Direct antibody measurement, using radioimmunoassay (RIA) or enzyme-linked immunosorbent assay, allows the separate determination of different isotype responses to vaccination. Solid phase assay techniques can be adapted to the measurement of antibodies to most antigens, and offer a reproducible sensitive system.

The ultimate *in vitro* function of a B cell is to produce Ig [106]. A variety of end-points can be used, including plaque-forming cell responses [107,108], enumeration of cells containing cytoplasmic Ig, or measurement of Ig in culture supernatants [109]. In studies of Ig synthesis by blood MNCs, there is a correlation between numbers of Ig secreting cells, and cumulative Ig secretion [110]. Any method is useful, and allows quantitation of amounts of total Ig of several isotypes [111]

In vitro cultures of blood MNCS may be unstimulated, or stimulated with polyclonal B cell activators. The baseline state of activity of the humoral immune system can be measured in unstimulated cultures, and the capacity of the circulating cells to respond to activation measured in stimulated cultures. Polyclonal B cell activators, including drugs and biologic agents, act in an antigen non-specific manner. Many substances have been used, including lipopolysaccharide [112], Nocardia water soluble mitogen [113], Staphylococcus aureus Cowan I [114] and Epstein-Barr virus [115], but the majority of work in this field has been done with pokeweed mitogen (PWM) [109]. This plant lectin is an extract of *Phytolacca americana* (pokeroot), and was accidentally discovered to cause plasma lymphoblastosis and hypergammaglobulinemia in man [116]. Several mitogenic proteins are present in the extract [117,118]. Predominantly IgG_1 is produced in response to PWM stimulation, which also constitutes the majority of serum IgG [119,120].

Cells secreting Ig in response to PWM stimulation *in vitro* appear after approximately 4 days, and peak on days 7 to 8 [121]. The cells have the appearance of large lymphoblasts [122]. Approximately 40-70 picograms of Ig can be synthesized per actively secreting lymphoblast per day [123]. Synthesized Ig remains in the culture supernatant in a cumulative fashion [124]. Althought there is great variability in the maximum amount of Ig produced, and in the pattern of isotypes, MNCs from most normal subjects respond to PWM with an increase in IgG, IgM and IgA in the range of 3 to 10 fold.

An adequate response to PWM stimulation is dependent on the presence of T cells [125,126,127,128] and monocytes [129] *in vitro*. The T cell helper activity resides in the $CD4^+$ subset [130,131,132], which acquires Ia antigens after stimulation [133]. The monocyte effect is mediated in part by intact cells [134], and in part by the soluble factor interleukin 1 (IL-1) [135,136,137]. Monocytes and T cells may also exert inhibitory influences on Ig synthesis in vitro. The optimal ratio of monocyte to lymphocyte *in vitro* is approximately 1:10; if this is increased to 3:10, almost complete inhibition of Ig synthesis occurs [138,139]. Activated lymphocytes from individuals with diseases associated with increased spontaneous Ig synthesis *in vitro* may be free from this inhibitory effect [140]. The $CD8^+$ subset of T cells also exerts a suppressor activity [141], probably acting on the other T cell subsets rather than directly affecting B cells [142].

Combined *in vitro* and *in vivo* studies after vaccination are useful in the evaluation of humoral immunity. It has been shown that after vaccination, MNCs that spontaneously secrete antibody appear in the blood stream [68,69,143,144,145]. These cells are a window on the *in vivo* response, which can then be followed by *in vitro* lymphocyte cultures for quantitation of specific antibody and total Ig. Although polyclonal B cell activators are usually used to stimulate B cells in an antigen non-specific manner, they may also be used in this system to determine the effect of microenvironmental alterations on the capacity of B cells that are antigenically primed *in vivo*.

Phenotypic analysis of circulating MNCs can also be relevant to the evaluation of humoral immunity . Monocytes, B cells, and subclasses of T cells can be counted. T cells are important in the antibody response to protein, and probably polysaccharide antigens. In the past, the standard measurement for enumerating T cells was their ability to bind sheep erythrocytes [97]. Presently, populations of T cells are studied more precisely with monoclonal antibodies [97]. These antibodies bind to surface receptors that distinguish functional

subpopulations of lymphocytes. The ratio of helper to suppressor cells, that is CD4:CD8, is frequently reported as an indication of immune aberrancy. In the interpretation of results, it must be borne in mind that these tests are usually performed on blood lymphocytes, which bear an unknown relationship to microenvironmental conditions at the actual site of host immune responses.

Finally, examination of the histology and functional capacity of immune tissues and cells from other sites can be performed for the complete evaluation of immunity [146]. These invasive procedures can evaluate the functional state of bone marrow, lymph nodes [147], spleen and gut lymphoid tissue.

In summary, a specific antibody response, measured *in vivo* or *in vitro*, to antigen encounter *in vivo* provides the most direct and comprehensive assessment of all the processes involved in humoral immunity. The functional state of some aspects of the process may be studied by enumerating B cells, testing for antibody and total Ig synthesis *in vitro* under a variety of conditions, and by phenotypic analysis of lymphocytes.

5. ACQUIRED DEFICIENCIES IN HUMORAL IMMUNITY IN SURGICAL PATIENTS

Defects in immunity may be either primary or secondary, that is congenital or acquired. Primary immunodeficiencies are viewed as defects in cellular differentiation, and are classified according to whether the most prominent defect is in neutrophil, T, or B cell function. More than 70 such syndromes have been described, but the cumulative prevalence of clinically important cases is only 1:10,000.

The practicing surgeon is therefore unlikely to encounter cases of primary immunodeficiency. Acquired defects in host defence mechanisms are encountered far more commonly in medical practice than all primary defects combined [148]. In fact, all surgical patients can be described as having some degree of acquired immunodeficiency in either local or systemic host defence mechanisms, or both [149].

Host defense mechanisms may be broadly divided into local and systemic categories. Local host defenses play an important role in the process of humoral immunity. The human body is a complex tube covered by skin and lined with mucosa. Various mechanical, cellular, microbiological and biochemical properties of these coverings produce local host defense

mechanisms. It is a breakdown of these local barriers that allows encounter between bacterial antigens and the humoral immune system. Phagocytic cells, complement and humoral and cell-mediated immune mechanisms constitute systemic immunity. There are major interactions at many levels between the various local and systemic immune processes.

Surgical patients are a heterogeneous group. Primary diagnosis, age, nutritional status, general condition on admission, clinical course in hospital, magnitude of surgery, complications and co-existing organ system dysfunction present a vast array of possible effects on immune function. Many of these effects may change dynamically during the course of an illness. The sum, and interaction, of individual disease processes produce the net effect on humoral immune function of the individual. Present knowledge of the likely impact on humoral immunity of certain disease processes that are important in surgical patients is presented. These are age, malnutrition, cancer, burns, trauma and surgery, and anesthesia. The impact of the adrenocortical response to stress on humoral immunity is also discussed. Finally, the influence of some diseases commonly present in surgical patients, uremia, liver disease, and diabetes mellitus, on antibody systems is reviewed.

5.1 AGE

Aging is a multi-system process of unknown etiology. Other disease processes occur concomitantly; for example the prevalence of malnutrition in the elderly is as high as 40 to 65% [150,151]. In aged man, there are abnormalities in humoral immune systems [152]. Serum Ig levels are normal [153] or increased [154,155]. Circulating B cell numbers are normal [156,157,158], but there may be a change in the distribution of antibody forming cells in the body, with an increase in the bone marrow [159]. There are abnormalities in regulatory T lymphocyte subsets in aged subjects, with a decrease in helper cells [160]. Lymphokine production in response to stimulation *in vitro* is diminished [161].

Tests of specific antibody responses have generally shown modestly reduced responses [162]. In studies of the response to TT, although young control groups were not always included, the increase in antibody titer was less than 10 fold after vaccination [163,164,165], which is an acceptable definition of a normal response [68]. This suggests reduced immunity. However, in other studies, the antibody response to TT was well-preserved [166,167]. In studies of both *in vivo* and *in vitro* production of anti-TT antibody, there was an age-related decline [168].

Responses to other antigens have been measured. A normal response was shown to a protein antigen, flagellin [169]. The response to a polysaccharide antigen was reduced [170,171], although this may be significant only for IgM rather than IgG and IgA [172]. A study on the effect of different routes of administration of antigen has suggested that antibody responses to antigen encounter via the mucosal-associated lymphoid system may be better preserved than those to systemic encounters [173].

There are abnormalities in Ig synthesis *in vitro* by MNCs from older subjects. Normal [156] or reduced [174] amounts of Ig are produced spontaneously. An intrinsic defect in aged B cells, with decreased T helper cells [155,175], and increased T suppressor cells [156,176], has been shown. These effects may be different for IgG compared to IgM [177]. In studies of antigen priming of B cells *in vitro*, cells from older subjects did not respond well [178,179].

5.2 MALNUTRITION

Malnutrition, defined by one of several sets of criteria [180], has been estimated to be present in up to 50 percent of surgical patients [181,182]. Obesity may also produce an acquired immunodeficiency [183,184]. Most human studies on the effect of malnutrition on immunity have been done on undernourished children in developing nations [97,185]. Although useful, these data may not be strictly applicable to surgical patients, because of the inability to rule out concomitant infection [186]. Furthermore, alterations in host defense may be different when protein loss predominates [187], compared to combined protein-calorie malnutrition or single nutrient deficiency [188,189,190,191,192].

Nonetheless, abnormalities of B cell function have been demonstrated, but are not generally as striking as T cell defects [97,193]. Serum Igs have been reported as low [194], normal [195,196] or high [197,198]. Animal studies have produced similarly inconsistent results [199,200]. B cell numbers in peripheral blood are normal [196].

Studies of specific antibody responses have not been consistently abnormal in malnourished man or animal. Using older assay techniques, sometimes difficult to interpret in the light of modern knowledge, antibody responses in man have been shown to be reduced to several antigens; *Salmonella typhosa* organisms [201], tobacco mosaic virus and fowl red cells [202]. Responses have been to normal to other antigens, including polio and smallpox vaccines [203], TT [204], measles [205], diptheria [206], and PPS [207,208].

Animal studies have been more extensive. In rats immunized with sheep red blood cells while protein deficient, a reduced plaque forming cell response [209], and serum antibody levels [210] were found. There was a dose response curve between varying degrees of protein deprivation and decreased antibody responses [211]. A similar dose-response effect was shown with another antigen, Brucella abortus [212]. Studies with TT showed that both short- [213] and long-term nutritional deprivation reduced humoral immunity [214]. Generally, responses to T dependent antigens are reduced, while they are normal or reduced to T independant antigens [212,215]. Refeeding is generally immuno-restorative [215,216], but less so after brief [213] compared to longer periods of malnutrition [214]. Improved antibody responses after nutritional supplementation have also been shown in man [217].

In summary, based predominantly on animal evidence, malnutrition reduces humoral immune responsiveness, at least to T cell dependant protein antigens. There is a variable effect on other measures of B cell function. Refeeding under certain circumstances is immunorestorative.

5.3 CANCER

Diminished humoral immune responses have been shown in patients with malignancies. The immune defects associated with solid versus hematological malignancies may be different [218]. Also, the effect of chemotherapy, radiation and malnutrition, present in as many as 80% of patients [219], are compounding factors.

Hodgkin's disease patients immunized with PPS produce low antibody levels, depending on tumor stage and treatment [220]. Patients with visceral malignancies produced less IgG and IgM antibody to flagellin, a protein antigen [169]. In patients more than three years after succesful cancer therapy, responses were nearly normal. Studies of the response to TT have produced conflicting results [221,222,223]. In the *in vitro* evaluation of B cell function, depressed PWM-induced Ig synthesis by blood MNCs has been shown [224]. This was not caused by deficient IL-1 or -2 production. Whether this represents a B or primarily T cell defect is not known.

Treatment of malignancy with chemotherapy or radiotherapy may further reduce humoral immunity. Chemotherapeutic agents cause a well known depression of host defence mechanisms, and reduced antibody responses are a part of this [225]. Also, radiation treatment

of patients with lymphoma, and head and neck cancer was associated with reduced antibody responses to PPS [226,227].

In summary, there is evidence of decreased humoral immunity in patients with malignancies. Whether this is primarily due to cancer, or associated illness, is unknown. It may be aggravated by anti-neoplastic therapy.

5.5 BURNS

The burn patient has suffered a severe disruption of both local and systemic host defense mechanisms. Serum concentrations of Ig fall rapidly after major burns [228,229], especially IgG [230], but recover by one to two weeks. This suggests fluid shifts and dilution as the central explanation. However, local concentrations of Ig may be depressed, allowing diminished anti-bacterial activity in the burn wound [231,232].

Specific antibody responses have been studied in man and animals. Using a polyvalent Pseudomonas vaccine, antibody responses were detected to most serotypes [233,234]. The response to vaccination following burn injury with a protein antigen, TT, was normal in one study [235], and reduced in another [236]. Defective antigen presentation by accessory cells has been shown following experimental thermal injury [237].

In animal studies, conflicting results have been reported. In some studies, responses to both a T dependant and T independant antigen were enhanced when animals were immunized soon after injury [238,239,240]. However, in another burned animal model using TT vaccination on the day of injury, antibody responses were severely reduced from 5.8 to 0.8 units of anti-TT/ml serum [241]. There is no apparent resolution to these conflicting results.

In *in vitro* studies of B cell function, lymphocytes from burned patients showed increased spontaneous Ig production, and no augmentation with mitogen stimulation [242]. Other *in vitro* abnormalities have been described, including the presence of a growth factor in burned skin that enhances macrophage-lymphocyte interactions during antibody formation [243].

In summary, most evidence favors a predominantly T cell defect in systemic immunity after thermal injury, with a secondary influence on the antibody response to T dependant antigens [244,245,246].

5.6 TRAUMA AND SURGERY

Mechanical trauma and surgery share some common features. They involve tissue injury, and the administration of blood products, antibiotics and anesthetics, all potentially immunosuppresive [97]. Both trauma and surgery can be graded in terms of severity, and share common general metabolic and endocrine reponses that may influence immune processes [247,248,249]. Immune defects in injured or operated patients may have multiple etiologies. Malnutrition is present in many, from 28% of valve recipients [250], to 80% of patients with advanced gastrointestinal cancer [97]. If not present pre-operatively, sepsis or malnutrition may occur after surgery, compounding the alterations in immune responsiveness.

Serum Ig levels and B cell numbers have been assessed after trauma or surgery. Total Ig levels fall after trauma [14,251] and cardiopulmonary bypass [252], but this is likely dilutional. Little change was seen after elective nephrectomy [253]. Major trauma was associated with an early mild decline in circulating B cells, with a return to normal in 4 to 5 days [254]. In studies on the effect of surgery, B cell numbers were either normal [255,256,257], or transiently decreased postoperatively [253,258,259].

There is little data from human studies on specific antibody responses after trauma or surgery. In an early report using diptheria toxoid in battle injured patients, responses were detected [260]. However, many of these subjects may have been immunized late after injury. Another study suggests depressed immunity. The antibody response to TT in injured Korean war soldiers was reduced in four of seven subjects [261]. In the three responders, there was no control for false positive passive increases in serum antibody due to transfusion. A study of the response to diptheria toxoid in patients operated for gastrointestinal malignancy showed decreased antibody production [262].

In animal studies, cholecystectomy decreased the antibody response to SRBCs given two hours after operation [263]. However, in other experiments, serum antibody titres to SRBCs given during laparotomy were increased [264]. If immunization was delayed for 24 hours, there was no enhancement [265]. In other animal models based on trauma to the extremity, there was either no effect on antibody responsiveness [266], or enhancement [267].

An interesting feature of *in vitro* B cell function has been examined after surgery or trauma in man. Lymphoblastoid cells spontaneously secreting Ig have been detected in the blood of

patients post-operatively [268]. The cause, and the nature of the secreted Ig, have not been determined. There is a synchronous depression in IL-2 production *in vitro* by MNCs [269,270], while IL-1 production is normal.

Splenectomy is a special surgical procedure in relation to immunosuppression. The spleen plays a major role in protecting against sepsis by acting as a phagocytic filter, and as an antibody forming organ [271]. The asplenic state is associated with an increased risk of rapidly fatal septicemia [272,273,274]. Since most post-splenectomy infections are from encapsulated organisms, attention has been focussed on the antibody response to polysaccharide antigens, and this has been shown to be reduced [275].

In summary, as has been previously concluded, the effects of trauma and surgery on antibody formation have not been well studied, especially in man [252,276]. An exception to this is splenectomy, after which there is a significant diminution in humoral immune responsiveness.

5.7 ANESTHESIA

The influence of anesthetic agents on immunity in man cannot be studied separately from surgery. Available data are therefore from animal studies.

The effect of anesthetic agents has been studied predominantly use plaque-forming cell techniques [277]. Immunization before or during anesthesia with several study antigens has shown either a decrease [264,278], no effect [256,263,279,280] or an increase in antibody producing cells [281]. No change in circulating B cells has been shown [256]. There is thus no clear demonstration that anesthetic agents produce any major consistent adverse effect on humoral immunity in animals.

In summary, based primarily on animal studies, anesthesia has little significant effect on humoral immunity [282].

5.8 CORTICOSTEROIDS

In man, the adrenocortical response to stress includes an increase in adrenal weight and cortical hypertrophy [283,284]. Increased production of cortisol from 1.5 to 6 times normal,

coupled with diminished hepatic metabolism, results in elevated circulating levels [285]. The normal serum concentration of cortisol is from 0.1 to $0.6*10^{-6}$M. With severe illness, this may increase approximately 5 fold [286]. This corresponds to the administration of approximately 50 mg of hydrocortisone every 6 hours [287]. In studies of trauma subjects [288] and severe bacterial infections [289], the highest cortisol levels of approximately 5 to 10 fold normal occurred in subjects that subsequently died . After minor surgery, cortisol increases are modest, but abdominal and thoracic procedures can cause 5 to 10 fold increases [290]. Levels return to normal 5 to 10 days later [277].

Study of the impact of elevated steroid levels relies on the administration of exogenous drug to experimental subjects, or its use *in vitro*. Human studies are most relevant because, unlike rodents, immunity in man is relatively steroid resistant [291,292].

The administration of exogenous steroids decreases serum Ig levels due to decreased synthesis and increased catabolism [293]. However, specific antibody responses to vaccination of steroid treated subjects have been reported as normal [294,295,296].

In vitro B cell function has been examined after *in vivo* steroid treatment. Using doses of exogenous prednisone *in vivo* that correspond approximately to the maximal daily production of corticosteroids in severely ill man, an enhancement of spontaneous Ig synthesis by blood MNCs cultured *in vitro* [297], and either a depression [297] or no effect [298] on the response to PWM stimulation, was shown . A similar effect was also produced by larger doses of steroid [299].

The effect of corticosteroids on lymphocyte function has also been examined using the drug *in vitro*. With a range of concentrations similar to *in vivo* levels, more than a log increase in the generation of otherwise unstimulated Ig secreting cells occurred [300]. This stimulating effect was both T cell and monocyte dependant. In PWM stimulated cultures, *in vitro* hydrocortisone increased the production of Ig in several studies [298,301.302,303,304]. This may be related to inhibition of suppressor cells by hydrocortisone [305]. There may be interactions with other hormones. An increase in spontaneous Ig synthesis by blood MNCs exposed *in vitro* to either epinephrine or physiologic concentrations of hydrocortisone [306] has been shown. The use of both hormones together *in vitro* extended the range of concentrations of epinephrine that were stimulatory.

As well as these effects on humoral immune processes, elevated levels of corticosteroids *in vivo* cause lymphocytopenia due to changes in lymphocyte traffic [307,308], decrease the accumulation of inflammatory cells at sites of infection, inhibit neutrophil lysosomal release, depress T cell proliferation, and inhibit several aspects of monocyte function [292,309,310]. Thus, apart from direct action on B cells, corticosteroids may affect humoral immunity in several indirect ways.

In summary, the adrenal response to stress produces a hormonal milieu that may increase Ig synthesis, but does not affect specific antibody responses.

5.9 OTHER DISEASE PROCESSES

Uremia is associated with significant defects in host defense mechanisms [311]. Many of these are similar to those found in malnutrition [312]. Serum levels of IgM and IgA are normal, but IgG is either normal [313] or increased [314]. B cell numbers in peripheral blood are decreased [315,316]. Data on antibody responses are conflicting. Some reports have shown normal responses to influenza vaccine [317,318], diptheria toxoid [319], and PPS [320,321]. Others report decreased antibody production to influenza vaccine [322], typhoid vaccine [323], and hepatitis B vaccine [324]. Improved antibody responsiveness to some antigens may be achieved by administering additional doses of antigen [325]. *In vitro* studies have shown decreased spontaneous Ig synthesis [326]. Responses to PWM stimulation are also reduced [327], in association with decreased IL-2 production [328]. Other *in vitro* studies indicate that there is a failure of monocyte-T cell interaction, and that some immune processes can be restored *in vitro* by the addition of IL-2 [329].

Liver disease is associated with an increased risk of sepsis. There is a contribution from concomitant illness, for example malnutrition. Serum Ig levels are frequently increased [330], and antibody responses to vaccination appear to be either normal or increased [331]. Spontaneous synthesis of Ig by blood MNCs is increased [332]. Response to PWM is either normal [333], increased [334], or defective. Reduced *in vitro* Ig synthesis has been described in alcoholic liver disease [332] and pre-hepatic transplant patients [335].

Diabetes mellitus may be associated with altered systemic host defense. However, B cell function is normal. Serum Igs [336] and numbers of circulating B cells are normal [337]. Antibody responses to typhoid vaccine [338], PPS [321,339], and staphylococcal toxoid [340] were

approximately normal. In MNCs from diabetics, there was no evidence of spontaneous activation of B cells *in vitro*, and response to PWM was normal [341].

6. STRATIFICATION

Heterogeneity in patient physiologic status, regardless of basic disease process, is increasingly recognized as an important variable in determining outcome of any acute illness [342]. Four stratification mechanisms have been used in the present study: DTH skin testing, a system of physiologic stratification known as the APACHE II score, a scale of severity of surgery, and a modified injury severity score (MISS).

Decreased DTH skin testing with recall antigens has been correlated with increased risk of sepsis and mortality in a study of 2202 surgical patients [14]. Several defects in host defence mechanisms have been associated with reduced DTH, including decreased neutrophil chemotaxis, serum inhibitors of chemotaxis, reduced in vivo inflammation, altered phagocytosis and killing, and decreased T cells. The cause of anergy in these patients is unkown, but its occurrence in several disease states suggests multifactorial mechanisms. It is therefore likely that not all patients with reduced DTH would show the same type and degree of acquired abnormalities in other tests of host defence mechanisms. Nonetheless, decreased DTH can be used as an indicator of reduced resistance to infection, thereby identifying a patient population that could be expected to show the most marked abnormalities in other immune systems.

Heterogeneity in general physiologic status is increasingly recognized as an important variable in determining outcome of acute surgical illness. Using the hypothesis that the severity of acute illness could be measured by quantifying the degree of abnormality of multiple physiologic variables, an acute physiology and chronic health evaluation score was developed that has a strong relationship to mortality rate [343,344]. A revised version (APACHE II) calculates a score based on assigning points for degree of deviation from normal of twelve commonly measured physiologic and biochemical variables [345,346]. They are temperature, blood pressure, heart rate, respiratory rate, oxygenation, arterial pH, serum sodium, potassium, and creatinine, and hematocrit, white blood cell count and Glasgow coma score. Additional points are given for advanced age and pre-existing organ system dysfunction. This scoring system both transcends diagnostic categories, and allows

additional discrimination of risk within diagnostic groups [347]. It is used in the present studies to stratify patients according to severity of illness.

The degree of surgery has a known influence on outcome. An expanded version of a previously developed scale has been used [6], and is presented in appendix 1.

The degree of tissue injury, and risk of mortality after traumatic injury may be quantified by an injury severity score. A scoring system has been developed that assigns points according to lists of injuries in various organ systems [348,349,350]. Classification of both blunt and penetrating injuries by the same scale is an acknowledged problem, because a penetrating injury to a given organ involves less tissue damage than a blunt injury with similar lethality. There is a linear relationship between mortality rate and MISS for scores greater than 15; below that, there is minimal mortality [348]. Thermal trauma is classified according to the percentage of second and third degree body burns.

In summary, the identification of patients with high risk for sepsis and mortality is an important goal, so that properly stratified patients may be studied to identify defects in host resistance mechanisms. Patients may be stratified according to severity of illness, and their group risk of sepsis determined by DTH testing.

MATERIALS AND METHODS

1. SUBJECTS

1.1 PATIENT SELECTION AND CLINICAL DATA COLLECTION

Surgical patients on the wards and intensive care unit of the Royal Victoria Hospital were studied. The patients represent a diagnostically heterogeneous group. Standard clinical care was provided without regard to study involvement, including various forms of nutrition. The majority of patients underwent a surgical procedure. The remainder either had inoperable disease, usually malignancy, or their illness resolved with non-operative management. Subjects with blunt, penetrating or thermal trauma were studied separately. Pregnant patients, or those receiving corticosteroids, chemotherapy or radiotherapy during the period of study, or for one year previously, were excluded from all studies.

Additional exclusion criteria were used in studies of specific antibody responses. For all antibody response studies, including trauma subjects, only patients surviving 2 weeks after vaccination were included. For TT immunization studies, no subject had received a TT booster in the previous 2 years. Beyond this criterion, no reliable information was generally available regarding the past history of vaccination. For PPS immunization studies, patients with splenectomy, lymphoid malignancy, renal failure requiring dialysis, or pneumococcal pneumonia were not studied. No subject had been previously vaccinated with PPS.

For non-trauma patients, subjects receiving blood products, except packed red blood cells, were excluded from studies of specific antibody responses. This is based on the measurement of anti-TT levels in non-immunized blood donors. The anti-TT content of plasma from 17 normal blood donors, not recently immunized, was measured. The range was 0.01 (the level of detectability of the assay) to 3.8 U/ml, the median was 0.14 and the geometric mean 0.20. Because of this antibody content, transfusion of plasma or whole blood could produce false increases in serum antibody, especially in subjects with very low pre-immunization levels.

Patients were followed prospectively for evolution of illness, surgery performed and outcome. *Major sepsis* was defined as the occurrence of a positive blood culture, cholangitis, or intracavitary abscess proved at surgery or autopsy, or confirmed by percutaneous aspiration using radiologic guidance. *Minor sepsis* was defined as wound infection with purulence, pneumonia, urinary tract infection, cellulitis or other non-major sepsis. *Outcome* was considered as alive or dead at time of discharge from hospital. *Death* was considered septic-related if sepsis was the stated cause or a contributing factor on the autopsy report or death certificate.

Clinical laboratory data, including arterial blood gas values, hemoglobin concentration, differential leukocyte counts, total serum proteins, serum albumin, beta and gamma globulin levels, IgG, IgM and IgA levels were obtained by routine hospital clinical laboratory procedures. Groups of healthy laboratory controls were simultaneously studied with patient groups for all experiments.

Studies were approved by the Ethics Review Committee of the Royal Victoria Hospital, and informed consent obtained.

1.2 CLASSIFICATION

Study subjects were classified on the basis of DTH skin testing, degree of surgery performed, APACHE II scores, and for trauma patients, by a modified injury severity score (MISS).

1.2.1 DELAYED TYPE HYPERSENSITIVITY SKIN TESTING

Skin tests were performed with antigens that individuals in the study geographic area were most likely sensitized to [14]. The antigens were Candida (1:100), mumps antigen (undiluted), purified protein derivative (5 TU), Trichophyton (100 PNU) and Varidase (100 U). A volume of 0.1 ml of each antigen was injected intradermally on the arm or forearm. The resulting diameter of induration was measured at 24 and 48 hours after injection. The average of the sum of the two greatest diameters of each antigen in mm was recorded. The skin test score (STS) was defined as the sum of these individual antigen reactions. For some analyses, a positive response to each antigen was defined as induration equal to or greater than 5 mm at either 24 or 48 hours after injection. Patients responding to two or more antigens were classified as reactive (R) and those responding to no antigens as anergic (A). Subjects responding to a single antigen were considered relatively anergic (RA), and although occuring sometimes in followup data, such subjects were not initially selected for any study. No skin tests were performed for the first 24 hours after operation, to avoid the effect of residual anesthetic agents. Trauma subjects were skin tested as soon as possible after admission, usually 24 to 48 hours.

Patients in each DTH class were subclassified. A walk-in (W) patient was defined as a subject admitted for elective surgery that could be delayed up to 3 weeks without adverse effect, and with no evidence of active infection on admission history and physical examination [12]. Subjects studied while in the Surgical Intensive Care Unit (all A) were designated I, and all other patients as hospitalized (H). The following subclasses were thus formed, WR, HR, WA, HA and IA. There were no IR patients.

1.2.2 DEGREE OF SURGERY

An arbitrary classification system was modified [351]. Four classes were defined, with progressive increase in severity from class 1 to 4. The operations grouped in each class are listed in appendix 1.

1.2.3 ACUTE PHYSIOLOGY AND CHRONIC HEALTH EVALUATION

To classify patients according to degree of derangement of physiological status, the Acute Physiology and Chronic Health Evaluation (APACHE II) score was calculated according to Knaus [345]. Points, from 0 to 4, are assigned on the basis of deviation from normal for each variable. The worst value in a given 24 hour period was used to calculate the score for that day. Additional points are given for advancing age and chronic illness. The sum is the APACHE II score. The range of possible scores is from 0 (no illness) to 71 (severe illness).

1.2.4 MODIFIED INJURY SEVERITY SCORE

Trauma patients were stratified on the basis of injury severity according to a MISS. Individual injuries were grouped into five anatomic regions; Neurologic, Neck and Face, Chest, Abdomen, and Pelvis & Extremities. Injuries in each region were assigned a severity score from 0 (no injury) to 5 (severe, life threatening, survival uncertain) based on clinical estimate of degree of injury and associated mortality. Observed injuries in study patients were assigned severity scores according to tables modified by the author (appendix 3) from Baker [348,349] and Mayer [350,352] and based on the Abbreviated Injury Scale of the American Medical Association Committee on Medical Aspects of Automotive Safety [353,354]. The MISS was calculated as the sum of the squares of the three highest of the greatest scores for each of the five anatomic regions.

1.3 VACCINES USED IN VIVO

Multiple lots of aluminum phosphate adsorbed TT (USP) were used (Lederle, Montreal, PQ and Institut Armand Frappier, Laval, PQ). Each dose of 0.5 ml contained 5 Limes flocculation units (Lf) of TT and 1.5 mg aluminum phosphate. Subjects were immunized

intramuscularly in the deltoid region. When human hyperimmune globulin was given to trauma patients, it was administered in the other arm.

For studies of anti-PPS response, Pneumovax vaccine was used (Merck, Sharp and Dohme, Kirkland, PQ). The 23 valent preparation contained 25 mcg each of 23 serotypes in each 0.5 ml dose (US nomenclature 1, 2, 3, 4, 5, 8, 9, 12, 14, 17, 19, 20, 22, 23, 26, 34, 43, 51, 54, 56, 57, 68, 70). Subjects were immunized intramuscularly in the deltoid region. No subject had been previously vaccinated with Pneumovax.

2. BLOOD MONONUCLEAR CELL METHODS

Cell culture methods were developed that provided optimal *in vitro* conditions for normal peripheral blood MNCs to produce Ig with and without stimulation by pokeweed mitogen. The following descriptions are of the methods used routinely. Whenever modifications were used, they are described as employed.

2.1 BLOOD MONONUCLEAR CELL ISOLATION AND CULTURE

Culture conditions are critical for satisfactory *in vitro* Ig synthesis by human MNCs [355]. To avoid the known mitogenic effect of fetal bovine serum (FBS) for human MNCs [356], a culture system was developed using defined media supplements of insulin, transferrin and selenium [357] (ITS), with minimal [FBS]. Following experimentation with blood taking methods, cell isolation procedures, cell concentration, culture medium composition, lots of FBS, culture plate type and manufacture, and kinetic studies, the following methods and conditions were found to be optimal for *in vitro* studies of antibody and Ig synthesis.

Peripheral blood was obtained in syringes (Becton Dickinson, Rutherford, NJ) containing 10 units preservative free heparin/ml [358] (Sigma, St Louis, MO, H-3125, porcine). Blood was immediately diluted with Hank's balanced salt solution (HBSS) with 0.01 M HEPES buffer and 1 unit heparin/ml (HBSS+H), layered on 4° C ficoll-sodium metrizoate (Ficoll-Paque, Pharmacia, Montreal, PQ, specific gravity = 1.070) and centrifuged at 400g for 30 min [359]. Interface MNCs, including most of the Ficoll-sodium metrizoate solution, were harvested and washed three times with HBSS+H. After incubating in heparin-free HBSS at 37° C in

5% CO_2 for one hour to remove cytophilic Ig [360], cells were washed twice more in HBSS. All media and cell preparations were maintained at 37° C continuously.

Differential counting of > 100 cells was performed in Turk's solution in a hemocytometer. On the basis of cell size and nuclear morphology, cells were counted as lymphocytes, monocytes or PMNs. Cell viability by trypan blue exclusion exceeded 95%. For each cell preparation, the calculated monocyte:lymphocyte ratio (M:L), and the degree of PMN contamination as a percentage of the number of lymphocytes, was recorded. For culture, MNC were suspended at $2*10^6$ lymphocytes/ml in the following medium: Roswell Park Memorial Institute 1640 supplemented with 2% FBS (FLOW laboratories, McLean, VA, lot 29101599), 0.01 M HEPES, 50 IU penicillin/ml, 50 mcg streptomycin/ml, (GIBCO, Grand Island, NY), 10 mM glutamine (Flow Laboratories, McLean, VA, 15-8-1-16), 5 mcg/ml bovine insulin, 5 mcg/ml iron saturated human transferrin and 5 ng/ml selenium (ITS Medium Supplement, Collaborative Research, Boston, MA). A single lot of FBS was used for all experiments. All solutions and media were sterilized with 0.22 micron filtration.

For routine MNC culture, triplicate wells of $0.4*10^6$ lymphocytes/well were incubated in round-bottom polystyrene microtiter plates (Linbro, Flow Laboratories, McLean, VA) at 5% $C0_2$ in a humidified 37° C incubator. Monocytes were not removed. Cultures were either spontaneous, i.e. not stimulated by polyclonal B cell activators, or stimulated with drugs or pokeweed mitogen. The final volume of all culture wells was 220 mcl. Wells with cells from control subjects were always included on each plate. After 10 days, supernatants were harvested into polyvinyl chloride (PVC) microtiter plates and stored at -20° C until assayed.

2.2 LECTINS AND DRUGS

Pokeweed mitogen, a lectin extract of *Phytolacca americana*, was used as a T cell dependent human B cell mitogen. Tested lots (GIBCO 670-5360, Grand Island, NY) were used in cultures at an optimal final concentration of 1:200. The lectin was dissolved in culture medium, filter sterilized, and added at the initiation of culture.

Hydrocortisone Na-succinate (Upjohn, lot K979) was used in variable final concentrations in *in vitro* cultures. The drug was dissolved in culture medium, filter sterilized, and added at the initiation of culture.

Mitomycin C (Sigma, St. Louis, MO) was used to treat isolated T cells for *in vitro* experiments. Cells were incubated in fresh mitomycin C at 50 mcg/ml HBSS at 37° C for 30 min, followed by triplicate washing.

Cycloheximide [361] and emetine [362] (Sigma, St. Louis, MO), protein synthesis inhibitors, were added at culture initiation in final concentrations of 10^{-5} and 10^{-4}M respectively.

As a general preservative, and to prevent sIg capping in some cell preparations [363], 0.025% Na azide was used (Sigma, St. Louis, MO).

2.3 B CELL METHODS

Surface Ig positive (sIg^+) cells in the blood were enumerated by two methods: binding to anti-human Ig coated polyacrylamide beads, and counting in the fluorescence activated cell sorter (FACS) after staining with anti-Ig.

For the polyacrylamide bead method, blood MNCs were harvested as above. After incubation to remove cytophilic Ig, cells were washed twice, suspended in standard culture medium with FBS added to final concentration of 30%. To identify phagocytic cells, the preparation was incubated with 1 micron polystyrene latex (L) particles (Dow Diagnostics, Shelburne, ON) at a ratio of approximately 50 beads:cell for 30 min at 37° C. After washing, cells were suspended in HBSS with 0.1% sodium azide at 4° C to prevent capping. An aliquot of anti-human Ig (heavy and light chains) coated polyacrylamide beads (Immunobeads, BioRad Laboratories, Richmond, CA) was added to give a ratio of 100 beads:cell. The suspension was centrifuged at 150 g for 3 min and incubated at 4° C for 10 min. The cells were resuspended, and fluorescein diacetate and toluidine blue added. Only viable MNCs were counted under light and fluorescent microscopy. On the basis of the ingestion of latex particles and the presence of 3 or more surface adherent polyacrylamide beads, cells were scored as macrophages (L^+), or sIg^+ or sIg^- lymphocytes. More than 200 lymphocytes were counted in each specimen. The surface of macrophages frequently showed some polyacrylamide bead binding, secondary to surface complexed Ig [364]. Mature PMNs were frequently non-viable, and could be distinguished on the basis of nuclear morphology. Band PMNs sometimes showed intense fluorescence staining, and could also be distinguished on the basis of nuclear morphology.

2.4 T CELL METHODS

T cell enumeration and separation were performed using sheep red blood cells (E), and subclass analysis with FACS staining.

To count and separate sheep red blood cell receptor positive cells (E^+), E were prepared with 2-aminoethylisothiouronium bromide [365] (AET, Sigma, St. Louis. MO). Fresh E were obtained in Alsever's solution from the same sheep for all experiments. After washing 4 times with normal saline (NS), the cells were suspended in 4 volumes of fresh 0.22 micron filtered 0.14M AET solution at pH 9. The cells were incubated at 37° C for 15 min with intermittent agitation, and washed with 4° C NS til clearing of the supernatant. Preparations were used within 5 days, and re-washed immediately before use.

To count E^+ cells, $1*10^6$ lymphocytes, isolated as described above, were incubated in siliconized glass tubes with 30% FBS and latex particles, as above. After washing 3 times with HBSS, the cells were suspended in 0.2 ml HBSS with 100 mcl FBS previously adsorbed with E and human type AB erythrocytes. One hundred mcl of AET-treated E at $300*10^6$/ml were added, the tubes incubated at 37° C for 15 min, centrifuged at 400g for 3 min, and incubated overnight at 4° C. The cells were gently resuspended, and after the addition of fluorescein diacetate to identify viable cells, >200 lymphocytes were counted. Cells were scored E^+ if there were 3 or more adherent E [366]. Results are shown as the % of positive lymphocytes.

To separate E^+ and E^- MNC, cell preparations were suspended in HBSS after serum-free incubation to remove cytophilic Ig. Samples were taken for differential counting and determination of percentage sIg^+, E^+ and L^+ cells. An equivalent volume of 4% AET-E in NS was added, incubated at 37° C for 15 min, and centrifuged at 200g for 10 min. After placing on ice for 60 min, the cells were gently resuspended and layered on 4° C ficoll-hypaque. After centrifugation at 400g for 30 min, interface and pellet cells were harvested separately, and red cells removed with hypotonic lysis. E^+ cells were treated with 50 mcg/ml mitomycin C for 30 min at 37° C, followed by triplicate washing. The E^- fraction was re-rosetted. The second E^+ fraction was discarded, and the E^- fraction used. All preparations were washed, samples were taken for differential counting and determination of E^+, sIg^+ and L^+ percentages, and cells suspended in culture medium.

2.5 MONOCYTE METHODS

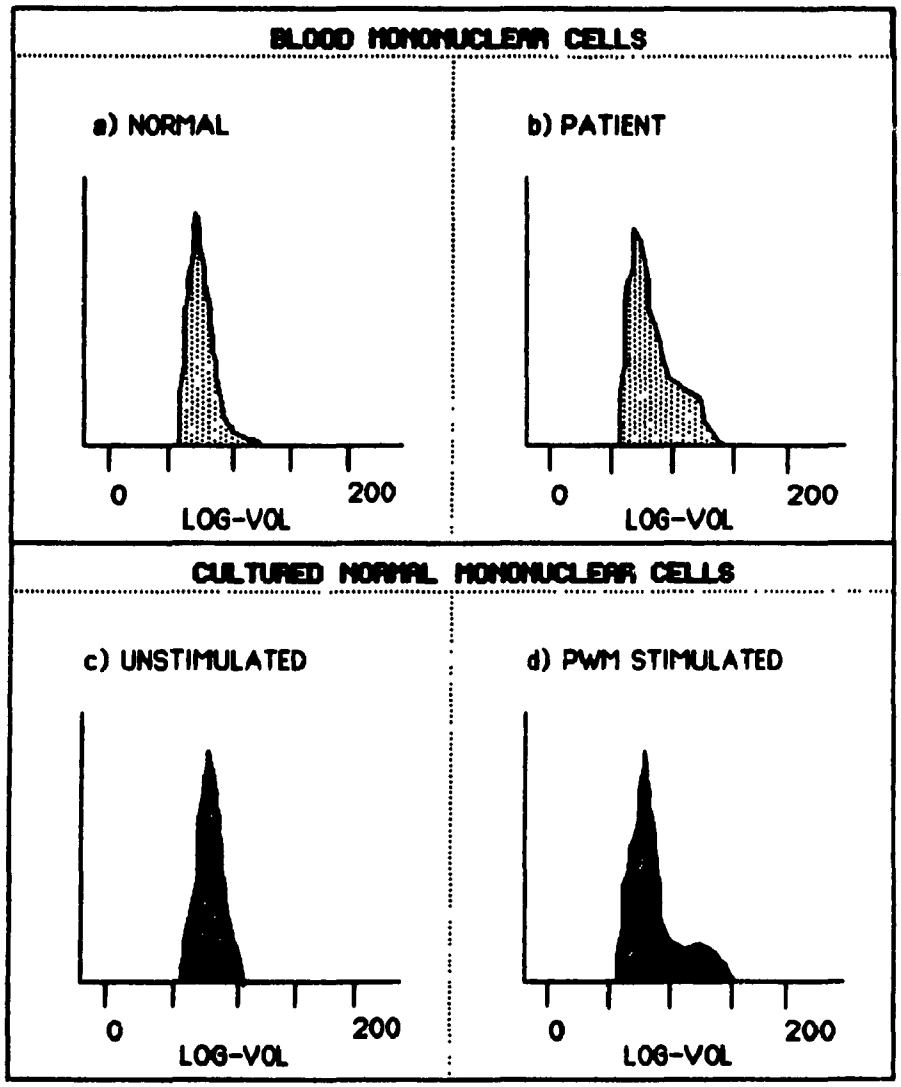
Monocytes were isolated with an adherence technique [367]. Flat polystyrene tissue culture flasks were pre-coated with 100% FBS for 2 hrs. After routine isolation procedures including serum free incubation to remove cytophilic Ig, whole MNC suspensions were incubated in the flasks for 1 hr at 37° C in 5% CO2 with 30% FBS. Non-adherent cells were decanted, and the flask rinsed with culture medium. Adherent cells were removed by the addition of 1 mM ethyl-diamino-tetra-acetic acid for 10 min with agitation. Cells were washed, and samples taken for determination of viability, morphology and L^+ percentage.

2.6 FLUORESCENCE ACTIVATED CELL SORTER ANALYSIS

Blood MNC were analysed for percentage of cells positive for several surface antigens. Cells were harvested according to the routine methods described above, including serum free incubation to remove cytophilic Ig. In standard culture medium, $1*10^6$ lymphocytes were placed in polystyrene microtubes. The tubes were filled to 1 ml with HBSS, centrifuged and the supernatant withdrawn, leaving 50 mcl. After resuspension, 50 mcl of human AB serum and an aliquot of stain were added, and the tubes incubated for 45 minutes on ice in the dark. The cells were washed three times with 1 ml HBSS with 0.025% sodium azide, suspended in 50 mcl, and fixed with 0.3 ml filtered 1% paraformaldehyde in PBS. After thorough resuspension and incubation at room temperature for 10 minutes, the cells were transferred to polystyrene tubes for storage at 4° C in the dark til assay. All stains were conjugated to fluorescein isothiocyanate (FITC), and a 5 mcl volume used. The stains used (Becton Dickinson, Mountain View CA), and the markers identified were: Leu-4 (T cell antigen [368]), Leu-2a (suppressor/cytotoxic T cell antigen [369]), Leu-3a (helper/inducer T cell antigen [370]), Leu-7 (natural killer T cell antigen [371]), Leu-M3 (monocyte/macrophage antigen [372]), and HLA-DR (B cells, monocytes, activated T cell antigen [373]). Affinity purified anti-human Ig was also used to identify sIg^+ cells, as described above. Control tubes were stained with FITC-Avidin (Becton Dickinson, Mountain View CA), using 25 mcl of 1:12.5 dilution in HBSS.

Cells were analyzed within a week using a Becton Dickinson FACS III Analyzer. Ten thousand cells were counted. The left volume gate was placed according to the log-vol plot at the left edge of the cell distribution, from 55 to 75. The right gate was left wide at 255, because of the frequent presence of large MNCs in patient preparations, seen as a long right

FIGURE 1: LOG-VOL PLOT OF FACS ANALYSIS OF FRESH, AND *IN VITRO* CULTURED, BLOOD MONONUCLEAR CELLS



The log-vol plots from the FACS III Analyzer are shown for representative fresh cell samples from a LC (a) and DTH A patient (b). As well, the plots of unstimulated (c) and PWM stimulated (d) cells after 5 days of *in vitro* culture are shown.

edge on the FACS log-vol plot (figure 1). The left fluorescence gate was 1, and the right selected on the avidin stained preparations to exclude >98% of cells. The right gate ranged from 84 to 101, with most preparations run at approximately 96.

3. SOLID PHASE RADIOIMMUNOASSAYS

The following descriptions are of the methods used routinely. Whenever modifications were used, they are described as employed.

Solid phase radioimmunassays (SPRIA) were developed for measurement of specific anti-TT and anti-PPS antibody in serum and in MNC culture supernatants. The assays were based on the the original methods of Zollinger [374,375] and Stevens and Saxon [376]. They utilize the general principle of non-specific adsorption of protein and polysaccharide molecules to plastic surfaces [377]. Experimentation with different materials, reagents and conditions showed the methods used to be optimal. The calculations used in analysis are based on the principles of direct and competitive solid phase RIAs [377].

Radiolabelled, isotype specific, affinity purified anti-human Ig was used in all assays. Goat anti-human IgG heavy chains (Zymed Lab, Burlingame, CA), IgM heavy chains (Cappel Laboratories, Cochranville, PA) and IgA heavy chains (Hyclone Sterile Systems, Logan, UT) were used. Antibody was radiolabelled with ^{125}I using a modification of Markwell and Fox [378]. The inside bottom of washed 5 ml borosilicate glass tubes was coated evenly with 10 micrograms of 1,3,4,6-tetrachloro-3a,6a-diphenylglycoluril [379] (IIa, Iodogen, Pierce Chemical Co., Rockwell IL, no.28600) by evaporation of the reagent in methylene chloride. Two hundred micrograms of antibody protein in 100 mcl of PBS-AZ at ph 7.4 (from 75 to 95% active antibody by manufacturer's immunoprecipation analysis) were reacted with 1 mCi of carrier free $Na^{125}I$, 10 mcl borate buffer at ph 8.4, and 10 mcl of 0.764 mcg/ml KI in borate buffered saline at ph 8.4 (final molar ratio of ^{125}I:^{127}I = 10:1) in a IIa coated tube at 4° C for 12 minutes under constant rotation. Unbound ^{125}I was removed by passage through a 10 ml Sephadex G25M column (Pharmacia, Montreal, PQ) equilibrated with 0.1% bovine serum albumin (BSA, RIA grade, Sigma Chemical Co., St. Louis, MO) in PBS-AZ, and prepared with 50 mcl of 0.2 M KI immediately before sample application. Specific activities of 1000 to 8000 counts per minute (CPM)/ng protein were obtained with less than 5% unbound ^{125}I (assuming 90% recovery of protein).

TABLE 1: ISOTYPE SPECIFICITY OF RADIOIMMUNOASSAY ANTIBODIES

	ANTIBODY SPECIFICITY		
ANTIGEN	**IgG**	**IgM**	**IgA**
IgG	**65**	1	3
IgM	2	**64**	3
IgA	1	1	**47**
BSA	1	1	1

Microtiter PVC wells were coated with purified human isotype-specific Ig, or BSA. Aliquots of radiolabelled affinity purified anti-human isotype-specific Ig were then incubated. The % radioactivity remaining bound after washing is shown. Cross reactive (to other isotypes) and non-specific (to BSA) binding was minimal for each radiolabelled antibody lot used.

Heavy chain specificity of the radiolabelled antibody was checked at least twice for each antibody lot by incubating aliquots in PVC microtiter plates previously coated with chromatographically purified human IgG, IgM, IgA (Cappel Laboratories, Cochranville, PA) or BSA (RIA grade, Sigma Chemical Co., St. Louis, MO). Binding of radiolabelled antibody of each isotype specific label to the corresponding solid phase immunoglobulin exceeded 40%, while cross-reactive or non-specific binding to other isotypes or BSA was less than 3% (table 1). Satisfactory purity of the immunoglobulin preparations was shown in control testing for assay of total immunoglobulin in culture supernatants. Lots of either Ig or anti-Ig with binding outside these specifications to other Ig isotypes, or to BSA, were not used.

3.1 ANTI-TETANUS TOXOID ANTIBODY ASSAY

Assay of serum for IgG anti-TT antibody content was performed as follows: 25 microliter aliquots of soluble TT (stock 2000 Lf/ml, 0.9 mg protein/ml, Connaught Laboratories, Toronto, ON) at 10 mcg/ml in PBS-AZ were incubated overnight in the wells of washed round bottom PVC plates (Dynatech, Alexandria, VA). After washing four times with PBS-AZ, 75 microliters of 1% BSA in PBS-AZ were incubated 2 hours. After washing four times, 25 microliter aliquots of serial log dilutions of serum samples in 0.1% BSA in PBS-AZ were placed in triplicate wells. An 18 hour incubation was followed by six washes. In a 30 mcl volume, 120 ng of radiolabelled affinity purified anti-human IgG were then placed in each well and incubated 18 hours. The wells were finally washed six times and individually counted in a Beckmann gamma-counter for remaining bound radioactivity. All incubations were at room temperature and all washes with PBS-AZ. The TT was routinely recovered, restored to original concentration ($OD_{280\ nm}$ = 0.164), and re-used.

The quantities of solid phase TT and radiolabel were shown to be in excess for the measured amounts of anti-TT antibody (figure 2). More than 90 ng of radiolabel/well at any [TT] produced a linear relationship between logarithm of antibody dilution and bound CPM, indicating an excess of both antigen and radiolabel. The use of greater quantities of radiolabel did not increase the resolution of antibody amounts, and less was inadequate.

A standard curve was produced for each experiment using a control post-immunization serum shown to contain 4 International Units (U) of IgG anti-TT/ml, hereafter referred to as units (U). This was determined by comparison assays with titered human Tetanus Immune Globulin (Connaught Laboratories, Toronto, ON, lot 15039-1, 164 mg IgG/ml by

radial immunodiffusion, 280 U/ml of tetanus anti-toxin by guinea pig neutralization test). This was known to contain only IgG by radial immunodiffusion and gel electrophoresis. The r^2 value for the linear portion of the curve, from log dilution 2 to 5, always exceeded 0.975 (table 2). In this area of the curve, there was an excess of solid phase antigen and radiolabelled antibody relative to the amount of anti-TT in the serum sample. Dilutions to the right of the linear portion have undetectable amounts of anti-TT present; those to the left have insufficient amounts of either solid phase antigen, radiolabelled antibody, or both. Serum and solid phase TT negative controls were included in each assay. Background binding produced a level of detectability of 0.001 U/ml.

Antigen specificity was demonstrated using soluble antigen studies. The addition of logarithmically graded amounts of soluble TT immediately after placing diluted serum in the assay wells produced a linear reduction in radiolabel binding (figure 3). Although some tapering occurs, binding could be reduced approximately to the level of serum negative control wells. The addition of BSA or keyhole limpet hemocyanin had no effect on binding.

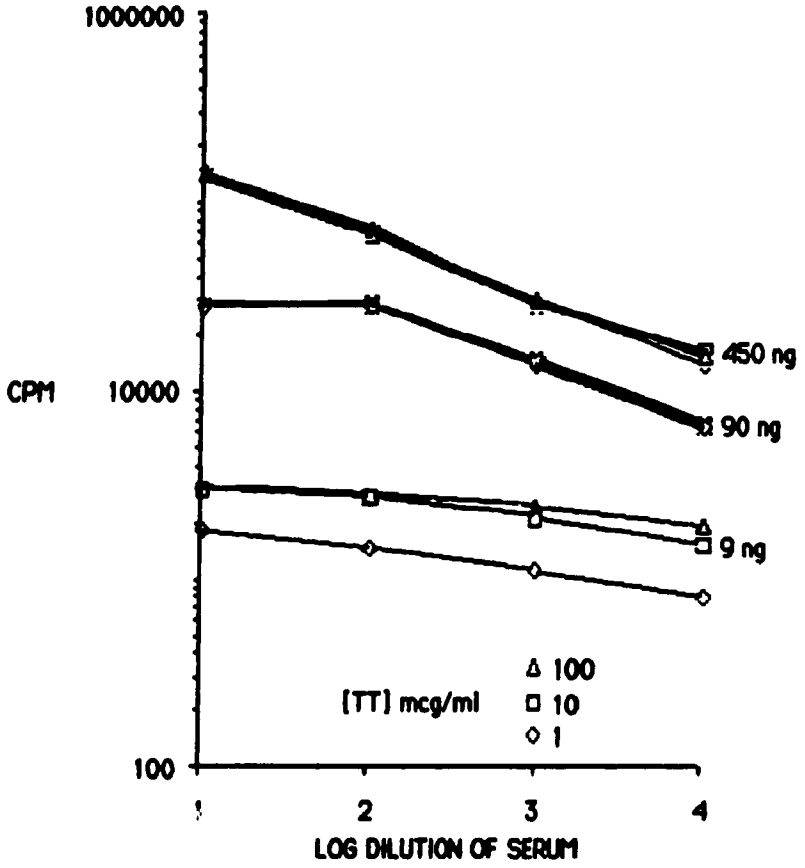
Reproducibility of the assay was determined by performing multiple standard curves on the same and different days, with concomitant repeated measurement of experimental samples (table 2).

For experimental sera, pre- and post-immunization samples from the same study subject were assayed simultaneously. Unknown sera were assayed at log dilutions from 1 to 4. Ten mcl of serum was required for antibody determination. Bound radioactivity in triplicate experimental wells was converted to U anti-TT/ml serum via a linear regression formula of log-log transformed data from the standard serum, using the computer program developed for this purpose (appendix 4). The arithmetic mean of triplicate assay wells at the experimental serum dilution nearest the midpoint of the linear regression line was taken as the antibody content.

The use of serum or plasma, either frozen and thawed or stored with 0.025% sodium azide at 4° C, had no effect on measured antibody content.

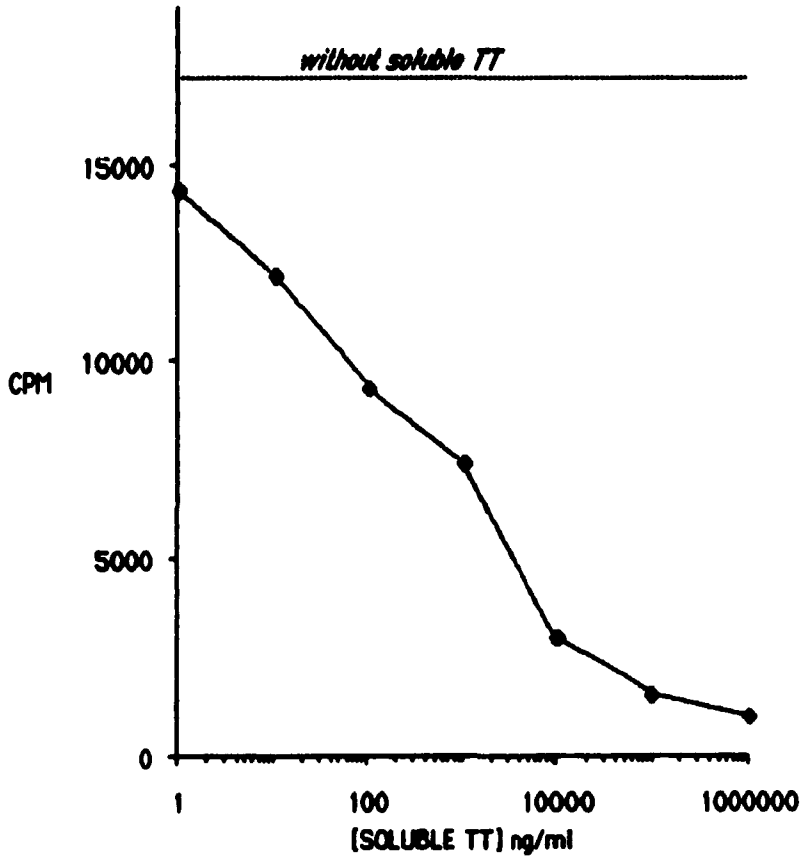
Experiments were performed to determine if there were detectable quantities of serum IgM anti-TT before or after immunization. Assays using radiolabelled anti-human IgM, and either plain diluted sera as above, or sera adsorbed with Staphylococcus aureus Cowan I to remove IgG, showed no significant binding above background.

FIGURE 2: ANTIGEN AND RADIOLABEL EXCESS IN ANTI-TETANUS TOXOID SOLID PHASE RADIOIMMUNOASSAY



The wells of microtiter plates were coated with soluble TT at 1, 10 or 100 mcg/ml PBS-AZ overnight. After secondary coating with BSA, and an 18 hour incubation of aliquots of diluted serum containing anti-TT (log dilutions 1 to 4), either 9, 90 or 450 ng of radiolabelled anti-human IgG were finally incubated, as indicated. The counts remaining bound to the solid phase after washing are shown.

FIGURE 3: SOLUBLE ANTIGEN INHIBITION OF SOLID PHASE RADIOIMMUNOASSAY FOR ANTI-TETANUS TOXOID



Microtiter plates were coated with soluble TT at 10 mcg/ml overnight. After secondary coating with BSA, 50 mcl of 1:1000 anti-TT serum were placed in the wells, followed immediately by 50 mcl of soluble TT in concentrations ranging from 0 to 1 mg/ml. After standard incubation and washing, an excess of radiolabelled anti-human IgG was incubated. The counts remaining bound to the wells after washing are shown. There is a dose response reduction in binding with soluble TT approximately to the level of serum negative control wells (CPM = 450). The addition of similar quantities of BSA had no effect.

TABLE 2: REPRODUCIBILITY OF ANTI-TETANUS TOXOID ANTIBODY SOLID PHASE RADIOIMMUNOASSAY

	STANDARD CURVE FORMULA				SERUM SAMPLE units anti-TT/ml serum			
EXPT	m	X	Y	r^2	A	B	C	D
1	1.54	9.7	2.07	.996	1.22	1.53	0.33	3.52
2	1.67	10.3	1.50	.997	1.29	1.54	0.47	3.63
3	1.66	10.3	1.65	.998	1.16	1.38	0.33	3.33
4	1.57	9.7	1.77	.975	1.38	1.70	0.38	3.97
5	1.60	9.9	1.63	.997	1.26	1.65	0.56	3.75
6	1.58	9.9	1.68	.989	1.27	1.92	0.37	4.11

Triplicate standard curves and assays of experimental samples A-D were performed on two separate days (experiment 1-3, and 4-6). For dilutions of the arbitrarily defined standard serum, linear regression was performed by least squares method with a computer program (appendix 4). Correlation coefficients were all greater than 0.97, and there was good agreement on the line formulas used to convert experimental CPM to units of anti-TT/ml serum. Variability in experimental sample values thus calculated was small.

Radioimmunoassay of culture supernatants for determination of specific anti-TT IgG was performed similarly to assay of serum except that undiluted culture supernatants were incubated in the wells in place of diluted serum. Bound radioactivity was quantified as $U*10^{-6}$ (mcU)/culture. The level of detectability was 0.01 mcU/culture. Geometric means of duplicate assay wells of triplicate culture supernatants were used. The same culture supernatants were assayed for content of anti-TT specific and total IgG.

3.2 DEFINITION OF IN VIVO ANTIBODY RESPONSE TO TETANUS TOXOID

Since there was variation in pre-immunization antibody levels, antibody responses were expressed as either differences or ratios [380]. The *in vivo* antibody response to TT was calculated as either the difference or the ratio between anti-TT content in day 14 post-, and pre-immunization sera. For contingency table analysis, a positive response was defined either as > = 0.1 new U IgG anti-TT/ml (day 14 post-immunization minus pre-immunization content), or as > = 10 fold increase in antibody (day 14 post-immunization divided by pre-immunization content).

Although there was usually a correlation in groups between the two methods of expressing the magnitude of an antibody response, each has a different implication. An antibody response is a biological amplification system, and as such may be expected to show a logarithmic pattern. This is best expressed as a ratio. However, a subject who has pre- and post-immunization antibody levels of 0.01 and 0.1 has produced the same ratio response as another with levels of 1 and 10, but only 1% of the amount of new antibody calculated as a difference. *In vivo* responses were therefore examined in both ways.

3.3 ANTI-PNEUMOCOCCAL POLYSACCHARIDE ANTIBODY ASSAY

A SPRIA for isotype specific anti-PPS antibody was adapted from that previously described for TT. Affinity purified anti-human IgG, IgM and IgA were used to assay each isotype separately. Briefly, 25 mcl aliquots of 1:10 dilution of Pneumovax in PBS-AZ at pH 7.4 were incubated overnight in the wells of microtiter plates. The antigen was recovered and re-used. Sequential incubations were then performed with 1% BSA, serial log dilutions of serum, and aliquots of radiolabelled anti-human IgG, IgM or IgA. Similar to the procedures for TT assay described above, the quantities of solid phase PPS and radio-labelled antibody

were in excess for the serum dilutions used. Antigen specificity was shown for each isotype using soluble antigen inhibition studies comparable to those for TT.

A standard curve was produced for each isotype using a post-immunization serum from an LC subject arbitrarily defined to contain 4 U of anti-PPS of each isotype/ml. Bound radioactivity in experimental wells was converted to U of isotype specific anti-PPS/ml serum via a linear regression formula of log-log transformed data from the standard serum using the computer program listed in appendix 4. Serum and PPS negative controls were included in each assay. Background binding produced a level of detectability of 0.01 U/ml. The arithmetic mean of duplicate assay wells at the experimental serum dilution nearest the midpoint of the linear regression line was taken as the antibody content for each isotype.

Serum samples from each donor were assayed at the same time. Thirty mcl of serum were needed for antibody determination, and unknown serum samples were assayed at log dilutions 1 to 4.

Radioimmunoassay of culture supernatants for IgG, IgM and IgA anti-PPS was performed similarly to assay of serum except that undiluted culture supernatants were incubated in the wells in place of diluted serum. Bound radioactivity was quantified as $U*10^{-3}$ (mU)/culture. The level of detectability was 0.002 mU/culture. Geometric means of duplicate assay wells of triplicate culture supernatants are shown. The same culture supernatants were assayed for content of specific anti-PPS and total Ig of each isotype.

3.4 DEFINITION OF IN VIVO ANTIBODY RESPONSE TO PNEUMOCOCCAL POLYSACCHARIDE

The *in vivo* serum antibody response was calculated for each isotype as the peak of anti-PPS/ml serum achieved between days 0 and 28 after immunization minus U anti-PPS/ml at day 0. For contingency table analysis, a positive response was defined as > = 2 fold increase (post-immunization divided by pre-immunization) in antibody between day 0 and 28 after vaccination.

3.5 CULTURE SUPERNATANT TOTAL IMMUNOGLOBULIN ASSAY

A solid phase competitive immunoradioassay (SPCIRA) was used to quantitate total IgG, IgM and IgA in culture supernatants. The wells of washed round bottom PVC microtiter plates were coated overnight in humidified chambers with 50 mcl of chromatographically purified human IgG (pooled normal sera), IgM (pooled myeloma) or IgA (pooled normal sera) (Cappel Laboratories, Cochranville, PA; IgM also from Jackson Research Laboratories, Cochranville, PA) at 10 mcg/ml in PBS-AZ [381]. After three washes with PBS-AZ, 75 mcl of 1% BSA in PBS-AZ were placed in the wells and incubated 2 hrs. The wells were washed three times, and 25 microliters of undiluted culture supernatant placed in them. This was followed immediately by 10 microliters of radiolabelled affinity-purified anti-human IgG, IgM or IgA at 2 mcg/ml in 0.1% BSA in PBS-AZ. After a two hour incubation at 37° C, the plates were washed six times with PBS-AZ and remaining bound radioactivity counted.

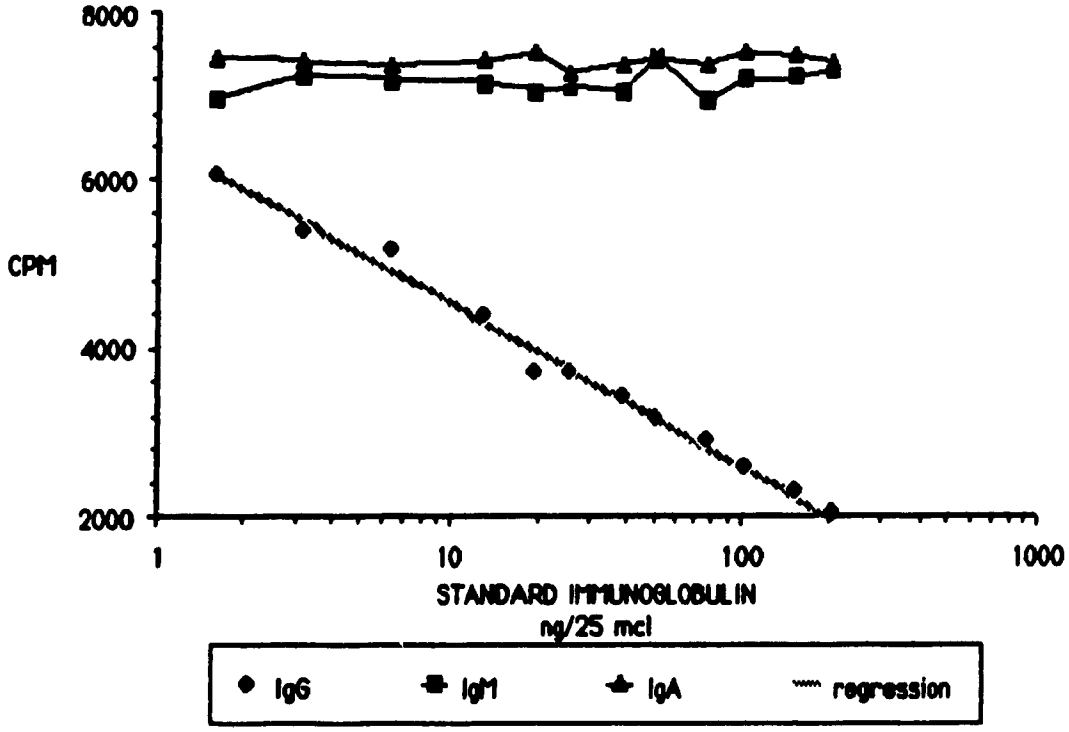
With each assay, a standard curve was prepared for each isotype using known concentrations of human IgG, IgM, and IgA diluted in culture medium. Bound radioactivity versus the log of Ig concentration was linear over the range of standard Ig concentrations used (figure 4). Bound CPM in experimental wells were converted to ng isotype specific Ig/culture via a linear regression formula from the standard curve, using the computer program listed in appendix 4. The level of detectability was 10 ng/culture. Individual supernatants were assayed in duplicate for each isotype. Data from duplicate assay wells and triplicate culture supernatants were averaged arithmetically to obtain the total amount of each isotype in ng/culture. When indicated, the same culture supernatants were assayed for content of total Ig of each isotype and antigen specific antibody, i.e. anti-TT or anti-PPS.

Heavy chain specificity was demonstrated by the failure of either of the two other isotypes to inhibit binding of the radiolabelled anti-IgG, -IgM, or -IgA to the relevant solid phase Ig isotype (figure 4). This also shows effective purity of the Ig preparations.

4. STATISTICAL ANALYSIS AND DATA PRESENTATION

Demographic data, skin test results, clinical laboratory data and analysis of cell surface phenotypes were averaged arithmetically, and the unpaired Student t-test used for group comparisons.

FIGURE 4: STANDARD CURVE AND DEMONSTRATION OF ISOTYPE SPECIFICITY FOR SOLID PHASE COMPETITIVE IMMUNORADIOASSAY OF TOTAL IMMUNOGLOBULIN IN CULTURE SUPERNATANTS



The wells of PVC microtiter plates were coated overnight with chromatographically purified human IgG. After secondary coating with BSA, standard dilutions of IgG in culture medium were placed in the wells. This was followed immediately by radiolabelled affinity-purified anti-human IgG, IgM, or IgA. After incubation and washing, bound CPM versus the log of the concentration of IgG was linear. Only IgG inhibited binding of the radiolabel to the solid phase IgG, demonstrating heavy chain specificity of the IgG and the anti-IgG. The linear regression formula for the IgG standard curve is $x = 10^{(3.345-CPM*0.000519)}$, with $r^2 = 0.99$. Similar experiments were performed to demonstrate purity of the other isotype Ig ant anti-Ig preparations.

For the RIAs, least squares regression was used for the standard curve. For each assay, the use of arithmetic and geometric means in the analysis of the raw data is noted above.

The *in vivo* antibody responses were defined as noted above. Group results were averaged as geometric means, and the data shown as mean x÷ SEM of the logarithms in base 10. For individual negative or zero values, the level of sensitivity for the appropriate assay was substituted in the calculations. Groups were compared using the Wilcoxon rank sum test (WRST), a nonparametric test for independent groups without normal distribution [382]. When definitions of positive or negative responses were used, groups were compared with contingency table analysis, assuming random samples and independent outcome. The X^2_x values are shown, with x degrees of freedom shown as the subscript. Even if expected counts were <5 per cell, Yates correction was used, as recommended by Conover [382]. For comparisons of more than 3 groups, a Kruskal-Wallis test, the nonparametric equivalent of analysis of variance, was done [382]. An overall statistical significance was obtained, and inter-group comparisons performed within the framework of the test.

For group averages of *in vitro* Ig data, arithmetic means $\pm$ SEM and geometric means x÷ SEM were used for total Ig and antigen-specific Ig quantities respectively. Arithmetic data were compared between groups with the unpaired Student t-test, and geometric data with WRST.

To determine the statistical significance of the difference between unstimulated and PWM stimulated synthesis of total Ig for each individual, unpaired Student t-tests were used. Although conceptually paired, the paired t-test produced statistical significance out of order with the apparent biological significance based on the variability in triplicate cultures from a given individual, and variability between individuals. Using the unpaired test produced a satisfactory analysis in that nearly all normal subjects gave significant positive, or normal, responses to PWM stimulation with this approach. Group response patterns were analyzed with contingency table analysis.

Comparisons between quantities of antigen specific Ig produced *in vitro* were done with the Wilcoxon signed rank test (WSRT).

To avoid assumptions regarding normal distribution of data, correlations were performed with the Spearman nonparametric method, and the degree of correlation stated as the r_s value.

In the graphic representation of data, error bars for arithmetic means are shown as $\pm$ SEM. Error bars are usually not shown for geometric means, since they add little meaning to data that may not be normally distributed. If used, $\pm$ the antilog of the (log mean $\pm$ SD of logs) in base 10 was shown.

For all statistical analyses, exact p values were noted whenever possible. A p value <0.05 was generally taken to indicate statistical significance.

RESULTS

1. SPECIFIC ANTIBODY RESPONSES

The humoral immune responses to a protein antigen, TT, and a polysaccharide antigen, PPS, were studied.

1.1 ANTIBODY RESPONSES TO TETANUS TOXOID

To investigate the antibody response to a protein antigen, *in vivo* and *in vitro* studies were done with TT. The day of immunization was day 0. Serum samples were obtained before vaccination and at intervals thereafter, and were assayed for IgG anti-TT antibody content. Peripheral blood was obtained before and after immunization for *in vitro* MNC culture. Culture supernatants were assayed for isotype specific anti-TT antibody and total IgG.

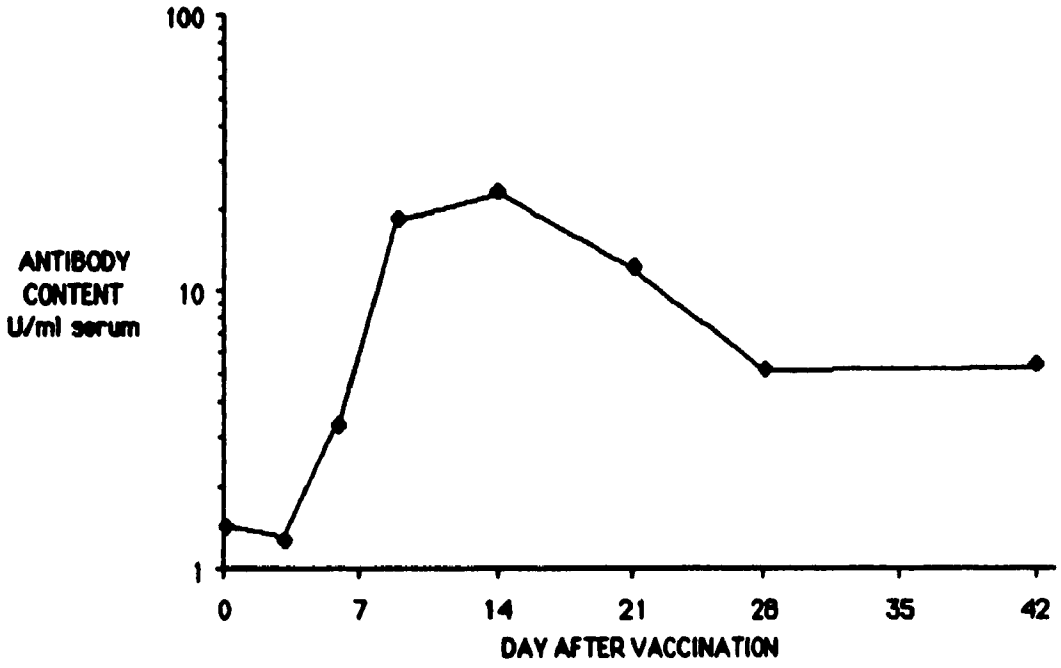
1.1.1 IN VIVO ANTIBODY RESPONSES TO TETANUS TOXOID

The *in vivo* antibody response response to TT immunization was studied in normal subjects and patients.

1.1.1.1 NORMAL IN VIVO ANTIBODY RESPONSES TO TETANUS TOXOID

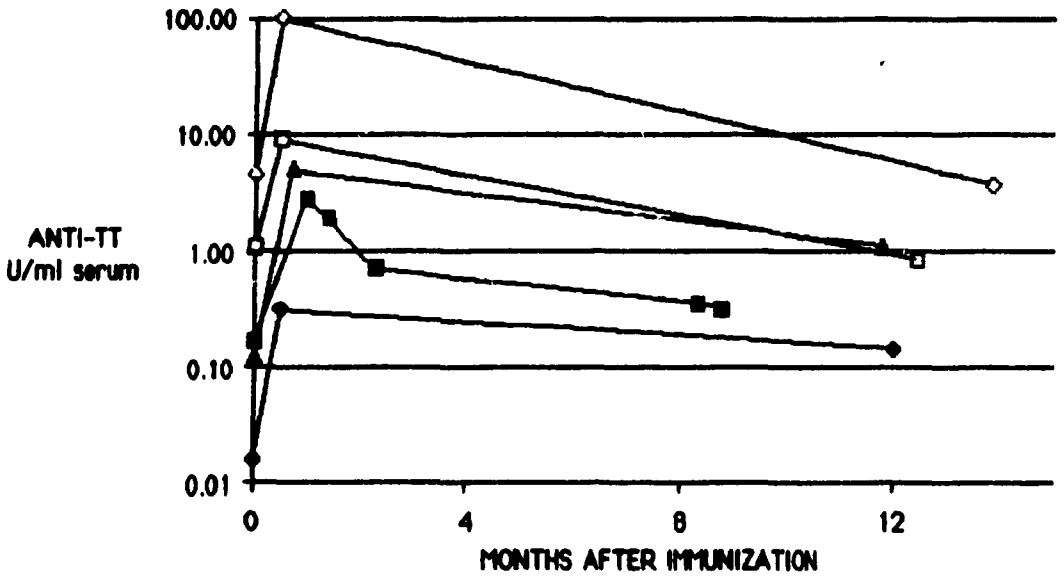
Four LC subjects were immunized with TT. The geometric mean levels of serum antibody at intervals from day 0 to 42 are shown in figure 5. After a lag phase of 3 days, antibody content begins to rise from a pre-immunization mean of 1.4 U/ml serum, reaching a peak at

FIGURE 5: NORMAL *IN VIVO* ANTIBODY RESPONSE TO TETANUS TOXOID



Four healthy LC subjects were immunized on day 0 with TT. The geometric mean antibody content in IU/ml serum at intervals after vaccination is shown.

FIGURE 6: LONG-TERM LEVELS OF SERUM ANTI-TETANUS TOXOID ANTIBODY IN NORMAL SUBJECTS AFTER VACCINATION



IgG anti-TT levels are shown before and at intervals up to 12 months after immunization in 5 LC subjects. A gradual decline is seen, although levels are maintained at greater than pre-immunization values up to a year after vaccination.

day 14. Levels decline therafter to a new plateau at day 28 to 42. For these individuals, the mean quantity of new U/ml serum at day 14 was 17.5. The mean ratio response was a 15.8 fold increase in antibody content. Although the peak serum content was reached at day 14, the maximal synthesis rate occurred between days 3 to 9, indicated by the steepest slope of the response curve (figure 5). During this period, there was an exponential increase in serum antibody levels, as shown by the relatively linear portion of the curve with a logarithmic abscissa.

In long-term follow-up of antibody content of serum many weeks after vaccination of LC subjects, a gradual decline was observed (figure 6). Antibody levels were maintained at higher than pre-immunization values at one year in some subjects but not in all.

1.1.1.2 PATIENT IN VIVO ANTIBODY RESPONSES TO TETANUS TOXOID

After classification by skin testing, subjects were immunized with TT, and the *in vivo* antibody response measured as either the ratio of day 14 to day 0 antibody levels, or as the difference between these two samples. Late serum samples, beyond day 14, were assayed when available.

Eighty-one subjects were studied, including 26 LC, 19 R and 36 A patients.

Demographic data, operation performed and outcome for R and A patients are listed in tables 3 and 4 respectively. Group average demographic and clinical laboratory data obtained on the day of vaccination are shown in table 5. The average age of both patient groups was greater than LC ($p = 0.001$, unpaired t tests). The duration and degree of surgery performed was similar among R and A. Skin test scores were significantly lower in both R and A groups than in LC ($p = 0.001$, unpaired t tests).

The degree of physiologic derangement was greater in all patients ($p = 0.001$ compared to LC). Those with reduced DTH reactivity were more ill than R ($p = 0.005$). The relative contributions of abnormalities in vital signs, gas exchange, biochemical and hematological parameters were all greater in A than in R patients (figure 7).

There were abnormalities in blood hematologic and protein values. The concentration of hemoglobin was lower in all patients ($p = 0.001$) but not different between R and A groups ($p = 0.08$). From LC to R to A, there was an increase in total leukocytes and monocytes,

TABLE 3: DEMOGRAPHIC DATA, DIAGNOSIS, OPERATION AND OUTCOME OF DTH REACTIVE PATIENTS VACCINATED WITH TETANUS TOXOID

CL	AGE	SX	STS	APE	DIAGNOSIS	OPERATION	DAY	DEG	TIME	SEP	OUTCOME
HR	77	M	37	10	Gastric Carcinoma	Gastrectomy	-1	3	170	-	Alive
HR	67	M	19	7	Esohageal Varices	Sclerotherapy	+4	1	95	-	Alive
HR	71	F	21	7	Duodenal Carcinoma	Duodenal Resection	-17	3	200	-	Alive
HR	52	M	33	2	Malunion	Internal Fixation	-240	2	195	-	Alive
HR	69	M	20	4	Ischemic Bowel	Bowel Resection	-9	3	140	-	Alive
HR	61	M	24	11	Cholecystitis	Cholecystectomy	-21	2	100	+	Dead
HR	59	M	37	3	Arterial Occlusion	Amputation	-99	2	50	-	Alive
WR	63	M	40	5	Pancreatic Carcinoma	-	-	-	-	-	Alive
WR	73	F	25	6	Rectal Carcinoma	Anterior resection	+4	3	215	-	Alive
WR	64	F	13	5	Renal Carcinoma	Nephrectomy	+3	4	240	-	Alive
WR	73	M	43	5	Colon Carcinoma	Anterior Resection	+4	3	200	-	Alive
WR	79	M	21	8	Rectal Carcinoma	Anterior Resection	-3	3	215	-	Alive
WR	33	F	45	0	Chronic Fistula	-	-	-	-	-	Alive
WR	75	M	32	10	Gastric Carcinoma	Partial Hepatectomy	+10	4	435	-	Alive
WR	53	F	22	4	Morbid Obesity	Gastroplasty	+8	2	145	-	Alive
WR	45	F	42	4	Gastric Carcinoma	Gastrectomy	+3	3	250	-	Alive
WR	41	F	75	4	Morbid Obesity	Gastroplasty	+3	2	185	-	Alive

Demographic data, STS (obtained just before vaccination), APACHE II scores (APE, calculated on the day of vaccination), diagnosis and operation performed are listed for R subjects. They are grouped according to sub-classification as either hospital (HR) or walk-in (WR) subjects at the time of vaccination. The degree (DEG) and duration (TIME, min) of operations are shown. The timing of surgery in relation to vaccination is shown in number of days the operation occurred after (+) or before (-) vaccination. Outcome was based on condition at hospital discharge, and the presence or absence of major sepsis (SEP) at any time during hospitalization.

TABLE 4: DEMOGRAPHIC DATA, DIAGNOSIS, OPERATION AND OUTCOME OF DTH ANERGIC PATIENTS VACCINATED WITH TETANUS TOXOID

CL	AGE	SX	STS	APE	DIAGNOSIS	OPERATION	DAY	DEG	TIME	SEP	OUTCOME
HA	90	M	0	15	Carcinoid	Bowel Resection	0	3	245	-	Alive
HA	71	F	0	5	Biliary Carcinoma	Biliary Bypass	-20	2	290	+	Alive
HA	77	M	0	6	Diverticulosis	Colectomy	+8	3	230	-	Alive
HA	81	M	3	7	Gastric Carcinoma	Gastrectomy	-2	3	180	-	Alive
HA	72	M	0	7	Rectal Carcinoma	Anterior Resection	+20	3	255	-	Alive
HA	56	M	0	5	Bowel Obstruction	Bowel Resection	-8	3	220	-	Alive
HA	78	F	0	18	Cholecystitis	Cholecystectomy	-2	2	85	-	Alive
HA	68	M	0	5	Abdominal Abscess	Laparotomy	-13	2	115	+	Alive
HA	76	F	0	5	Metastatic Carcinoma	Laparotomy	-75	2	75	-	Alive
HA	79	M	3	9	Metastatic Carcinoma	Lung Biopsy	-22	2	45	-	Dead
HA	19	M	0	0	Osteomyelitis	-	-	-	-		Alive
HA	79	F	0	7	Pancreatic Carcinoma	Pancreatoduodenectomy	-30	4	360	+	Dead
HA	58	F	0	13	Diverticulitis	Hemicolectomy	+2	3	255	+	Dead
HA	84	F	0	14	Choledocholithiasis	Choledochoduodenostomy	+3	3	180	-	Alive
HA	72	F	0	10	Pancreatic Carcinoma	Gastroenterostomy	-11	2	180	-	Dead
IA	51	M	0	9	Cholecystitis	Cholecystectomy	+1	2	125	+	Dead
IA	74	M	0	9	Cholecystitis	Cholecystectomy	-9	2	130	+	Dead
IA	55	F	0	10	Gastric Carcinoma	Esophagogastrectomy	-9	4	275	+	Alive
IA	62	F	0	8	Radiation Colitis	Total Colectomy	-53	4	215	+	Dead
IA	60	M	0	10	Pharyngeal Carcinoma	Radical Neck Dissection	-30	1	75	+	Alive
IA	67	M	0	12	Aortoenteric Fistula	Laparotomy	-7	2	105	+	Alive
IA	52	M	0	11	Mediastinitis	Aortocoronary Bypass	-13	4	560	+	Dead
IA	49	M	3	12	Hepatic Cirrhosis	Portocaval Shunt	-14	4	420	-	Alive
IA	58	F	0	24	Pericarditis	Pericardiectomy	+2	3	110	+	Alive
IA	55	M	3	12	Mediastinitis	Aortocoronary Bypass	-15	4	240	+	Dead
IA	43	M	0	11	Pancreatic Carcinoma	Pancreatoduodenectomy	-20	4	365	+	Dead
IA	62	M	0	15	Mediastinitis	Thoracotomy	-14	3	230	+	Alive
WA	75	F	0	7	Colon Perforation	Colectomy	-15	3	235	-	Alive
WA	51	F	0	3	Colon Carcinoma	Closure Colostomy	+21	3	365	-	Alive
WA	81	F	3	6	Colon Carcinoma	Anterior Resection	+12	3	155	-	Alive
WA	68	F	6	7	Gastric Carcinoma	-	-	-	-	-	Dead
WA	80	M	0	8	Pancreatic Pseudocyst	-	-	-	-	-	Alive
WA	67	M	0	7	Pancreatic Carcinoma	Laparotomy	+9	1	50	-	Dead
WA	69	F	6	6	Reflux Esophagitis	-	-	-	-	-	Alive
WA	54	F	0	4	Lymphoma	Biopsy	-7	1	46	-	Alive
WA	60	F	0	3	Colon Carcinoma	Hartmann's Procedure	+10	3	155	-	Alive

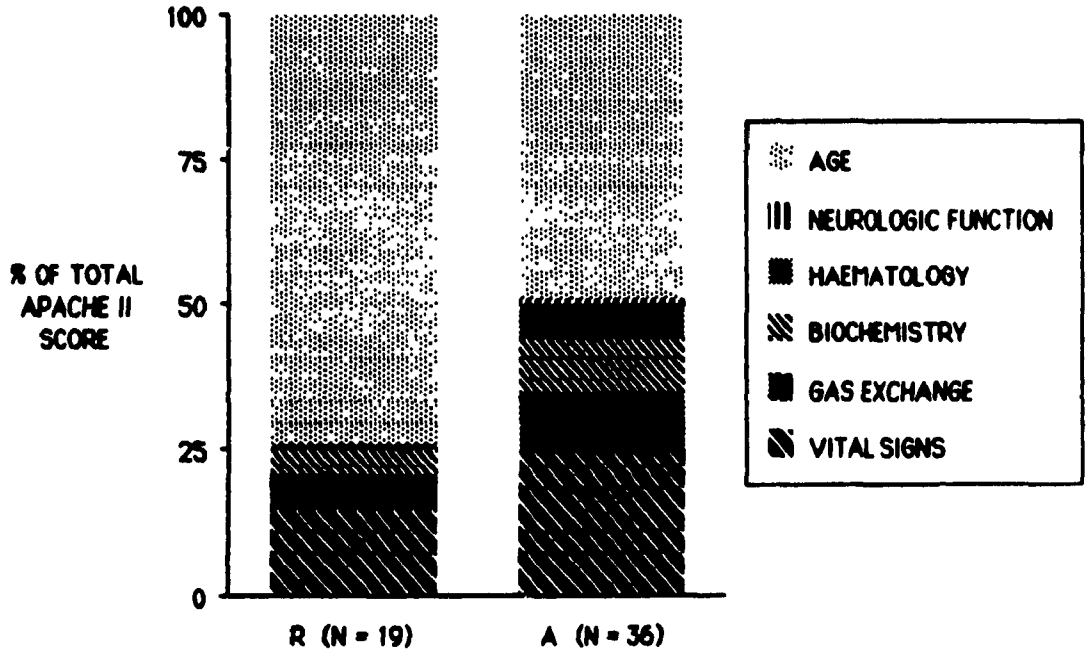
Demographic data, STS (obtained just before vaccination), APACHE II scores (APE, calculated on the day of vaccination), diagnosis and operation performed are listed for A subjects. They are grouped according to sub-classification as either hospital (HA), intensive care (IA), or walk-in (WA) anergics at the time of vaccination. The degree (DEG) and duration (TIME, min) of operations are shown. The timing of surgery in relation to vaccination is shown in number of days the operation occurred after (+) or before (-) vaccination. Outcome was based on condition at hospital discharge, and the presence or absence of major sepsis (SEP) at any time during hospitalization.

TABLE 5: DEMOGRAPHIC DATA, DEGREE AND TIME OF SURGERY, CIRCULATING BLOOD CELL COUNTS, AND SERUM PROTEIN LEVELS OF PATIENTS VACCINATED WITH TETANUS TOXOID

	LAB CONTROL	REACTIVE	ANERGIC
N	26	19	36
AGE (years)	41 ± 3	*62 ± 3	*67 ± 2
DEGREE OPERATION	-	0-4, 3	0-4, 3
TIME OPERATION (min)	-	189 ± 23	205 ± 23
STS (mm)	42 ± 2	*31 ± 3	*#1 ± 1
APACHE II	1.2 ± 0.3	*5.4 ± 0.7	*#8.9 ± 0.8
HEMOGLOBIN (g/l)	143 ± 3	*119 ± 6	*110 ± 3
LEUKOCYTES (cells/mm^3)	6096 ± 265	*8642 ± 1148	*13077 ± 1685
LYMPHOCYTES (cells/mm^3)	2056 ± 133	*1510 ± 212	*1226 ± 148
MONOCYTES (cells/mm^3)	348 ± 32	768 ± 302	*1085 ± 437
TOTAL PROTEIN (g/l)	74 ± 1	*63 ± 2	*#57 ± 1
ALBUMIN (g/l)	44 ± 1	*31 ± 1	*28 ± 1

Demographic and clinical laboratory data, collected at the time of immunization, are shown. The range, and median degree of operation were not significantly different. Other data are shown as arithmetic means ± sem. * indicates $p < 0.05$ compared to LC, # indicates $p < 0.05$ compared to R (unpaired t-tests).

FIGURE 7: APACHE II SCORES IN DTH REACTIVE AND ANERGIC PATIENTS



APACHE II scores were determined for DTH R and A patients. The relative contributions of points in each category of physiologic variables to the total is showm.

and a decrease in lymphocytes. Total protein and serum albumin levels were reduced among patients, but not significantly different between R and A subjects ($p = 0.12$).

There was a difference in outcome among the patient groups. The incidence of major sepsis was 1/19 R , and 15/36 A patients ($X^2_1 = 7.99, p = 0.005$). One R , and 10 A patients died ($X^2_1 = 3.94, p = 0.05$). Nine of the patient deaths were related to sepsis. Both sepsis and mortality rates were higher in patients with higher APACHE II values.

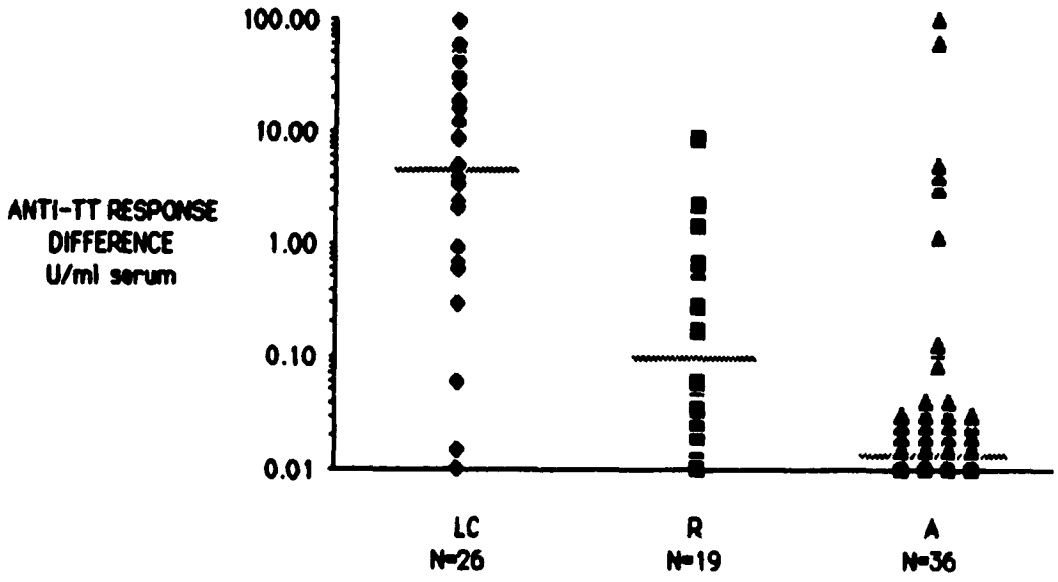
There was a reduction in antibody responsiveness among all the patients compared to LC, worst among those with reduced DTH reactivity. Whether calculated as differences (figure 8) or ratios (figure 9), the pattern of individual responses and means was similar. The average anti-TT responses are shown in table 6. There were significant differences between all the groups ($p = 0.0005$ for R or A compared to LC, and $p = 0.002$ for R versus A, WRST). There were similar differences when responses were calculated as ratios. There were significant differences between all the groups ($p = 0.002$ for R or A compared to LC, and $p = 0.04$ for R versus A, WRST).

There were also differences in response rates using arbitrarily defined criteria (table 6). Responses were considered normal if there was > 0.1 new U/ml serum, or > a 10 fold increase in antibody content. Eighty-eight % of LC produced more than 0.1 U/ml, compared to 47% and 25% for R and A subjects respectively ($X^2_2 = 24.4, p = 0.0005$). Using fold increase, positive responses rates were 65, 47 and 19 for LC, R and A respectively ($X^2_2 = 13.7, p = 0.001$).

There were differences among subclasses of patients. The individual pre-, and day 14 post-immunization anti-TT levels in subclasses of R and A subjects are shown in figures 10 and 11. Means and response rates are listed in table 6. Among the R patients, responses were better in the WR compared to HR class calculated as a difference ($p = 0.008$, WRST, and $X^2_1 = 6.7, p = 0.009$), or a ratio ($p = 0.02$, WRST, and $X^2_1 = 2.8, p = 0.10$). Among the three A subclasses, there were no significant differences between the groups (all $p > 0.07$, WRST and X^2_2).

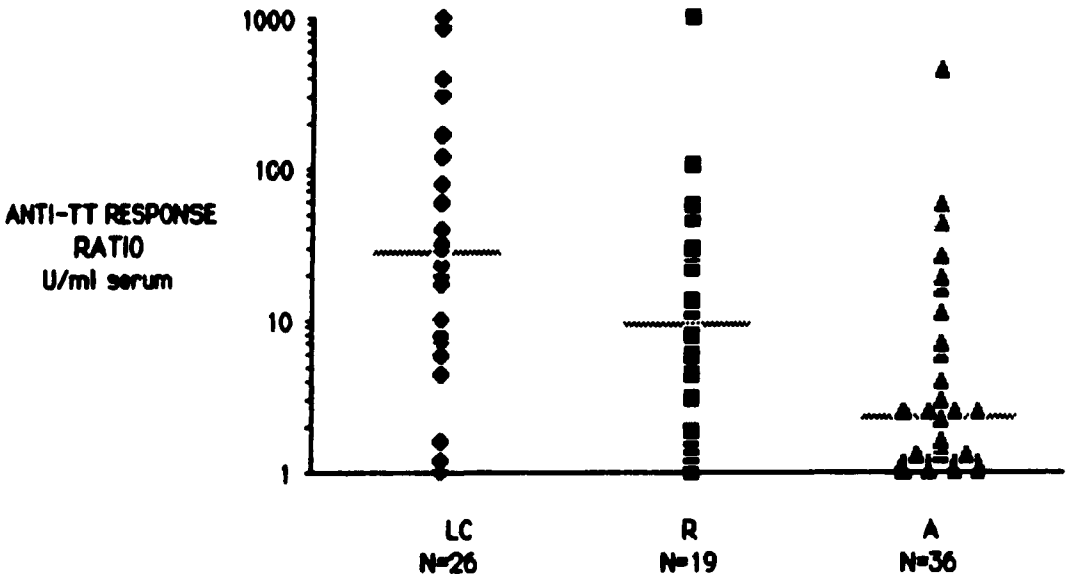
Patient groups with the same subclassification, but different skin test categories, were compared. These groups had similar clinical definitions, and were indistinguishable except on the basis of skin tests. Walk-in R and A patients had similar clinical definitions, and degree of physiologic abnormality (APACHE II scores 5.2 ± 0.8 and 5.7 ± 0.6 for R and A,

FIGURE 8: PATIENT *IN VIVO* ANTIBODY RESPONSE (DIFFERENCE) TO TETANUS TOXOID



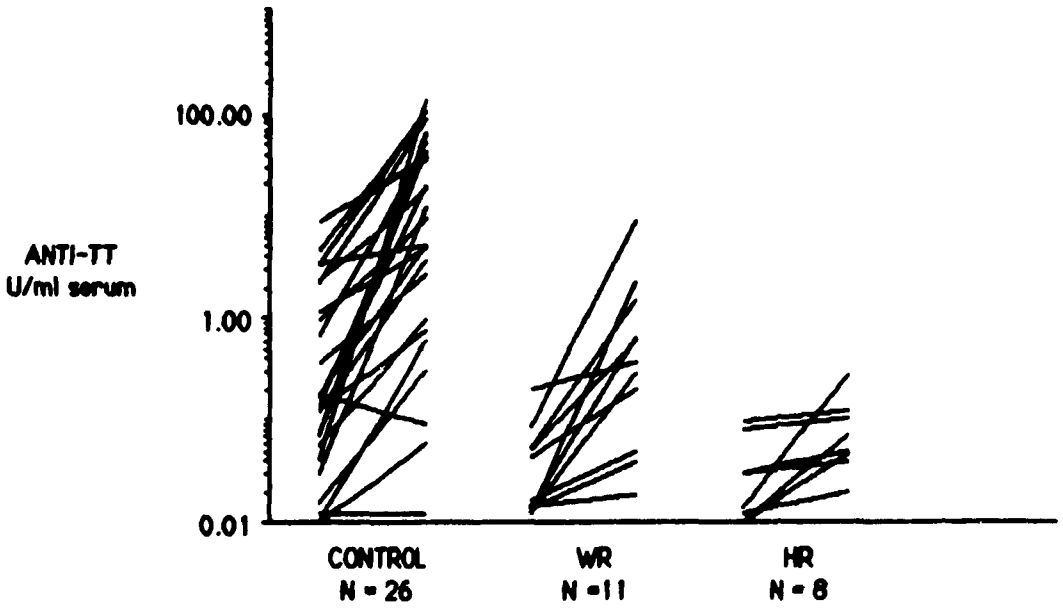
Individual antibody responses to TT vaccination of different groups of subjects are shown. The response was defined as the difference between day 14 post-, and pre-vaccination serum anti-TT content. The geometric means are indicated by the horizontal bars. There are statistically significant differences between all group comparisons ($p = 0.0005$ for R or A compared to LC, $p = 0.002$ for A compared to R, WRST).

FIGURE 9: PATIENT *IN VIVO* ANTIBODY RESPONSE (RATIO) TO TETANUS TOXOID



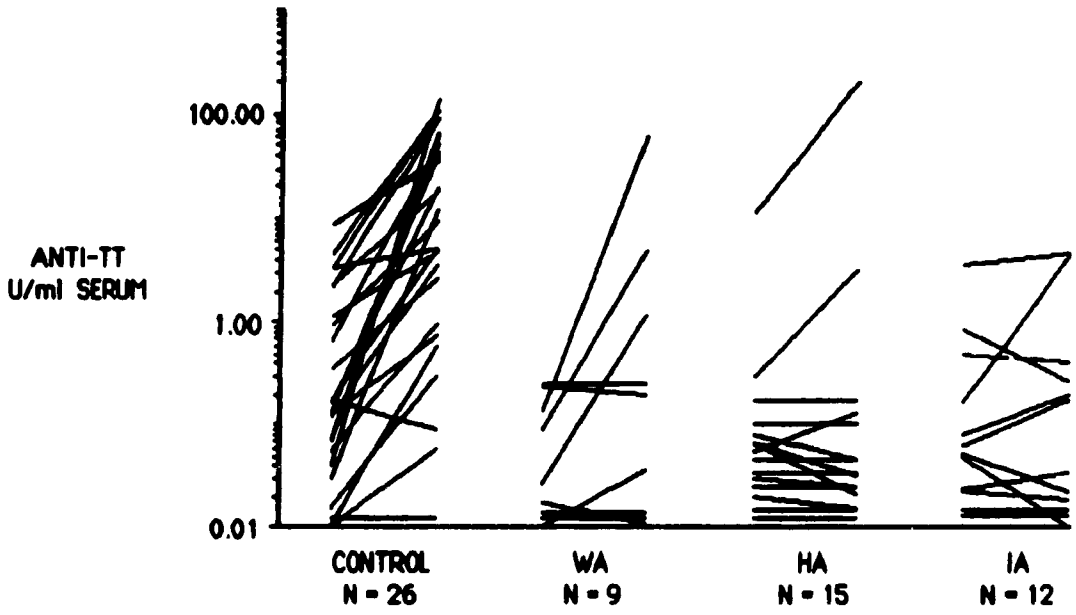
Individual antibody responses to TT vaccination of different groups of subjects are shown. The response was defined as the ratio of day 14 post-, to pre-vaccination serum anti-TT content. The geometric means are indicated by the horizontal bars. There are statistically significant differences between all group comparisons ($p = 0.002$ for R or A compared to LC, $p = 0.04$ for A compared to R, WRST).

FIGURE 10: *IN VIVO* ANTIBODY RESPONSES TO TETANUS TOXOID IN DTH REACTIVE PATIENT SUBGROUPS



Antibody responses to TT vaccination of different subgroups of DTH R subjects are shown as a line connecting the pre-, and post-immunization levels of anti-TT for each subject. When calculated as a difference or ratio, there were statistically significant differences between the responses of WR and HR groups ($p = 0.008$, $p = 0.02$, WRST).

FIGURE 11: *IN VIVO* ANTIBODY RESPONSES TO TETANUS TOXOID IN DTH ANERGIC PATIENT SUBGROUPS



Antibody responses to TT vaccination of different subgroups of DTH A subjects are shown as a line connecting the pre-, and post-immunization levels of anti-TT for each subject. When calculated as a difference or ratio, there were no statistically significant differences between the responses of WA, HA or IA groups ($p > 0.07$, WRST).

TABLE 6: *IN VIVO* ANTIBODY RESPONSES TO VACCINATION WITH TETANUS TOXOID

CLASS	N	DIFFERENCE mean	x±	sem	RATIO mean	x±	sem	POSITIVE RESPONSES DIFFERENCE N	(%)	RATIO N	(%)
CONTROL	**26**	**4.39**	**x±**	**0.27**	**28.35**	**x±**	**0.19**	**23**	**(88)**	**17**	**(65)**
WR	11	0.26	x±	0.29	20.39	x±	0.29	8	(73)	7	(64)
HR	8	0.02	x±	0.30	3.50	x±	0.24	1	(13)	2	(25)
ALL R	19	***0.09**	x±	0.23	***9.40**	x±	0.20	9	(47)	9	(47)
WA	9	0.01	x±	0.01	5.50	x±	0.34	3	(33)	2	(22)
HA	15	0.01	x±	1.12	2.09	x±	1.54	2	(13)	3	(20)
IA	12	0.01	x±	0.17	1.28	x±	0.17	4	(33)	1	(8)
ALL A	36	***#0.01**	x±	0.26	***#2.26**	x±	0.12	9	(25)	7	(19)

In vivo antibody responses to TT vaccination were calculated as either the difference or the ratio between pre-, and day 14 post-immunization serum anti-TT levels. The geometric means x± sem (log base 10) are shown. * indicates $p < 0.05$ compared to LC, and # $p < 0.05$ compared to ALL R (WRST). A positive response was defined as either > = 0.1 new units/ml serum (DIFFERENCE) or > = 10-fold increase in antibody (RATIO).

p = 0.65, unpaired t-test). Althought the antibody responses were better among the R patients (table 6, figures 10 and 11), the differences were not statistically significant (p > 0.07 for all WRST and X^2_1). For the hospitalized subclasses, HR and HA, the APACHE II scores were insignificantly higher in the HA group compared to HR (8.9 $\pm$ 1.3 and 6.0 $\pm$ 1.2, p = 0.18, unpaired t-test). The antibody responses were similar between these two groups (p > 0.05 for all WRST and X^2_1).

The effect of surgery during the course of the immune response was examined by comparing responses of subjects operated on or not soon after immunization. There was substantial patient variability, but surgery did not have a consistent effect on antibody responses, even if performed during the first week after vaccination (data not shown).

There was no relationship between age and antibody responses in the LC group (r_s = -0.22, p = 0.6), or in the patient group (r_s = -0.05, p = 0.7).

Pre-immunization anti-TT levels were somewhat lower in DTH reactive patients, but generally comparable among all patient, and control subjects (figure 10 and 11).

In summary, antibody responses to a protein antigen are reduced among all patients, especially those with reduced DTH reactivity. Skin test reactive patients who are immunized while pre-operative, produced the best responses.

1.1.1.3 RELATIONSHIP OF DTH TESTING, PHYSIOLOGIC ASSESSMENT AND OUTCOME TO IN VIVO ANTIBODY RESPONSES TO TETANUS TOXOID

Because of the trends in anti-TT responses among patient groups, relationships were sought between the STS and APACHE II score, and anti-TT responses. Only patients with APACHE II scores available on the day of vaccination were included, 27 R and 14 A. The skin test data immediately preceding vaccination was used for patient classification. The ratio of anti-TT antibody levels on day 14/day 0 was used as the antibody response.

Forty one subjects were studied. The age range was 41 to 90, with median 68 and mean $\pm$ SD of 65 $\pm$ 12. Anti-TT responses ranged from 1 (no increase) to 2200 fold increases. The geometric mean antibody response x÷ SEM was 3.39 x÷ 0.93 fold increase. Positive responses, defined as greater than a ten fold increase in antibody, were shown by 11/41 (27%). The

APACHE II scores ranged from 2 to 24, with median 7 and mean $\pm$ SD of 8 $\pm$ 4. The STS ranged from 0 to 75, with median 3 and mean $\pm$ SD of 11 $\pm$ 17.

Within the skin testing subgroups, the APACHE II scores varied. Among the anergic patients requiring intensive care (IA, N = 9), the mean $\pm$ SD APACHE II score was 13.0 $\pm$ 4.8. In comparably well elective pre-operative patients who were, however, similarly anergic (WA, N = 7), the mean score was 5.7 $\pm$ 1.6. In skin test reactive elective pre-operative patients (WR, N = 8), the mean score was comparable at 5.8 $\pm$ 2.2.

The total hospital sepsis rates and mortality were 12/41 (29%) and 10/41 (24%) respectively. Five deaths were related to sepsis. Sepsis and mortality increased with increasing physiologic derangement. Dividing the patients into three groups based on APACHE II scores from 0-5, 6-10 and > = 11, the rates of sepsis were 2/12 (17%), 4/20 (20%) and 6/9 (67%) respectively (X^2_2 = 7.83, p = 0.02). Similarly, the mortality rates were 0/12 (0%), 6/20 (30%) and 5/9 (56%) (X^2_2 = 6.17, p < 0.05).

Dividing the patients into antibody responders and non-responders, the rates of sepsis were 1/11 (9%) and 11/30 (37%) respectively (X^2_1 = 1.77, with Yates, p = 0.18). Mortality rates were 1/11 (9%) and 9/30 (30%) for antibody responders and non-responders respectively (X^2_1 = 0.94, with Yates, p = 0.33).

Dividing the patients into anergic or reactive on the basis of DTH testing, the rates of sepsis were 11/27 (41%) and 1/14 (7%) respectively (X^2_1 = 5.03, p = 0.03). Mortality rates were 9/27 (33%) and 1/14 (7%) for DTH non-responders and responders respectively (X^2_1 = 3.43, p = 0.06).

By non-parametric correlation, there was a statistically significant negative relationship between APACHE II score and antibody response (r_s = -0.445, p = 0.004), such that the more ill patients produced less antibody. Similarly, there was a negative correlation between APACHE II scores and STS (r_s = -0.376, p = 0.02). Finally, there was a positive correlation between STS and antibody responses (r_s = 0.471, p = 0.002).

There was no correlation between age and APACHE II scores, STS or antibody responses (r_s = 0.143, 0.030, -0.035, p = 0.62, 0.84 and 0.82 respectively).

1.1.2 IN VITRO SPECIFIC AND TOTAL IMMUNOGLOBULIN SYNTHESIS AFTER TETANUS TOXOID VACCINATION

Blood MNCs were isolated before and at intervals after TT immunization, and cultured under standard conditions. After 10 days, culture supernatants were assayed for cumulative content of specific anti-TT and total IgG. Data are shown according to the day of MNC harvesting.

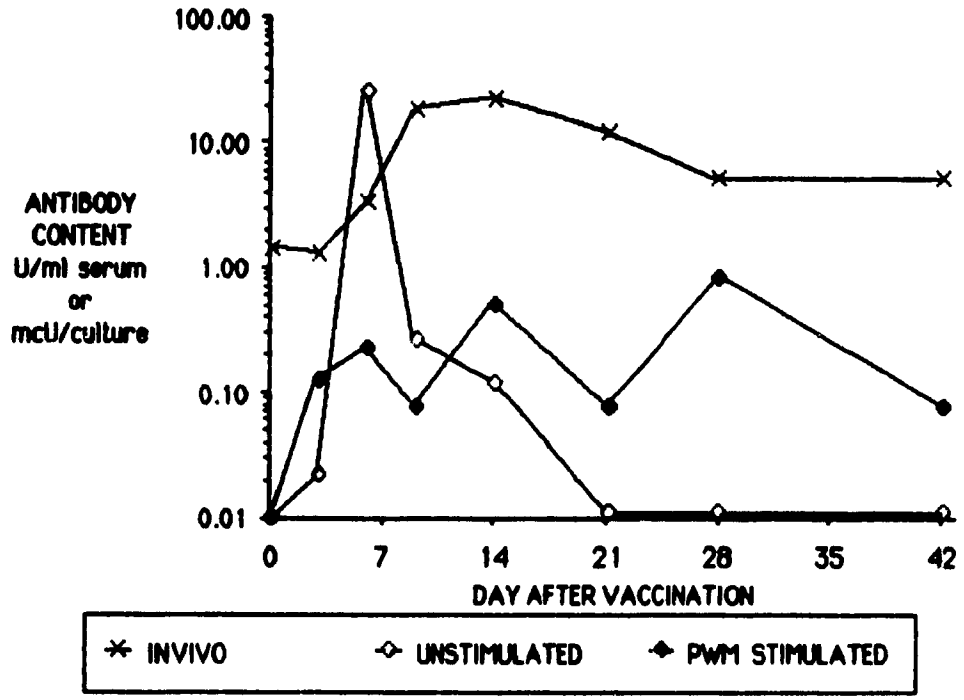
1.1.2.1 NORMAL IN VITRO SPECIFIC AND TOTAL IMMUNOGLOBULIN SYNTHESIS AFTER TETANUS TOXOID VACCINATION

Four LC subjects were initially studied. After immunization, blood MNCs were harvested at intervals from days 0 to 42 and cultured *in vitro* under standard conditions for 10 days. Cultures were either unstimulated, or stimulated with PWM.

In unstimulated cultures, no subject produced detectable quantities of antibody *in vitro* prior to immunization. Detectable antibody synthesis began 3 days after vaccination and peaked at day 6 (figure 12). Lesser quantities of antibody were produced by MNC harvested 9 and 14 days after immunization. From day 21 onward, there was no detectable spontaneous *in vitro* antibody production. Spontaneous synthesis peaked during the period of maximal increase in serum antibody levels, from days 4 to 8, and ceased when serum antibody levels were stable or declining. The addition of 10^{-4}M emetine to the cultures at initiation reduced supernatant antibody content to undetectable levels (data not shown). A time course experiment showed that most of the specific antibody was produced in the first 2 days of culture. At this time, 2.2 mcU were present in the supernatant, compared to 2.9 on day 4 and 10 (data not shown).

In PWM stimulated cultures, no antibody was produced before vaccination. Stimulation caused a significant decrease in specific antibody synthesis on day 6 in all individuals (figure 12). This was during the time of peak spontaneous antibody synthesis *in vitro*. Specific antibody synthesis could be induced by PWM after spontaneous synthesis had ceased, beyond day 21. Although on average, some antibody was always produced in such late cultures, quantities varied widely, and positive responses were not consistently present among different subjects.

FIGURE 12: NORMAL ANTI-TETANUS TOXOID ANTIBODY SYNTHESIS *IN VITRO* AFTER VACCINATION *IN VIVO*



After TT vaccination of 4 LC subjects on day 0, blood MNCs were harvested at intervals, and cultured *in vitro* in either unstimulated, or PWM stimulated, cultures under standard conditions for 10 days. Serum, and culture supernatants were assayed for amounts of IgG anti-TT. Geometric mean amounts of IgG anti-TT produced *in vitro*, and serum antibody levels (*IN VIVO*), are shown.

The same culture supernatants were assayed for cumulative content of total IgG. Spontaneous IgG synthesis increased from minimal pre-immunization levels to a peak at day 10 to 14 with a gradual decline thereafter (figure 13). Compared to spontaneous anti-TT IgG synthesis, the peak of total IgG production occurred later. In PWM stimulated cultures, there was an early increase in synthesis, followed by a decrease that was maximal at day 10, the same time as the peak for spontaneous synthesis. Quantities of PWM-induced total IgG, however, always exceeded spontaneous synthesis.

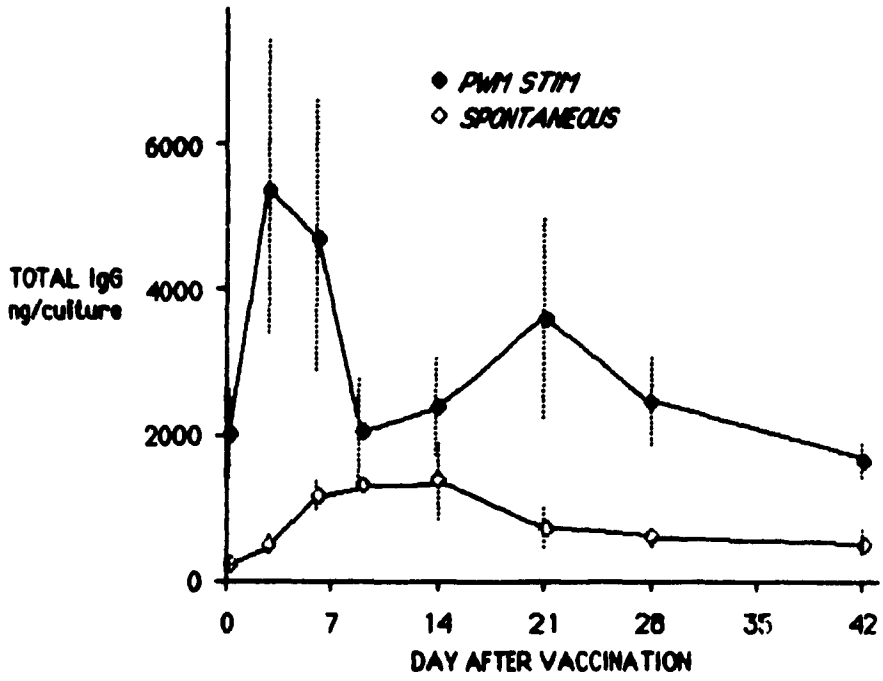
An extended study of 9 LC subjects on days 0, 6 and 14 after immunization further defined the *in vitro* response to *in vivo* TT vaccination. No subjects produced large amounts of antibody before immunization (figure 14). Spontaneous synthesis of anti-TT was greatest on day 6. At this time, PWM significantly reduced antibody synthesis ($p = 0.01$, WSRT). Two subjects were reduced to undetectable levels. On day 14, PWM increased synthesis for nearly all individuals ($p = 0.01$, WSRT), and produced a positive response in cultures from 2 subjects with negative unstimulated cultures.

There were also changes in total IgG production. Before vaccination, all subjects produced more IgG with PWM stimulation than without (figure 15). On day 6, MNCs from 3 subjects produced similar or less total IgG with PWM. The pattern was normal again on day 14, with all subjects responding to PWM stimulation.

The amount of specific antibody present in supernatants after TT vaccination contributes little to the total IgG quantity. The weight of specific antibody in culture supernatants may be approximated by calculation using the hyperimmune globulin originally used to define the units. Ten mcg of IgG represents approximately 1 U of anti-TT. The maximum amount of anti-TT produced in culture, 2030 mcU, thus constitutes only 2 ng of total IgG. As this is much less than was produced in either PWM or unstimulated cultures, the majority of the supernatant IgG represents non-specific Ig.

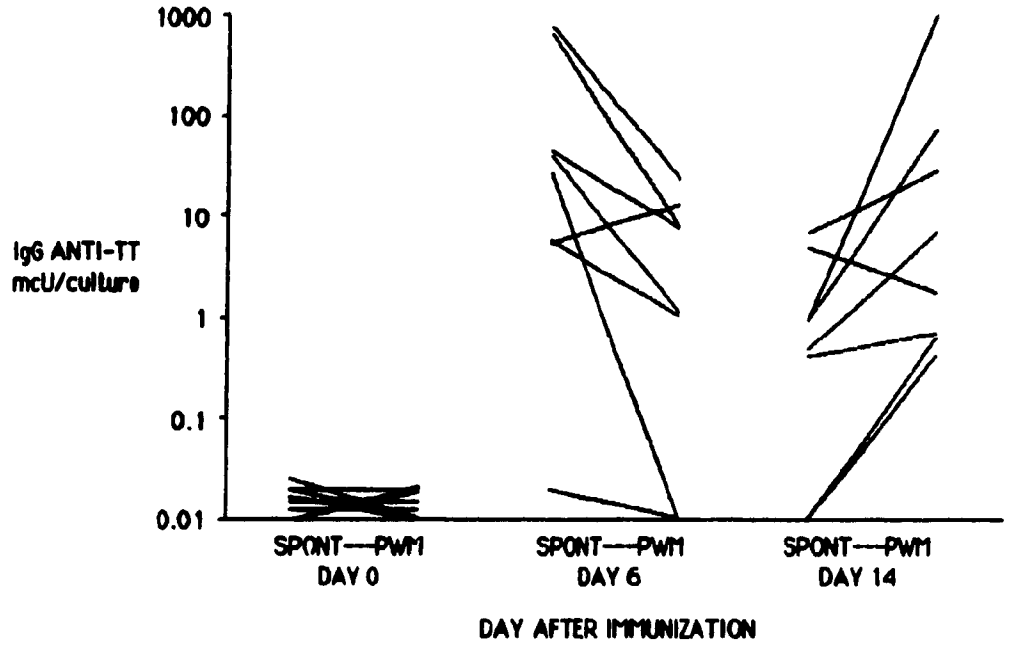
In summary, there were MNCs in the blood that spontaneously produce specific antibody *in vitro* after *in vivo* vaccination. The kinetics suggest that this was a biologic correlate of the *in vivo* response. Stimulation with PWM reduced this, but was able to induce the synthesis of specific antibody by circulating MNCs later after immunization.

FIGURE 13: NORMAL TOTAL IgG SYNTHESIS *IN VITRO* AFTER TETANUS TOXOID VACCINATION *IN VIVO*



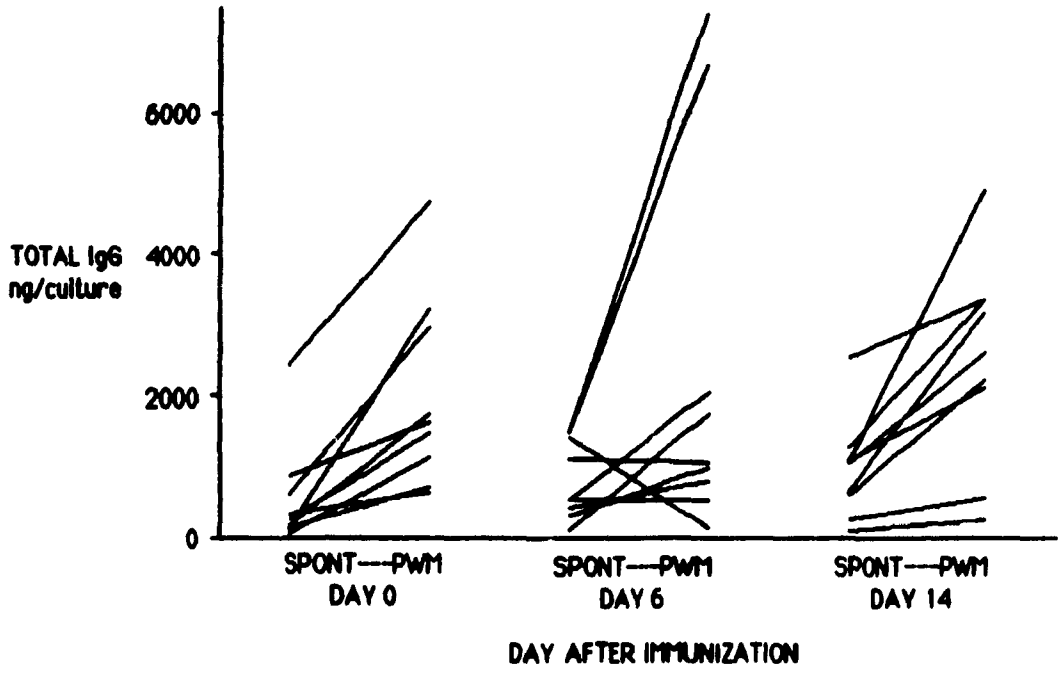
After TT vaccination of 4 LC subjects on day 0, blood MNCs were harvested at intervals, and cultured *in vitro* in either unstimulated, or PWM stimulated, cultures under standard conditions for 10 days. Culture supernatants were assayed for amounts of total IgG. Arithmetic means $\pm$ SEM are shown.

FIGURE 14: EFFECT OF POKEWEED MITOGEN ON NORMAL ANTI-TETANUS TOXOID ANTIBODY SYNTHESIS *IN VITRO* AFTER VACCINATION *IN VIVO*



After TT vaccination of 9 LC subjects on day 0, blood MNCs were harvested on days 0, 6 and 14, and cultured *in vitro* in either unstimulated (SPONT), or PWM stimulated (PWM), cultures under standard conditions for 10 days. Culture supernatants were assayed for amounts of IgG anti-TT. Amounts of IgG anti-TT produced *in vitro* are shown as a line for each individual connecting the amounts of anti-TT produced with, and without PWM. Stimulation with PWM significantly reduced anti-TT synthesis in day 6 cultures, and increased it in day cultures ($p = 0.01$, $p = 0.01$, WSRT),

FIGURE 15: EFFECT OF POKEWEED MITOGEN ON NORMAL TOTAL IMMUNOGLOBULIN SYNTHESIS *IN VITRO* AFTER TETANUS TOXOID VACCINATION *IN VIVO*



After TT vaccination of 9 LC subjects on day 0, blood MNCs were harvested on days 0, 6 and 14, and cultured *in vitro* in either unstimulated (SPONT), or PWM stimulated (PWM), cultures under standard conditions for 10 days. Culture supernatants were assayed for amounts of total IgG. This is shown as a line for each individual connecting the quantity of total IgG produced with, and without PWM.

1.1.2.2 PATIENT IN VITRO SPECIFIC AND TOTAL IMMUNOGLOBULIN SYNTHESIS AFTER TETANUS TOXOID VACCINATION

Peripheral blood MNC from 5 R and 4 A subjects were cultured *in vitro* on days 0, 6 and 14 after TT immunization. Culture supernatants were assayed for total and specific IgG content.

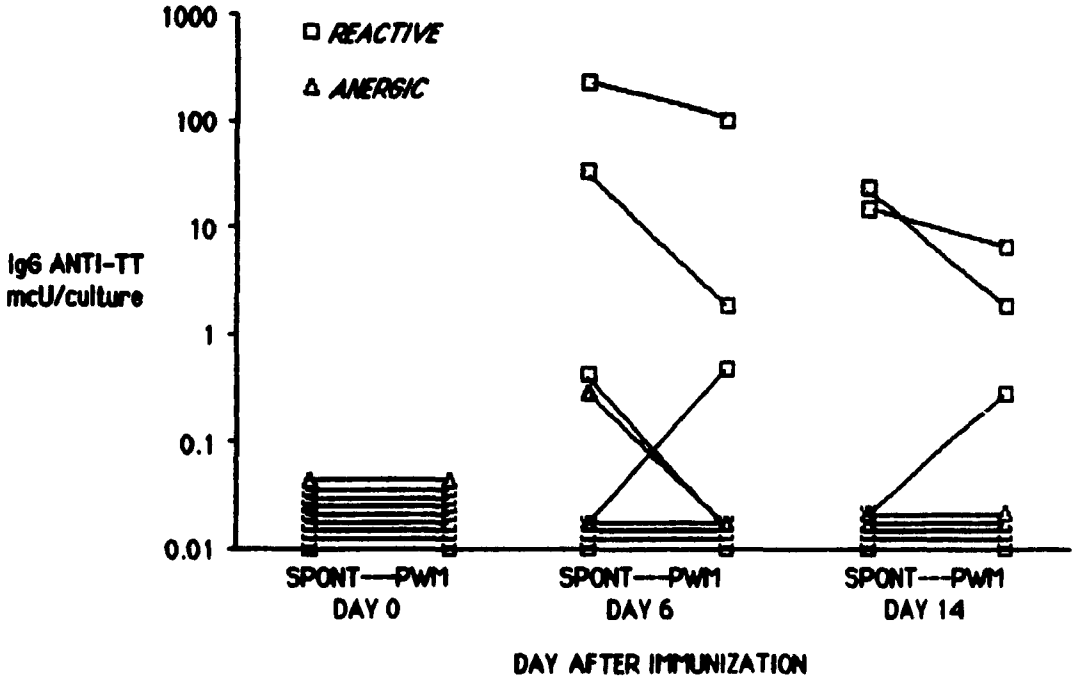
Spontaneous synthesis of anti-TT IgG followed a similar pattern to *in vivo* antibody responses. Before immunization, no subject produced antibody *in vitro* (figure 16). Similar to *in vivo* data, there was a graded response between LC, R, and A. On day 6, 7 of 9, 3 of 5 and 1 of 4 LC, R, and A subjects, respectively, produced detectable quantities of anti-TT *in vitro*. The geometric mean antibody amounts were 7.34, 0.78 and 0.02 mcU/culture for LC, R and A groups. The only A subject whose cells produced antibody *in vitro* at day 6 was also the only A subject in this group to show a normal *in vivo* response, 1.1 new U/ml serum at day 14.

In PWM stimulated cultures, less antibody was produced on day 6 by MNCs from most patients, similar to LC subjects. On day 14, only 3 patients had positive cultures for anti-TT, and in 2 of these, PWM decreased synthesis, unlike LC subjects. Apart from the single occasion noted above, lymphocytes from this and all other A subjects did not produce specific antibody *in vitro* in either unstimulated or PWM stimulated cultures at any time before or after TT immunization.

The failure of specific antibody production *in vitro* was not due to a total failure of IgG synthesis. All subjects produced substantial amounts of total IgG after vaccination (figure 17). At all times after immunization, R patients tended to produce more, and A subjects the same or less, non-specific IgG with PWM stimulation compared to spontaneous synthesis.

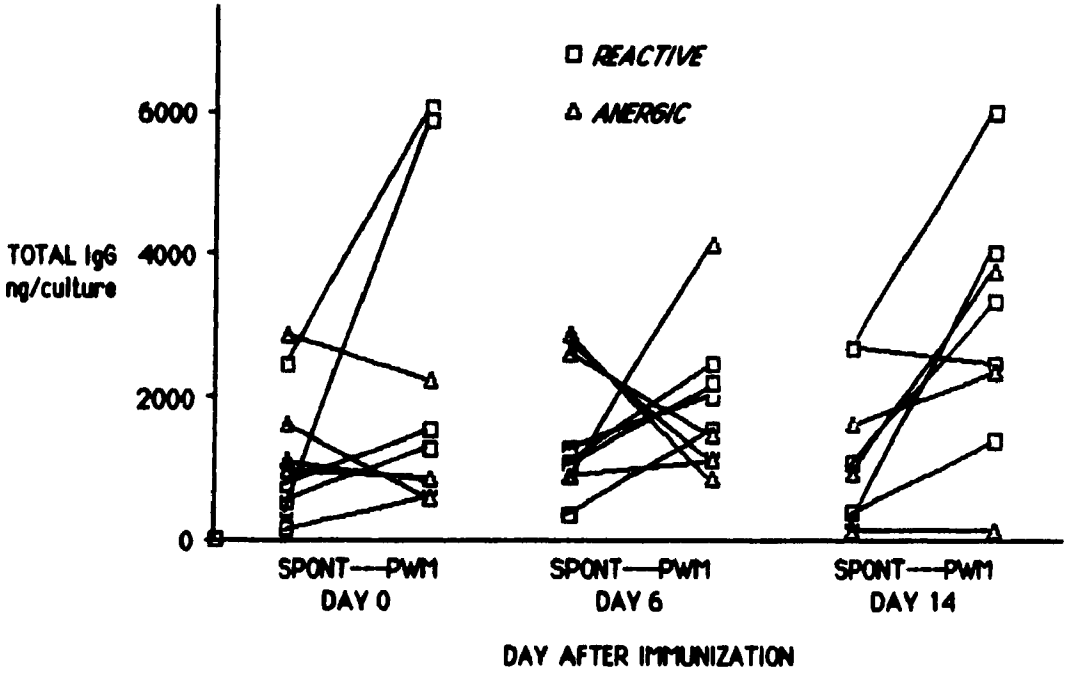
Data from a representative anergic subject is shown in table 7. This 75 year-old female was admitted for investigation of diarrhea. The final diagnosis was metastatic carcinoid syndrome. She was anergic to skin testing on admission and remained so throughout hospitalization. Laparotomy was performed on day 15 after TT immunization. Peripheral blood MNC harvested at day 0, 6 14 and 20 failed to produce detectable quantities of antibody *in vitro* in either unstimulated or PWM stimulated cultures. The *in vivo* antibody response was similarly modest, only 0.03 U/ml serum.

FIGURE 16: EFFECT OF POKEWEED MITOGEN ON PATIENT ANTI-TETANUS TOXOID ANTIBODY SYNTHESIS *IN VITRO* AFTER VACCINATION *IN VIVO*



After TT vaccination of 5 R and 4 A patients on day 0, blood MNCs were harvested on days 0, 6 and 14, and cultured *in vitro* in either unstimulated (SPONT), or PWM stimulated (PWM), cultures under standard conditions for 10 days. Culture supernatants were assayed for amounts of IgG anti-TT. Amounts of IgG anti-TT produced *in vitro* are shown as a line for each individual connecting the amounts of anti-TT produced with, and without PWM.

FIGURE 17: EFFECT OF POKEWEED MITOGEN ON PATIENT TOTAL IMMUNOGLOBULIN SYNTHESIS *IN VITRO* AFTER TETANUS TOXOID VACCINATION *IN VIVO*



After TT vaccination of 5 R and 4 A patients on day 0, blood MNCs were harvested on days 0, 6 and 14, and cultured *in vitro* in either unstimulated (SPONT), or PWM stimulated (PWM), cultures under standard conditions for 10 days. Culture supernatants were assayed for amounts of total IgG. This is shown as a line for each individual connecting the quantity of total IgG produced with, and without PWM.

TABLE 7: ANERGIC PATIENT EXAMPLE OF ANTIBODY RESPONSE TO TETANUS TOXOID

DAY POST-TT VACCINATION	IN VIVO ANTI-TT IgG U/ml serum	IN VITRO ANTI-TT IgG mU/culture UNSTIM	IN VITRO ANTI-TT IgG mU/culture STIM	IN VITRO TOTAL IgG ng/culture UNSTIM	IN VITRO TOTAL IgG ng/culture STIM
0	0.01	<0.01	<0.01	785	1520
6	0.01	<0.01	<0.01	831	4105
14	0.04	<0.01	<0.01	382	1386
20	0.02	<0.01	<0.01	2674	1917

A 75 year old WA female was immunized with TT on day 0. Surgery (colectomy) was performed on day 15. Antibody levels to TT were measured in sera before and after immunization. Blood MNCs were cultured using standard conditions, and supernatants assayed for content of anti-TT specific and total IgG. Cell cultures were either unstimulated (UNSTIM), or stimulated with PWM (STIM).

In summary, spontaneous *in vitro* specific antibody synthesis in patients was related to the serum antibody response. Stimulation with PWM was unable to induce specific antibody synthesis in patients who did not respond *in vivo* to vaccination.

1.1.2.3 CORRELATION BETWEEN IN VIVO AND IN VITRO RESPONSES TO TETANUS TOXOID

Several observations suggested a relationship between *in vivo* antibody responses and *in vitro* spontaneous antibody synthesis in normal subjects and patients. Peak *in vitro* synthesis occurred at the time of maximal rate of appearance of serum antibody, and ceased when *in vivo* levels stopped increasing. Only patients who responded *in vivo* produced specific antibody *in vitro*. Using rank correlation, a quantitative relationship was thus identified between peak antibody responses *in vivo* measured at day 14, and peak spontaneous antibody production *in vitro* at day 6 ($N = 18$, $r_s = 0.79$, $p = 0.001$). Correlating antibody production *in vitro* at day 14 instead of day 6 with *in vivo* antibody responses yielded a much poorer relationship ($r_s = 0.39$, $p = 0.2$). In cultures stimulated by PWM, there was a better correlation between *in vitro* antibody synthesis and eventual serum antibody responses on day 14 ($r_s = 0.57$, $p = 0.03$), than on day 6 ($r_s = 0.51$, $p = 0.04$).

Spontaneous *in vitro* antibody synthesis early after vaccination may thus be considered a biological manifestation of the *in vivo* response to antigen encounter.

1.2 ANTIBODY RESPONSES TO PNEUMOCOCCAL POLYSACCHARIDE

To investigate the antibody response to a polysaccharide antigen, *in vivo* and *in vitro* studies were done with PPS vaccine (PPS). The day of immunization was day 0. Serum samples were obtained before vaccination and at intervals thereafter, and were assayed for IgG, IgM and IgA anti-PPS antibody content. Peripheral blood was obtained before and after immunization for *in vitro* MNC culture. Culture supernatants were assayed for isotype specific anti-PPS antibody and total IgG, IgM and IgA.

1.2.1 IN VIVO ANTIBODY RESPONSES TO PNEUMOCOCCAL POLYSACCHARIDE

1.2.1.1 NORMAL IN VIVO ANTIBODY RESPONSES TO PNEUMOCOCCAL POLYSACCHARIDE

Four LC subjects with a mean age of 34 were immunized with PPS. Figures 18 and 19 show the mean normal isotype specific serum antibody response to vaccination. After a lag period of 3-5 days, during which IgG anti-PPS levels appeared to decline, antibody levels began to increase. Although the period of exponential rise in serum antibody content was from approximately day 6 to 10 for all isotypes, the relative positions of the curves indicated that IgM is produced earliest, followed by IgA and IgG in order from early to late (arrow, figure 19). Peak levels are achieved by day 8 for IgM, and by days 9 and 14 for IgA and IgG. The geometric mean fold increase in antibody content at peak levels was 4.6, 8.3 and 46.1 fold for IgG, IgM and IgA. The geometric mean new U/ml serum at peak levels were 4.8, 4.5 and 4.3 for IgG, IgM and IgA.

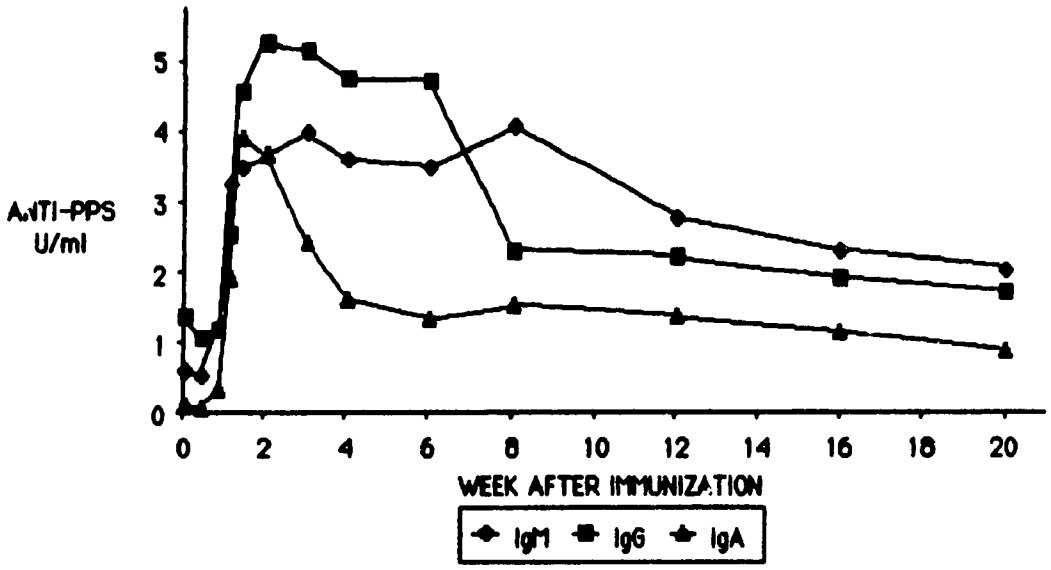
After the peak, there was an early sharp decline in IgA, followed by IgG after 6 weeks (figure 18). The decline in IgM is more gradual. After 4 weeks, the plateau antibody levels were 3.6, 6.2 and 18.6 fold higher than pre-immunization for IgG, IgM and IgA. The number of remaining new U/ml serum was 3.5, 3.1 and 1.8 for IgG, IgM and IgA. After 20 weeks, the plateau antibody levels had dropped to 1.3, 3.6 and 10.2 fold higher than pre-immunization for IgG, IgM and IgA. IgG anti-PPS levels had returned to approximately pre-immunization values, while 1.6 and 1.0 new U/ml in excess of pre-immune quantities remained for IgM and IgA.

In summary, after PPS vaccination there is a period of exponential increase in serum antibody levels from day 6 to 10. The isotypes appear in the blood, and peak, in order with IgM first, followed by IgA and IgG.

1.2.1.2 PATIENT IN VIVO ANTIBODY RESPONSES TO PNEUMOCOCCAL POLYSACCHARIDE

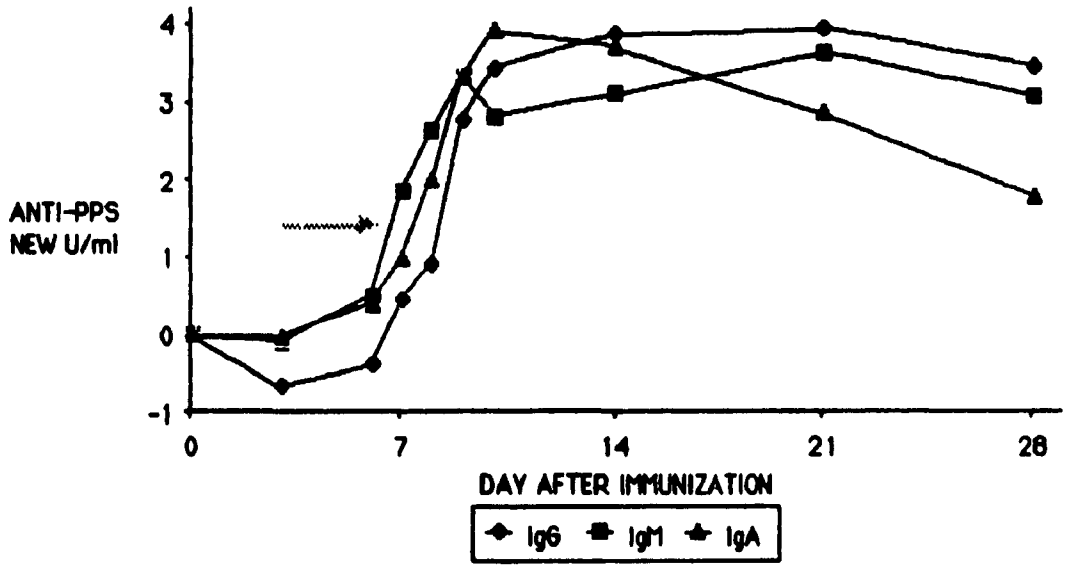
Control subjects and patients classified according to skin test were studied. Eight C, 5 R (4 W and 1 H) and 7 A (2 W, 1 H and 4 I) subjects were studied. Table 8 lists the demographic data, diagnosis, surgery performed and outcome for each patient. The median and mean

FIGURE 18: NORMAL *IN VIVO* ANTIBODY RESPONSE TO PNEUMOCOCCAL POLYSACCHARIDE



Geometric mean serum IgG, IgM and IgA anti-PPS levels are shown before and at intervals up to 20 weeks after immunization of 4 LC subjects.

FIGURE 19: EARLY NORMAL *IN VIVO* ANTIBODY RESPONSE TO PNEUMOCOCCAL POLYSACCHARIDE



Geometric mean serum IgG, IgM and IgA anti-PPS levels are shown before, and during the early period after immunization of 4 LC subjects. New units/ml serum were calculated for each isotype by subtracting pre-vaccination antibody levels from the levels after vaccination. The arrow indicates the period of exponential increase in serum antibody levels, where the order of appaearance of the different isotypes can be discerned; IgM first, then IgA and IgG.

ages ± SEM were 44, 45 ± 7, 62, 60 ± 6 and 70, 68 ± 4 for C, R and A subjects respectively. For skin testing done just before vaccination, the average STS ± SEM were 36 ± 6, 35 ± 13 and 0 ± 0 for C, R and A groups. In followup skin testing, there was no significant trend in either patient group.

To assess physiologic imbalance and degree of illness, APACHE II scores were calculated on the day of, and at intervals after vaccination. The individual APACHE II scores calculated on the day of vaccination are shown in table 8. There were statistically significant differences between A and both C and R groups ($p = 0.001$ and 0.04 respectively, WRST), but not between C and R ($p = 0.08$, WRST). In followup evaluation during the course of the study, scores of both A and R patients showed little change, especially those at lower levels (figure 20). This indicated a relatively stable, but abnormal, physiologic state during the course of the study.

Although there were differences in hematological values between study groups, there was no change due to vaccination. Day 8 after vaccination was selected because of maximal in vivo antibody synthesis at this time. Patients tended to have higher leukocyte and neutrophil counts, and lower lymphocyte counts, worst in the A group. This was due to changes in variability rather than absolute changes in cell counts. There were no significant differences within each group in hematological values on day 0 and day 8. Differential cell counting showed no remarkable trend in numbers of monocytes, eosinophils, or basophils.

Total protein, albumin, and beta globulins were measured pre-vaccination and on days 8 and 28 post-vaccination. Before vaccination, total protein levels ± SEM were 7.2 ± 0.2, 7.2 ± 0.2, and 6.4 ± 0.4 for C, R and A groups (all $p > 0.05$, unpaired t-tests). Serum albumin in A subjects was significantly less than in C or R: 2.7 ± 0.2 compared to 4.2 ± 0.2 and 3.9 ± 0.3, respectively ($p = 0.0005$, $p = 0.004$, unpaired t-tests). Beta globulins were comparable in all groups at 1.1 ± 0.1, 1.1 ± 0.1 and 1.0 ± 0.1. There were no trends in concentrations of these serum proteins after vaccination in any group.

Immunoglobulin levels were also measured, and were elevated in patients (table 10). Serum gamma globulin, IgG, IgM and IgA concentrations were consistently higher in the R group than C, and highest in A, but not significantly so except for IgA levels in A compared to C at day 28 ($p = 0.04$, unpaired t-test). There was no trend in the Ig levels after vaccination.

TABLE 8: DEMOGRAPHIC DATA, DIAGNOSIS, SURGERY AND OUTCOME IN SUBJECTS VACCINATED WITH PNEUMOCOCCAL POLYSACCHARIDE

CONTROL SUBJECTS

CL	AGE	SX	STS	APE
LC	59	F	31	3
LC	27	M	31	0
LC	69	M	32	6
LC	53	F	35	2
LC	69	M	33	5
LC	23	F	20	0
LC	33	M	25	0
LC	27	M	72	0

DTH REACTIVE SUBJECTS

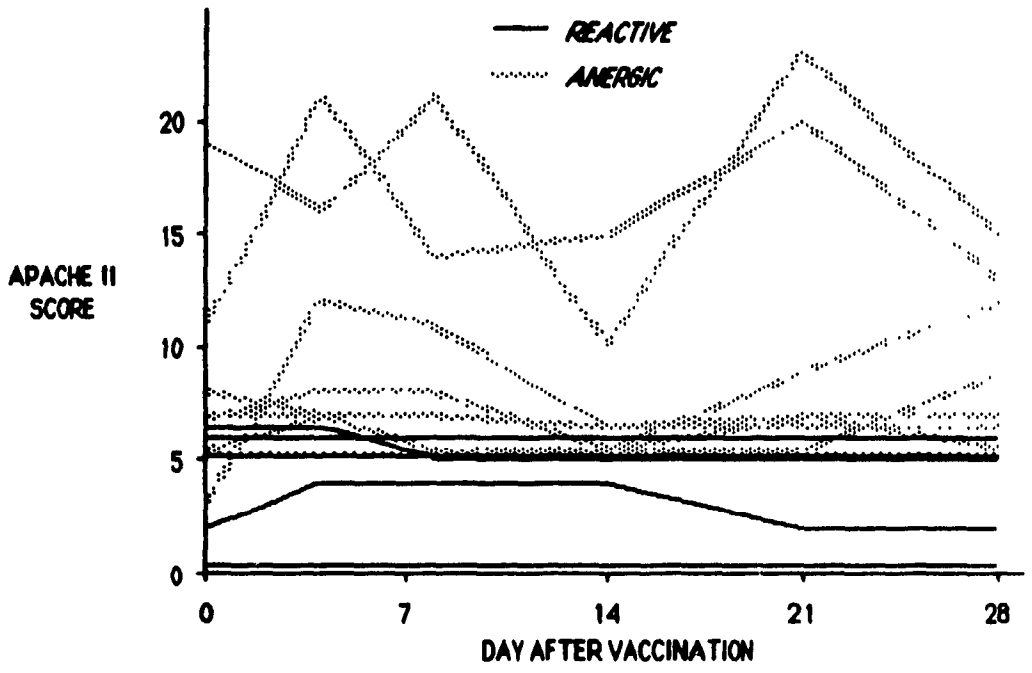
CL	AGE	SX	STS	APE	DIAGNOSIS	OPERATION	DAY	DEG	SEP	OUTCOME
HR	48	M	73	2	Biliary Fistula	Fistula Closure	-42	3	+	Alive
WR	76	F	10	6	Colon Carcinoma	Hemicolectomy	+7	3	-	Alive
WR	42	M	36	0	Cellulitis	-	-	-	-	Alive
WR	70	M	10	5	Pancreatic Carcinoma	Pancreaticoduodenectomy	+29	4	-	Alive
WR	62	M	49	6	Incisional Hernia	Herniorraphy	+6	1	-	Alive

DTH ANERGIC SUBJECTS

CL	AGE	SX	STS	APE	DIAGNOSIS	OPERATION	DAY	DEG	SEP	OUTCOME
HA	67	F	0	6	Colonic Stricture	Sigmoid Colectomy	-15	2	+	Alive
IA	70	M	0	19	Post-operative Stroke	Aortocoronary Bypass	-36	4	-	Dead
IA	47	M	0	3	Pharyngeal Carcinoma	-	-	-	-	Alive
IA	73	M	0	11	Mediastinitis	Aortic Valve Replacement	-13	4	+	Alive
IA	79	M	0	8	Gastric Obstruction	Gastrojejunostomy	+24	2	+	Dead
WA	73	F	0	5	Ampullary Stenosis	Sphincteroplasty	+20	3	-	Alive
WA	70	M	0	7	Ampullary Carcinoma	Pancreaticoduodenectomy	+3	4	+	Dead

Demographic data, STS (obtained just before vaccination), APACHE II scores (APE, calculated on the day of vaccination), diagnosis and operation performed are listed for R subjects grouped according to sub-classification. The degree (DEG) of operations is shown. The timing of surgery in relation to vaccination is shown in number of days the operation occurred after (+) or before (-) vaccination. Outcome was based on condition at hospital discharge, and the presence or absence of major sepsis (SEP) at any time during hospitalization.

FIGURE 20: EVOLUTION OF APACHE II SCORES AFTER PNEUMOCOCCAL POLYSACCHARIDE VACCINATION



Five DTH R and 7 A patients were vaccinated with PPS on day 0. The APACHE II scores were calculated on the day of vaccination, and at intervals thereafter. Both groups had abnormal physiologic conditions at the time of vaccination, worse in the A patients. There was little change over the 4 week period during study of the antibody response.

TABLE 9: CIRCULATING BLOOD CELL COUNTS BEFORE AND AFTER PNEUMOCOCCAL POLYSACCHARIDE VACCINATION

	LEUKOCYTE	NEUTROPHIL	LYMPHOCYTE	MONOCYTE	EOSINO	BASO
DAY 0						
C	5693 ± 671	3243 ± 526	1743 ± 52	563 ± 138	253 ± 97	53 ± 18
R	6964 ± 1344	4830 ± 946	1470 ± 363	472 ± 100	114 ± 54	14 ± 7
A	11427 ± 1768	8913 ± 1698	1190 ± 249	607 ± 69	262 ± 64	43 ± 15
DAY 8						
C	5590 ± 684	3035 ± 491	1915 ± 159	320 ± 51	195 ± 44	53 ± 8
R	6534 ± 1389	4846 ± 1210	*1038 ± 173	342 ± 81	116 ± 9	70 ± 35
A	*10000 ± 992	*7635 ± 1032	*1095 ± 164	*#743 ± 103	190 ± 39	28 ± 7

Clinical laboratory data were collected before (DAY 0) and after (DAY 8) immunization of 8 C, 5 R and 7 A subjects. Data are shown as arithmetic means ± SEM of cells/mm^3. Utilizing data from the same day, * indicates $p < 0.05$ compared to LC, and # $p < 0.05$ compared to R (unpaired t-tests). There were no significant differences within each group comparing pre- to post-immunization values.

TABLE 10: SERUM IMMUNOGLOBULIN LEVELS BEFORE AND AFTER PNEUMOCOCCAL POLYSACCHARIDE VACCINATION

	GAMMA GLOBULINS	IgG	IgM	IgA
DAY 0				
CONTROL	10.3 ± 1.0	8.9 ± 0.8	1.1 ± 0.4	2.0 ± 0.3
REACTIVE	12.4 ± 1.3	11.4 ± 1 2	1.1 ± 0.2	2.6 ± 0.6
ANERGIC	15.4 ± 3.5	14 3 ± 2.7	1.8 ± 0.5	3.8 ± 0.7
DAY 8				
CONTROL	11.5 ± 0.1	13.3 ± 3.4	0.5 ± 0.3	1.94 ± 0 2
REACTIVE	12.0 ± 1.9	9 1 ± 1.7	1.1 ± 0 3	2.10 ± 0 6
ANERGIC	12.7 ± 1.9	12 3 ± 2.1	1.5 ± 0.4	2 90 ± 0.6
DAY 28				
CONTROL	11.3 ± 1.4	10 4 ± 1.4	1.0 ± .5	2.2 ± .2
REACTIVE	12.4 ± 2.0	11 4 ± 1.9	1 9 ± .4	2.8 ± 5
ANERCIC	15.7 ± 1.4	12 8 ± 1 6	1.8 ± .3	*4 4 ± .7

The arithmetic mean and SEM of serum concentration of gamma globulins, IgG, IgM and IgA on the day of vaccination (DAY 0) and on days 8 and 28 are shown in g/l. Eight control, 5 reactive and 7 anergic patients were studied. * indicates $p < 0.05$ compared to control group (unpaired t test).

Serum antibody responses to PPS were normal in both groups of patients. The pre-and maximum post-immunization levels of isotype specific antibody are shown for each subject in figures 21 (IgG), 22 (IgM) and 23 (IgA). For all isotypes, the pre- and post-vaccination levels were comparable for all groups (all $p > 0.10$, WRST). Generally, as indicated by the slope of the lines for each class within the isotype, IgA responses were of the greatest relative magnitude, followed by IgG and IgM.

The average group data are shown in table 11. An isotype specific response was calculated for each subject as either the ratio or the difference between pre-, and maximal post-vaccination levels. There are no statistically significant differences between any groups for any isotype (all $p > 0.12$, WRST).

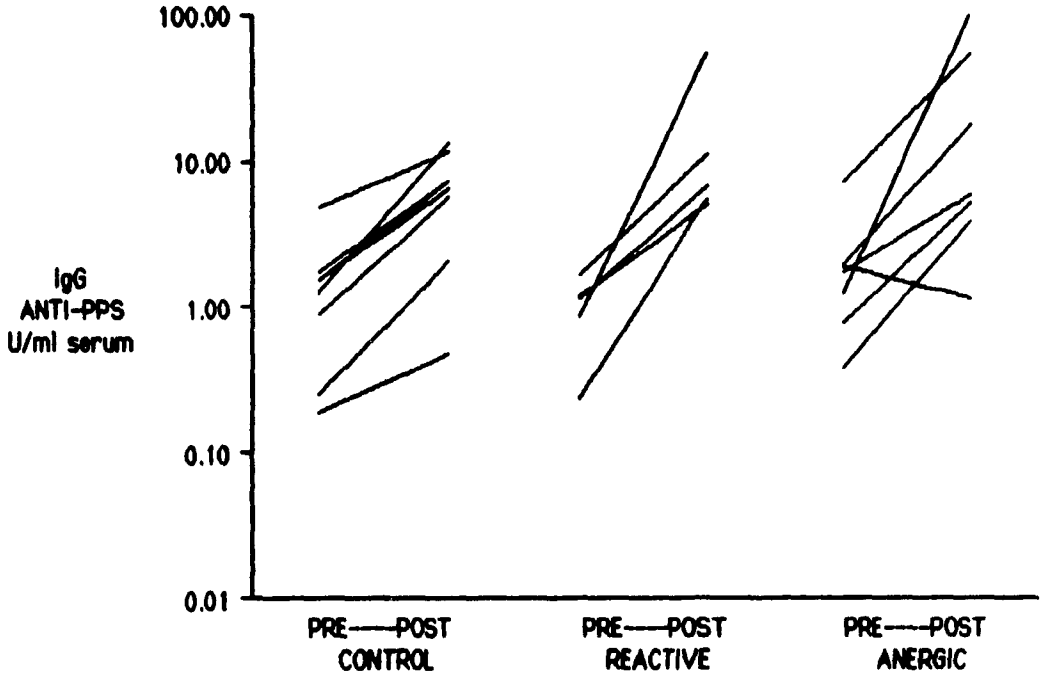
Arbitrarily defining $>= 2$ fold increase in antibody content as a positive response, the groups were also similar. Nearly all subjects (table 12) responded to vaccination ($X^2_2 <= 0.81$ with Yates, $p >= 0.67$, for all isotypes). A single A subject failed to produce antibody of any isotype after vaccination. The pre-vaccination levels of all isotypes were normal in this subject. Another A subject, and a single R subject, produced modest IgM responses.

1.2.1.3 RELATIONSHIP OF DTH TESTING, PHYSIOLOGIC ASSESSMENT AND OUTCOME TO IN VIVO ANTIBODY RESPONSES TO PNEUMOCOCCAL POLYSACCHARIDE

A single subject failed to produce antibody of any isotype after vaccination. This patient was immunized as an IA patient 13 days post-aortic valve replacement complicated by low cardiac output, renal failure, jaundice and sternal dehiscence secondary to mediastinitis. After a prolonged ICU course with high APACHE II scores, the patient was eventually discharged well. The best overall responses in the entire group were produced by another A subject. This patient was immunized as a WA 3 days before pancreatoduodenectomy for ampullary carcinoma. The post-operative course was complicated by an abdominal abscess and gastrocutaneous fistula, and the patient expired 2 months after surgery of septic-related complications. On an individual level, there is thus no relationship between antibody response to polysaccharide antigens, and the ability of the host to deal effectively with a major septic challenge.

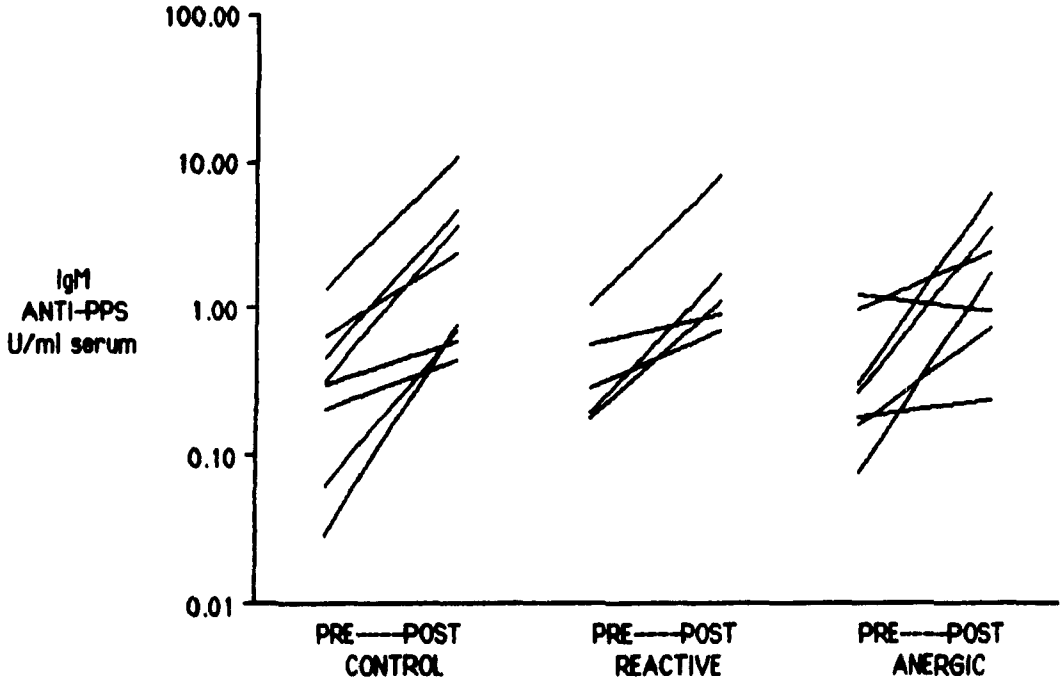
No trend in antibody responses was evident in relation to timing of surgery, age or sex.

FIGURE 21: PATIENT *IN VIVO* IgG ANTIBODY RESPONSE TO PNEUMOCOCCAL POLYSACCHARIDE



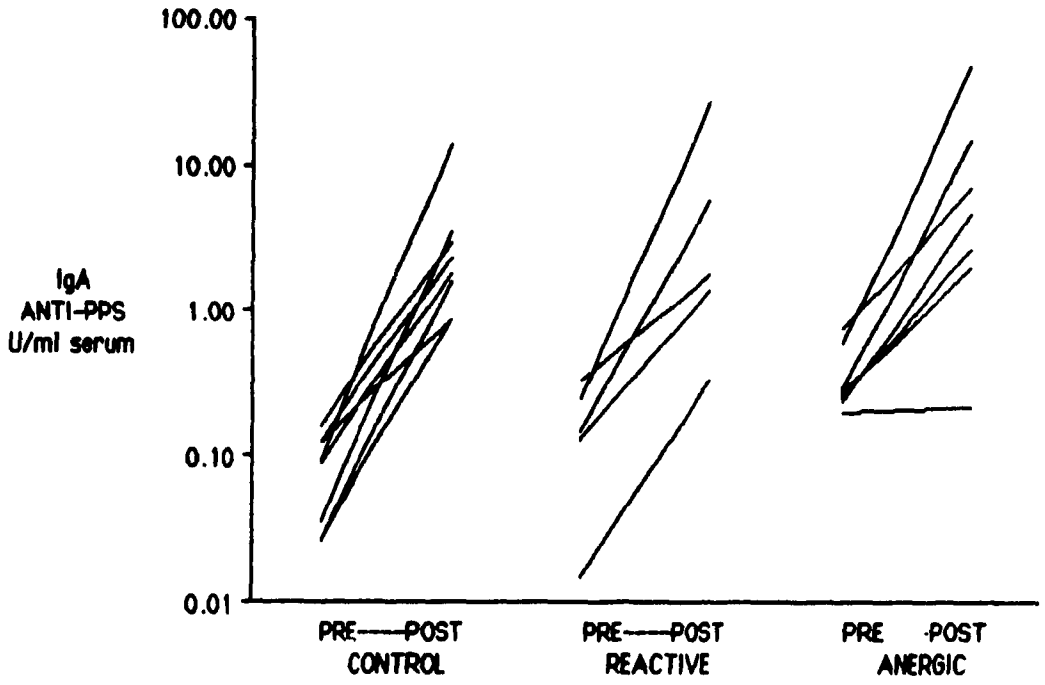
Eight LC, 5 R and 7 A subjects were immunized with PPS on day 0. The serum IgG anti-PPS is shown for each subject as a line connecting the pre-immunization level to the maximal amount of antibody detected within 28 days post-vaccination. Pre-, and post-vaccination serum IgG anti-PPS levels were comparable for all groups (all $p > 0.10$, WRST).

FIGURE 22: PATIENT *IN VIVO* IgM ANTIBODY RESPONSE TO PNEUMOCOCCAL POLYSACCHARIDE



Eight LC, 5 R and 7 A subjects were immunized with PPS on day 0. The serum IgM anti-PPS is shown for each subject as a line connecting the pre-immunization level to the maximal amount of antibody detected within 28 days post-vaccination. Pre-, and post-vaccination serum IgM anti-PPS levels were comparable for all groups (all $p > 0.10$, WRST).

FIGURE 23: PATIENT *IN VIVO* IgA ANTIBODY RESPONSE TO PNEUMOCOCCAL POLYSACCHARIDE



Eight LC, 5 R and 7 A subjects were immunized with PPS on day 0. The serum IgA anti-PPS is shown for each subject as a line connecting the pre-immunization level to the maximal amount of antibody detected within 28 days post-vaccination. Pre-, and post-vaccination serum IgA anti-PPS levels were comparable for all groups (all $p > 0.10$, WRST).

TABLE 11: PATIENT *IN VIVO* ANTI-PNEUMOCOCCAL POLYSACCHARIDE ANTIBODY RESPONSES

ISOTYPE	IgG	IgM	IgA
RATIO			
CONTROL	4.89 x÷ 0.08	6.86 x÷ 0.14	33.02 x÷ 0.16
DTH REACTIVE	12.17 x÷ 0.22	4.36 x÷ 0.14	25.21 x÷ 0.22
DTH ANERGIC	7.08 x÷ 0.24	4.91 x÷ 0.22	12.62 x÷ 0.24
DIFFERENCE			
CONTROL	3.82 x÷ 0.18	1.30 x÷ 0.20	2.17 x÷ 0.14
DTH REACTIVE	9.10 x÷ 0.21	1.07 x÷ 0.23	2.48 x÷ 0.32
DTH ANERGIC	4.83 x÷ 0.49	0.56 x÷ 0.38	2.79 x÷ 0.40

The antibody response for 8 control, 5 DTH R and 7 A subjects was calculated as either the ratio or the difference between the peak post-immunization antibody level achieved within 28 days, and pre-immunization values. Geometric means x÷ SEM are shown. There were no statistically significant differences among the groups in either table ($p > 0.05$, WRST).

TABLE 12: *IN VIVO* POSITIVE RESPONSE RATES TO PNEUMOCOCCAL POLYSACCHARIDE VACCINATION

GROUP	N	IgG	IgM	IgA
CONTROL	8	8	8	8
DTH REACTIVE	5	5	4	5
DTH ANERGIC	7	6	5	6

The number in each group of control, DTH reactive and DTH anergic subjects showing a positive response (greater than a 2 fold increase in antibody) in each isotype is shown. There are no statistically significant differences among the groups ($X^2_2 <= 0.81$, $p >= 0.67$ for all isotypes).

1.2.2 IN VITRO SPECIFIC AND TOTAL IMMUNOGLOBULIN SYNTHESIS AFTER PNEUMOCOCCAL POLYSACCHARIDE VACCINATION

Before and at intervals after vaccination, blood MNCs were harvested and cultured *in vitro* under standard conditions for 10 days. The cultures were either unstimulated, for study of spontaneous synthesis, or stimulated with PWM. The supernatants were assayed both for cumulative content of anti-PPS specific, and total, IgG, IgM and IgA.

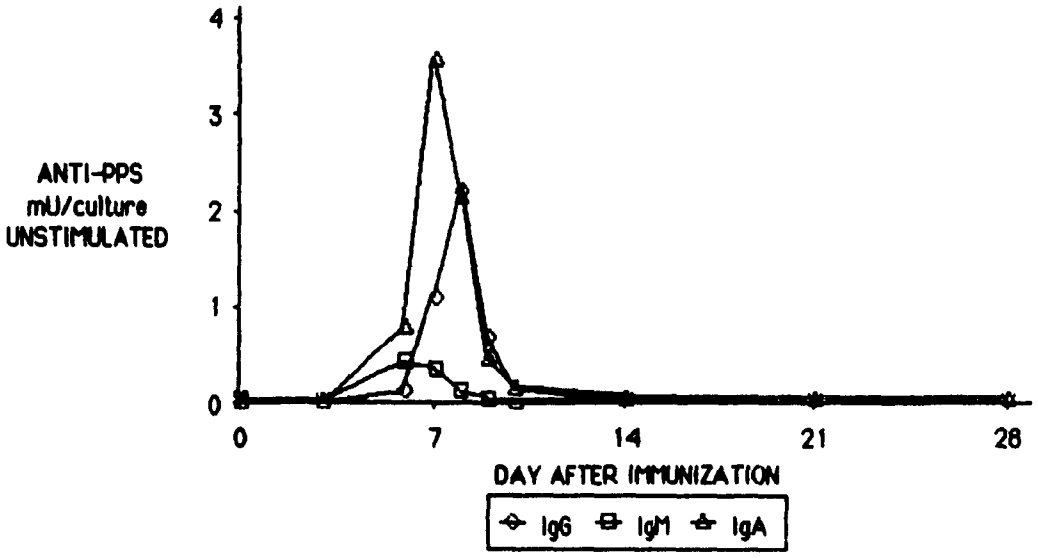
1.2.2.1 NORMAL IN VITRO SPECIFIC AND TOTAL IMMUNOGLOBULIN SYNTHESIS AFTER PNEUMOCOCCAL POLYSACCHARIDE VACCINATION

Four LC subjects were initially studied. Before and at 3 days after vaccination, no subjects produced antibody in unstimulated blood MNC cultures. Spontaneous antibody secretion then began, with a mean peak on days 6, 7 and 8 for IgM, IgA and IgG respectively (figure 24). The order of these peaks paralleled the appearance of isotype-specific antibody in the blood of the same subjects (figure 19). Lesser quantities of antibody were produced by MNC after the peaks, with no detectable spontaneous *in vitro* synthesis from day 14 onward. The addition of 10^{-4}M emetine at the initiation of cultures eliminated detectable antibody synthesis.

The amounts of specific antibody produced spontaneously by blood MNCs were insufficient to account for the total amount of new antibody in the serum. Considering the cultured cells to be representative of the circulating population at any given time, and assuming production of the maximum amount of IgG anti-PPS detected in 10 day *in vitro* cultures each day for a week *in vivo*, less than 3% of the total amount of new antibody in the serum could be accounted for.

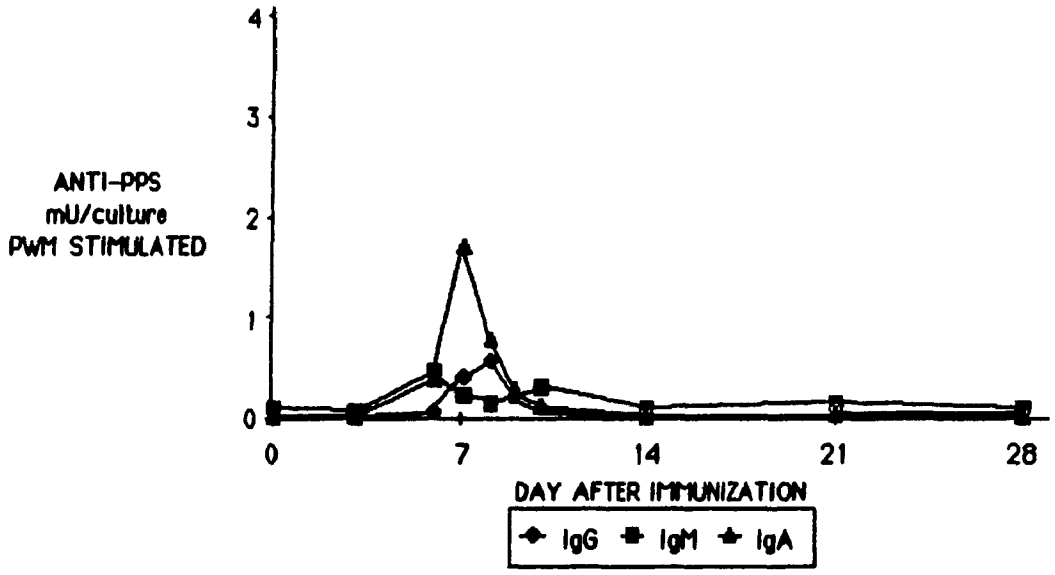
In PWM stimulated cultures, peak isotype specific synthesis followed the same temporal pattern as spontaneous synthesis. Maximal quantities of IgM, IgA and IgG anti-PPS were produced on days 6, 7 and 8 respectively (figure 25). Little IgM was produced compared to IgG and IgA. PWM stimulation reduced specific IgG and IgA synthesis on all occasions (figures 26 and 28). After day 14, when spontaneous synthesis had stopped, PWM stimulated cultures failed to produce IgG or IgA antibody. For IgM, PWM stimulation did not decrease synthesis during peak spontaneous production (figure 27). After spontaneous

FIGURE 24: NORMAL SPONTANEOUS ANTI-PNEUMOCOCCAL POLYSACCHARIDE ANTIBODY SYNTHESIS *IN VITRO* AFTER VACCINATION *IN VIVO*



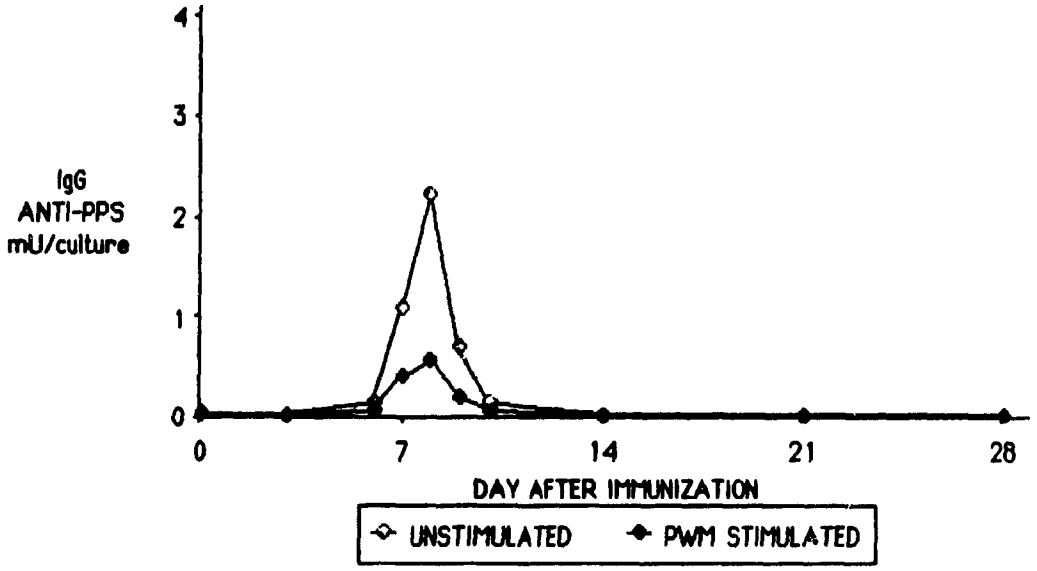
After PPS vaccination of 4 LC subjects on day 0, blood MNCs were harvested at intervals, and cultured *in vitro* in unstimulated cultures under standard conditions for 10 days. Culture supernatants were assayed for amounts of IgG, IgM and IgA anti-PPS. Geometric means are shown. There are differences between the isotypes in the amounts produced (IgA > IgG > IgM), and in the order of peak appearence (IgM > IgA > IgG)

FIGURE 25: NORMAL PWM STIMULATED ANTI-PNEUMOCOCCAL POLYSACCHARIDE ANTIBODY SYNTHESIS *IN VITRO* AFTER VACCINATION *IN VIVO*



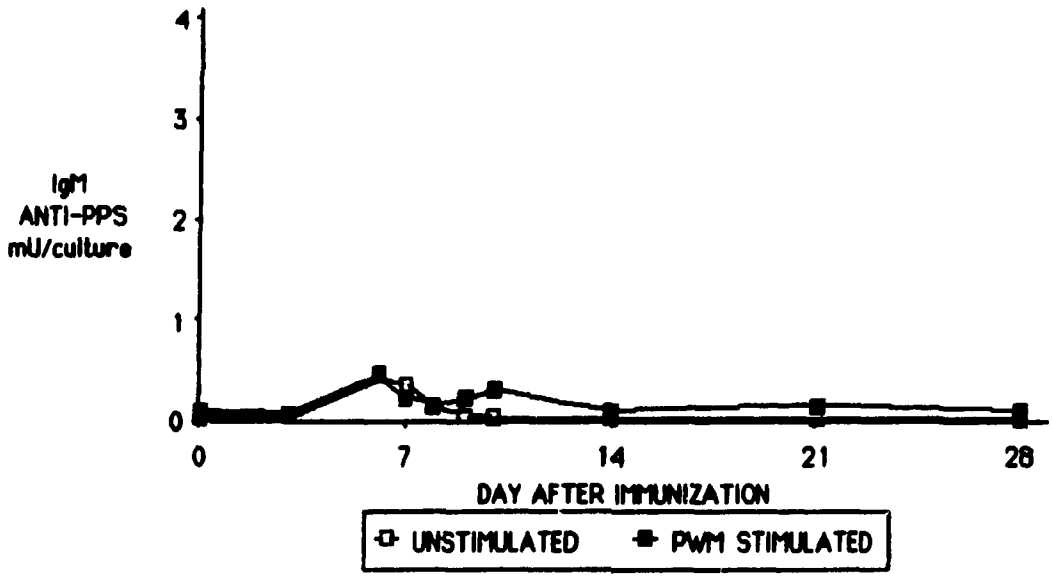
After PPS vaccination of 4 LC subjects on day 0, blood MNCs were harvested at intervals, and cultured *in vitro* in PWM stimulated cultures under standard conditions for 10 days. Culture supernatants were assayed for amounts of IgG, IgM and IgA anti-PPS. Geometric means are shown. PWM stimulation did not change the kinetics of isotype appearance, and after spontaneous synthesis had ceased, caused the sythesis of small amounts of only IgM anti-PPS.

FIGURE 26: NORMAL IgG ANTI-PNEUMOCOCCAL POLYSACCHARIDE ANTIBODY SYNTHESIS *IN VITRO* AFTER VACCINATION *IN VIVO*



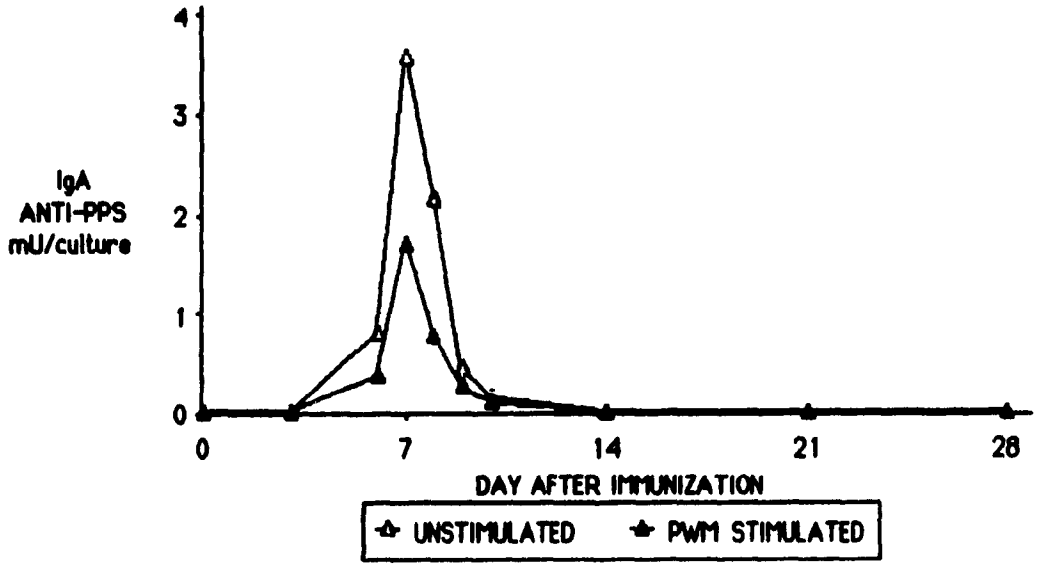
After PPS vaccination of 4 LC subjects on day 0, blood MNCs were harvested at intervals, and cultured *in vitro* in unstimulated, or PWM stimulated cultures under standard conditions for 10 days. Culture supernatants were assayed for amounts of IgG anti-PPS. Geometric means are shown. PWM stimulation reduced the amount of IgG anti-PPS during the period of spontaneous synthesis.

FIGURE 27: NORMAL IgM ANTI-PNEUMOCOCCAL POLYSACCHARIDE ANTIBODY SYNTHESIS *IN VITRO* AFTER VACCINATION *IN VIVO*



After PPS vaccination of 4 LC subjects on day 0, blood MNCs were harvested at intervals, and cultured *in vitro* in unstimulated, or PWM stimulated cultures under standard conditions for 10 days. Culture supernatants were assayed for amounts of IgM anti-PPS. Geometric means are shown. PWM stimulation had no effect on the synthesis of IgM anti-PPS during the period of spontaneous synthesis.

FIGURE 28: NORMAL IgA ANTI-PNEUMOCOCCAL POLYSACCHARIDE ANTIBODY SYNTHESIS *IN VITRO* AFTER VACCINATION *IN VIVO*



After PPS vaccination of 4 LC subjects on day 0, blood MNCs were harvested at intervals, and cultured *in vitro* in unstimulated, or PWM stimulated cultures under standard conditions for 10 days. Culture supernatants were assayed for amounts of IgA anti-PPS. Geometric means are shown. PWM stimulation reduced the amount of IgA anti-PPS during the period of spontaneous synthesis.

synthesis had stopped, stimulated cultures continued to produce small but detectable amounts of IgM anti-PPS.

The same culture supernatants were assayed for total isotype specific Ig. The baseline level of spontaneous IgG synthesis is higher than IgA or IgM (figure 29). During the time of peak spontaneous *in vitro* synthesis of specific antibody, there were peaks of total IgG and IgA synthesis in unstimulated cultures (figure 29). The IgA peak occurred on day 6, and the IgG on day 7, both one day earlier than maximal specific antibody synthesis *in vitro* (figure 24). Little change occurred in spontaneous total IgM synthesis.

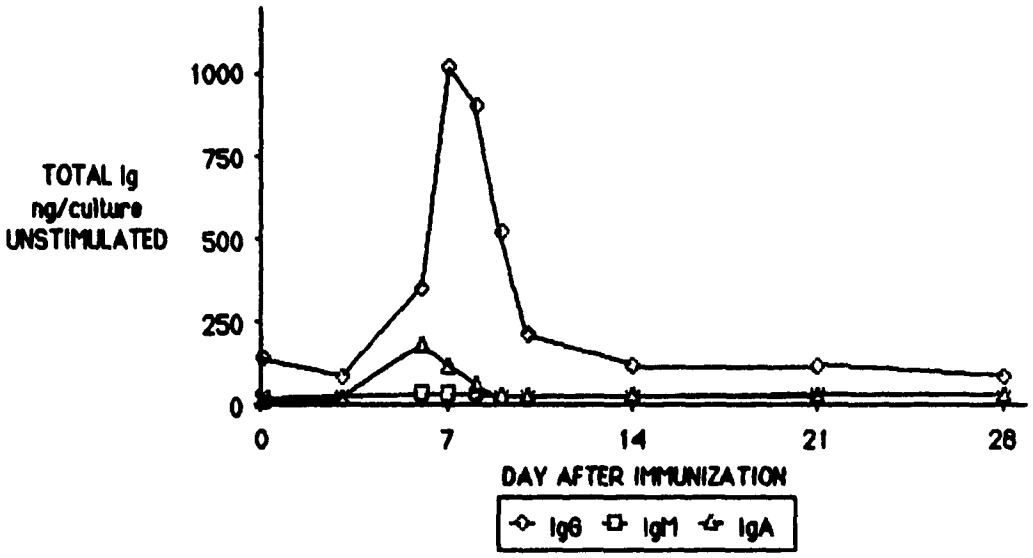
In PWM stimulated cultures, again most IgG was produced, followed by IgA and IgM (figure 30). Approximately day 7, at the time of maximal spontaneous specific and total Ig synthesis *in vitro*, there was a nadir in the PWM stimulated synthesis of all three isotypes. This returned to baseline levels within a few days. For a short time, however, less total IgG (figure 31) and IgA (figure 33) was produced by stimulated compared to unstimulated cells, a reversal of the normal response. For IgM, this reversal did not occur, although there was a similar nadir in stimulated synthesis (figure 32).

In summary, circulating MNCs produce specific antibody *in vitro* after *in vivo* vaccination. The kinetics and isotype pattern of *in vitro* synthesis parallel serum antibody levels. There are differences among the isotypes in the pattern of both total and specific immunoglobulin synthesis after vaccination, and in the response to PWM stimulation. Generally, IgG and IgA are comparable to each other, while IgM was different.

1.2.2.2 PATIENT IN VITRO SPECIFIC AND TOTAL IMMMUNOGLOBULIN SYNTHESIS AFTER PNEUMOCOCCAL POLYSACCHARIDE VACCINATION

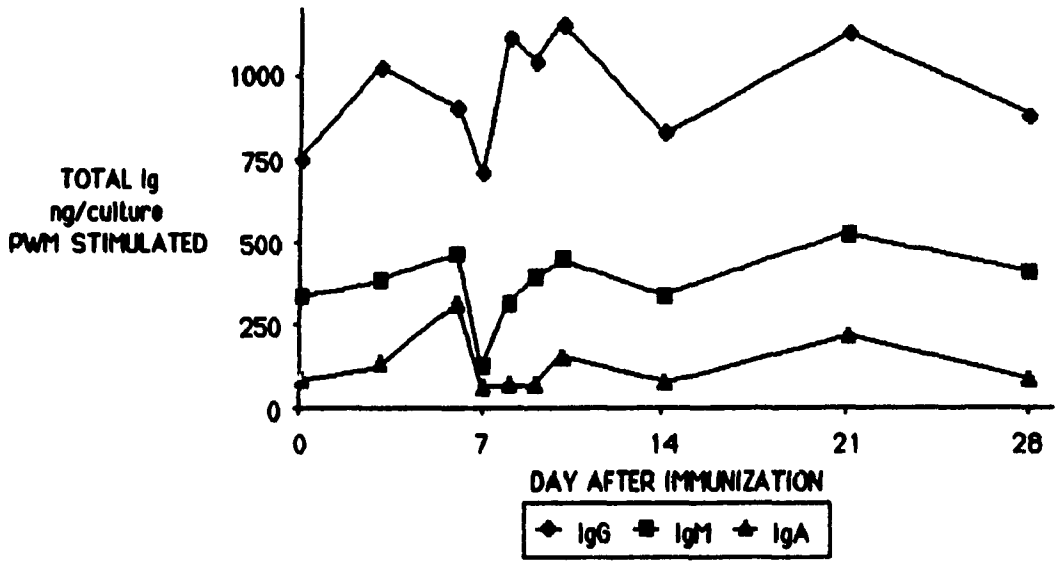
Blood MNCs from 8 LC, 5 R and 7 A patients were harvested on days 0, 6 and 8 after PPS vaccination. These days were selected as the most likely to give peak *in vitro* synthesis of specific antibody for the three isotypes, based on the data from normal subjects above. For subsequent analysis, all PWM stimulated and unstimulated data for each individual for each isotype was taken from either day 6 or 8, selected on the basis of peak spontaneous synthesis of antibody for that isotype in that individual.

FIGURE 29: NORMAL SPONTANEOUS TOTAL IMMUNOGLOBULIN SYNTHESIS *IN VITRO* AFTER PNEUMOCOCCAL POLYSACCHARIDE VACCINATION *IN VIVO*



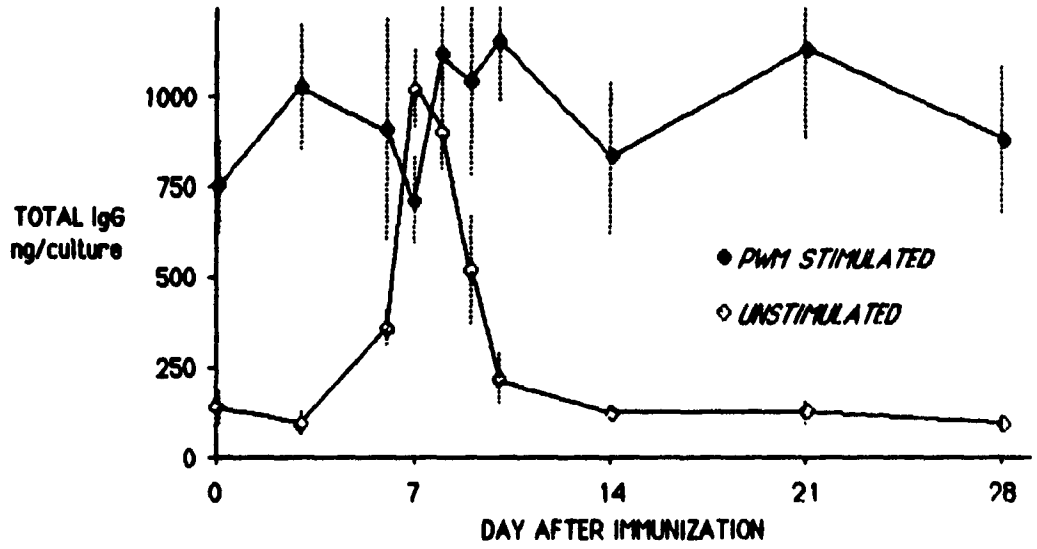
After PPS vaccination of 4 LC subjects on day 0, blood MNCs were harvested at intervals, and cultured *in vitro* in unstimulated cultures under standard conditions for 10 days. Culture supernatants were assayed for amounts of total IgG, IgM and IgA. Geometric means are shown. There are peaks of IgG and IgA synthesis, that occur the day before the peak synthesis of specific anti-PPS of these isotypes.

FIGURE 30: NORMAL PWM STIMULATED TOTAL IMMUNOGLOBULIN SYNTHESIS *IN VITRO* AFTER PNEUMOCOCCAL POLYSACCHARIDE VACCINATION *IN VIVO*



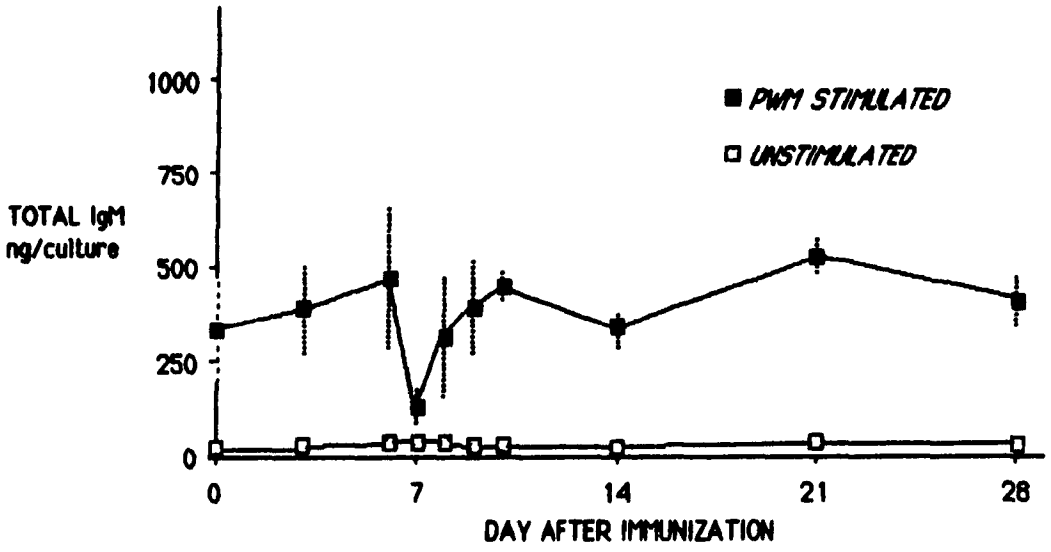
After PPS vaccination of 4 LC subjects on day 0, blood MNCs were harvested at intervals, and cultured *in vitro* in PWM stimulated cultures under standard conditions for 10 days. Culture supernatants were assayed for amounts of total IgG, IgM and IgA. Geometric means are shown. There was a nadir in synthesis for all isotypes, at approximately the time of peak spontaneous specific antibody synthesis (figure 24).

FIGURE 31: NORMAL TOTAL IgG SYNTHESIS *IN VITRO* AFTER PNEUMOCOCCAL POLYSACCHARIDE VACCINATION *IN VIVO*



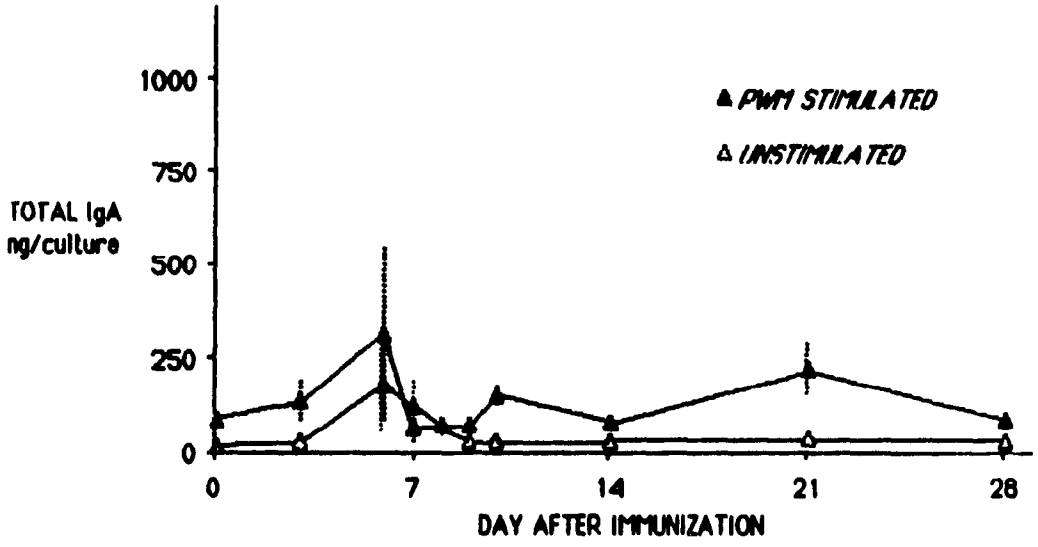
After PPS vaccination of 4 LC subjects on day 0, blood MNCs were harvested at intervals, and cultured *in vitro* in unstimulated, or PWM stimulated cultures under standard conditions for 10 days. Culture supernatants were assayed for amounts of total IgG. Arithmetic means ± SEMs are shown. PWM stimulation reduces total IgG synthesis below spontaneous levels at the time of peak spontaneous production after vaccination.

FIGURE 32: NORMAL TOTAL IgM SYNTHESIS *IN VITRO* AFTER PNEUMOCOCCAL POLYSACCHARIDE VACCINATION *IN VIVO*



After PPS vaccination of 4 LC subjects on day 0, blood MNCs were harvested at intervals, and cultured *in vitro* in unstimulated, or PWM stimulated cultures under standard conditions for 10 days. Culture supernatants were assayed for amounts of total IgM. Arithmetic means $\pm$ SEMs are shown. PWM stimulated IgM synthesis was reduced, but not below spontaneous levels, at the time of peak spontaneous production after vaccination.

FIGURE 33: NORMAL TOTAL IgA SYNTHESIS *IN VITRO* AFTER PNEUMOCOCCAL POLYSACCHARIDE VACCINATION *IN VIVO*



After PPS vaccination of 4 LC subjects on day 0, blood MNCs were harvested at intervals, and cultured *in vitro* in unstimulated, or PWM stimulated cultures under standard conditions for 10 days. Culture supernatants were assayed for amounts of total IgA. Arithmetic means $\pm$ SEMs are shown. PWM stimulation reduces total IgA synthesis to spontaneous levels at the time of peak spontaneous production after vaccination.

In vitro synthesis of specific antibody was comparable among the groups. In unstimulated cultures, no subject produced significant amounts of antibody *in vitro* before immunization. Table 13 shows the mean peak quantities of specific antibody produced *in vitro* after vaccination on either day 6 or 8. Although there was a general trend for R subjects to produce less than C, and A less than R, there were no statistically significant differences between the groups (all $p > 0.10$, WRST).

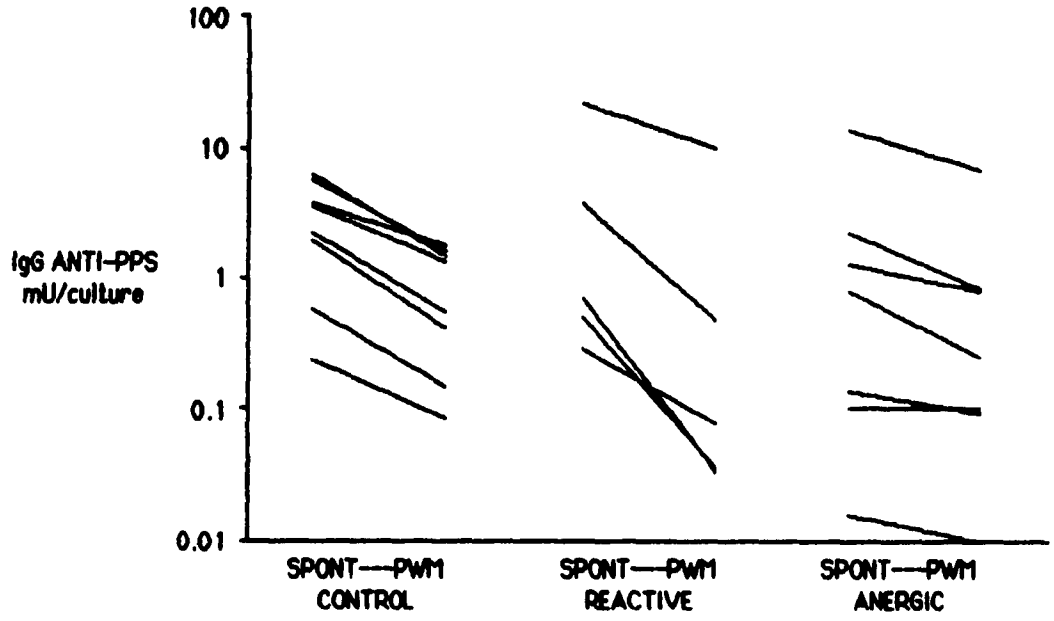
In PWM stimulated cultures, there was sporadic synthesis of specific antibody by MNC harvested before vaccination. One LC subject produced detectable quantities of IgM and IgG anti-PPS, while an R patient produced small amounts of IgM and IgA. The trend noted above of patients to produce less antibody *in vitro* than C was less evident. The only statistically significant difference was between C and R synthesis of IgA anti-PPS ($p = 0.04$, WRST).

During peak spontaneous antibody synthesis, PWM stimulation decreased synthesis of IgG and IgA anti-PPS, but not IgM. For all groups, differences were significant for IgG and IgA ($p < 0.05$, or < 0.10 for R due to statistical limitation, WSRT). For IgG, MNCs from every subject in all groups produced less anti-PPS when stimulated (figure 34). For IgA, all subjects except 2 A patients had a similar negative response to PWM (figure 36). For IgM anti-PPS, a different pattern occurred (figure 35). The magnitude of the differences were smaller than for IgG or IgA. Only 3/8, 2/5 and 5/7 subjects in C, R and A groups respectively produced less antibody with PWM ($p = 0.04$ for A only, WSRT).

The temporal pattern of maximal specific antibody synthesis *in vitro* was examined. A similar pattern was observed among all groups; most subjects produced more specific IgG on day 8 than 6, more IgM on day 6 than 8, and mixed distribution of peaks for IgA. This parallels the observations on the timing of the peak *in vitro* synthesis of the various isotypes in the initial LC experiments; day 6, 7 and 8 respectively for IgM, IgA and IgG.

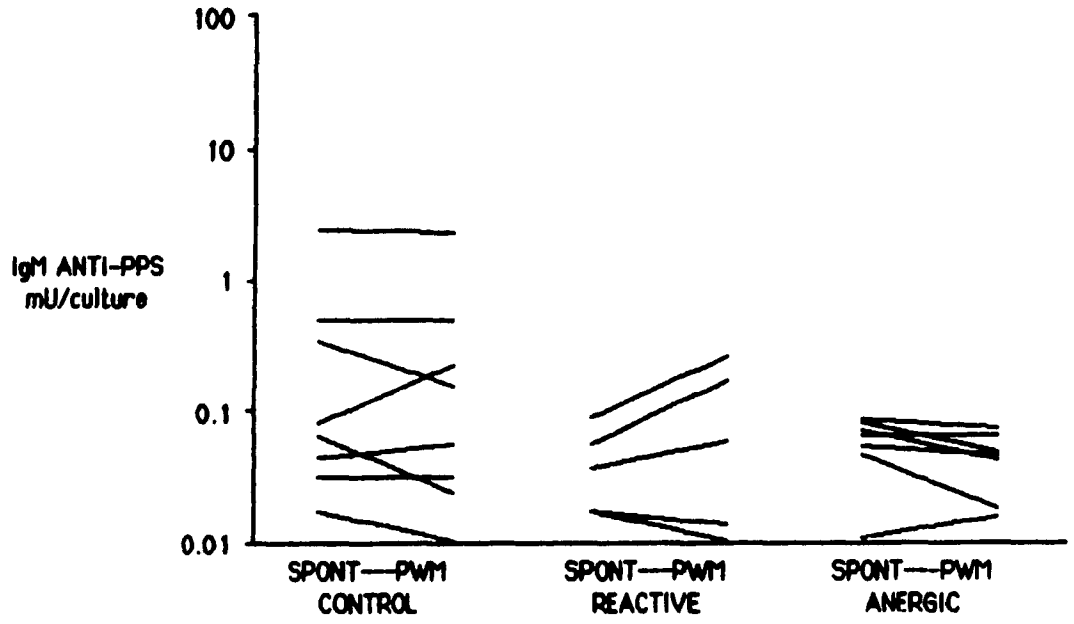
The same culture supernatants were assayed for cumulative total IgG, IgM and IgA content. The data are shown in figures 37, 38 and 39, and in table 14 as quantity of each isotype, and the sum of all three isotypes. The post-vaccination data are taken from the day of peak spontaneous antibody synthesis for each isotype in each individual. In unstimulated cultures, there were no statistically significant differences between the groups before or after vaccination. The trend, however, was for MNC from patients to produce more total IgG and IgA, but not IgM, than C subjects in pre-vaccination cultures. Anergic subjects produced

FIGURE 34: PATIENT IgG ANTIBODY SYNTHESIS AFTER PNEUMOCOCCAL POLYSACCHARIDE VACCINATION *IN VIVO*



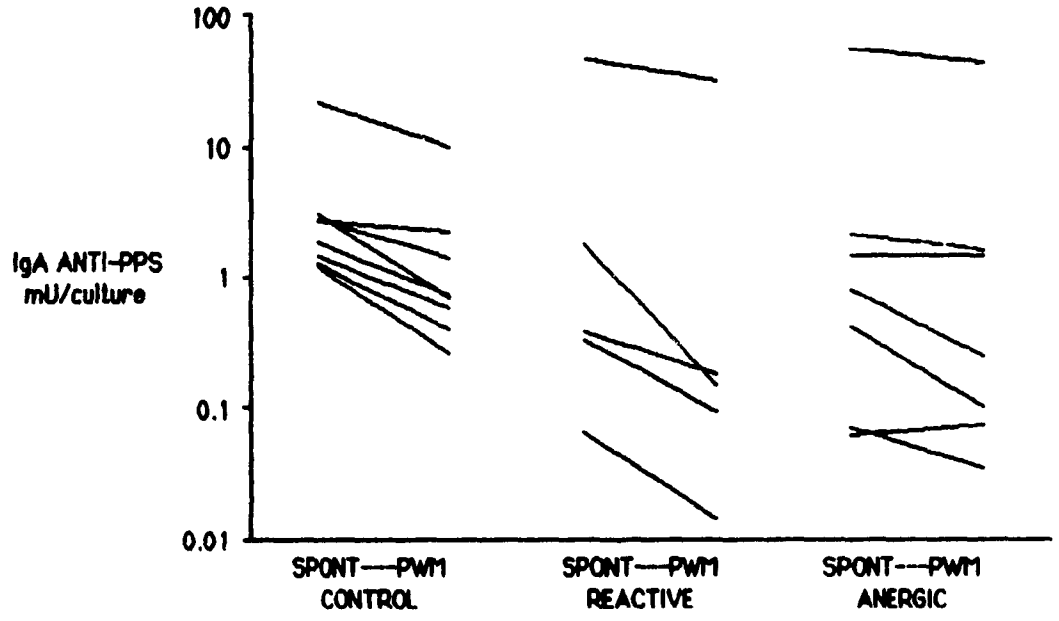
Eight LC, 5 R and 7 A subjects were immunized with PPS on day 0. Blood MNCs were harvested on days 6 and 8, and cultured *in vitro* for 10 days. Culture supernatants were assayed for content of IgG anti-PPS. The maximum on either day 6 or 8 was selected. The data are shown for each subject as a line connecting the amounts produced either without (SPONT) or with PWM stimulation (PWM). Similar quantities of IgG anti-PPS were produced by all groups ($p > 0.10$, WRST). Stimulation reduced the synthesis in all groups ($p < 0.05$, WSRT).

FIGURE 35: PATIENT IgM ANTIBODY SYNTHESIS AFTER PNEUMOCOCCAL POLYSACCHARIDE VACCINATION *IN VIVO*



Eight LC, 5 R and 7 A subjects were immunized with PPS on day 0. Blood MNCs were harvested on days 6 and 8, and cultured *in vitro* for 10 days. Culture supernatants were assayed for content of IgM anti-PPS. The maximum on either day 6 or 8 was selected. The data are shown for each subject as a line connecting the amounts produced either without (SPONT) or with PWM stimulation (PWM). Although IgM anti-PPS was decreased some in patients, the differences were not significant ($p > 0.10$, WRST). Stimulation had no effect ($p > 0.10$, WSRT).

FIGURE 36: PATIENT IgA ANTIBODY SYNTHESIS AFTER PNEUMOCOCCAL POLYSACCHARIDE VACCINATION *IN VIVO*



Eight LC, 5 R and 7 A subjects were immunized with PPS on day 0. Blood MNCs were harvested on days 6 and 8, and cultured *in vitro* for 10 days. Culture supernatants were assayed for content of IgA anti-PPS. The maximum on either day 6 or 8 was selected. The data are shown for each subject as a line connecting the amounts produced either without (SPONT) or with PWM stimulation (PWM). Similar quantities of IgA anti-PPS were produced by all groups ($p > 0.10$, WRST). Stimulation reduced the synthesis in all groups ($p < 0.05$, WSRT).

TABLE 13: *IN VITRO* ANTI-PNEUMOCOCCAL POLYSACCHARIDE ANTIBODY SYNTHESIS AFTER VACCINATION *IN VIVO*

ISOTYPE	N	IgG	IgM	IgA
UNSTIMULATED				
CONTROL	8	1.99 x± 0.18	0.12 x÷ 0.25	2.57 x÷ 0.14
DTH REACTIVE	5	1.50 x± 0.35	0.04 x÷ 0.14	0.91 x÷ 0.48
DTH ANERGIC	7	0.49 x± 0.37	0.05 x÷ 0.12	0.81 x÷ 0.38
PWM STIMULATED				
CONTROL	8	#0.57 x± 0.18	0.11 x÷ 0.27	#0.95 x÷ 0.18
DTH REACTIVE	5	#0.21 x± 0.47	0.05 x÷ 0.29	*#0.25 x÷ 0.56
DTH ANERGIC	7	#0.25 x± 0.36	0.03 x÷ 0.10	#0.48 x÷ 0.40

Blood MNC were harvested from immunized subjects 0, 6 and 8 days after vaccination, and cultured either with or without PWM. No subjects produced significant amounts of specific antibody *in vitro* on day 0 (data not shown). For post-immunization data, either day 6 or 8 was selected for each isotype in each subject on the basis of maximal spontaneous specific antibody synthesis, and all data for that isotype taken from these cultures. Geometric means x± SEM are shown (* indicates p = 0.04 compared to C group, WRST).

The effect of PWM on synthesis of specific Ig was compared by signed rank testing for each isotype in each subject group. For IgG and IgA, PWM stimulation significantly reduced synthesis for all groups (# indicates $p < 0.05$ for comparison with unstimulated results for same isotype and group, WSRT). The pattern for IgM was variable among the groups; little overall effect was present.

most. After vaccination, there were 3 to 9 fold increases in IgG and IgA synthesis among the groups, and less than 2 fold increases in IgM. In PWM stimulated cultures, the only statistically significant difference was between A and both C and R groups for IgM synthesis ($p = 0.01$ and 0.02 respectively, unpaired t-test). Generally, all groups produced more of all isotypes in stimulated cultures after vaccination than before, although these differences were not large.

Before vaccination, there were differences in the pattern of total Ig synthesis in response to PWM stimulation. Patients with reduced DTH reactivity generally gave insignificant responses of all isotypes to stimulation (figures 37, 38 and 39 for IgG, IgM and IgA). This was in contrast to LC MNCs, which always produced more of all isotypes with PWM. Skin test R patients showed an intermediate pattern.

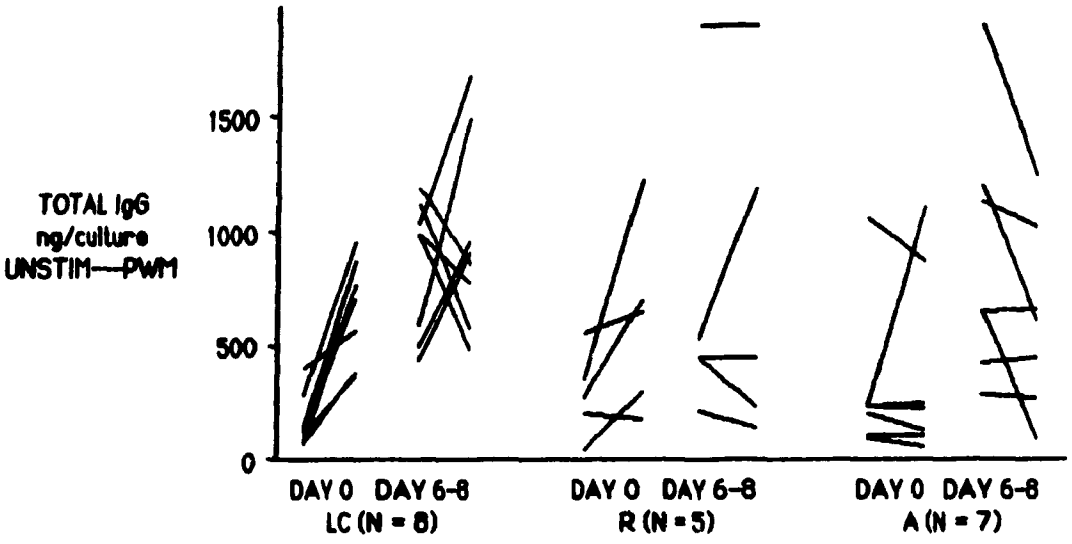
The effect of vaccination on PWM stimulated total Ig synthesis was different for each isotype. In all groups after vaccination, PWM decreased total IgG synthesis in several individuals (figure 37). IgM synthesis was not affected by vaccination (figure 38). Nearly all LC and R subjects continued to show positive responses to PWM, while A subjects remained unresponsive. For IgA synthesis, patterns were not markedly different from pre-vaccination, apart from a general increase in synthesis (figure 39)

In summary, quantities of anti-PPS produced *in vitro* by patient MNCs were normal. PWM stimulation decreased IgG and IgA antibody synthesis, but not IgM. Vaccination caused a change in the pattern of total Ig synthesis. Generally more total Ig was produced spontaneously. Stimulation with PWM caused a decrease in synthesis of IgG by post-vaccination cells. This occurred to a lesser degree with IgA, and not at all with IgM.

1.2.2.3 CORRELATION BETWEEN IN VIVO AND IN VITRO RESPONSES TO PNEUMOCOCCAL POLYSACCHARIDE

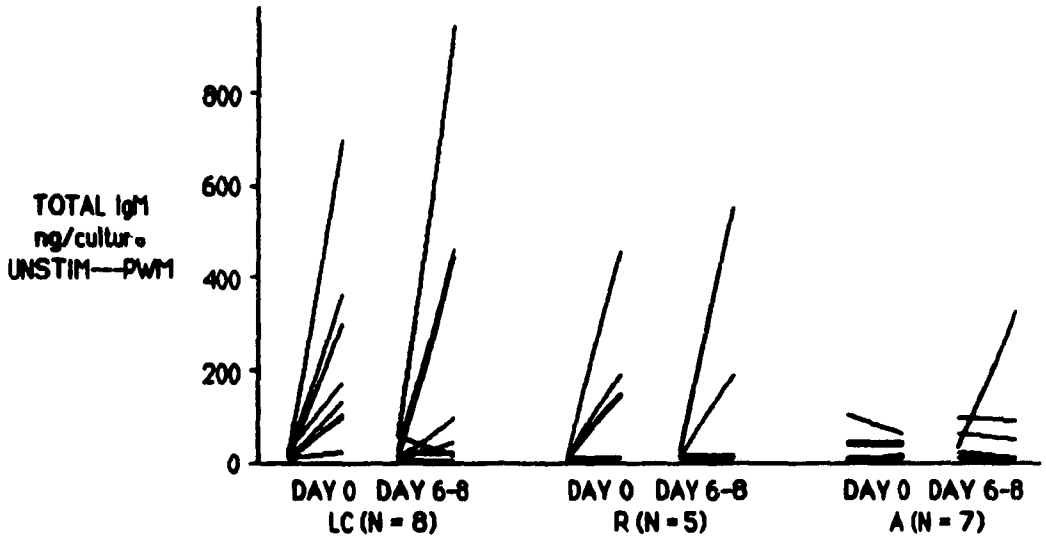
Kinetic and quantitative relationships were identified between *in vivo* antibody responses and *in vitro* antibody synthesis after vaccination. Peak *in vitro* synthesis occurred during the time of maximal rise in serum antibody levels. The order of appearance of the isotypes was also related in that IgM appeared first both *in vivo* and *in vitro*, followed by IgA and IgG. To determine the quantitative relationship, the antibody response was defined as the difference between peak post-immunization and pre-immunization samples. Data from peak

FIGURE 37: PATIENT TOTAL IgG SYNTHESIS AFTER PNEUMOCOCCAL POLYSACCHARIDE VACCINATION *IN VIVO*



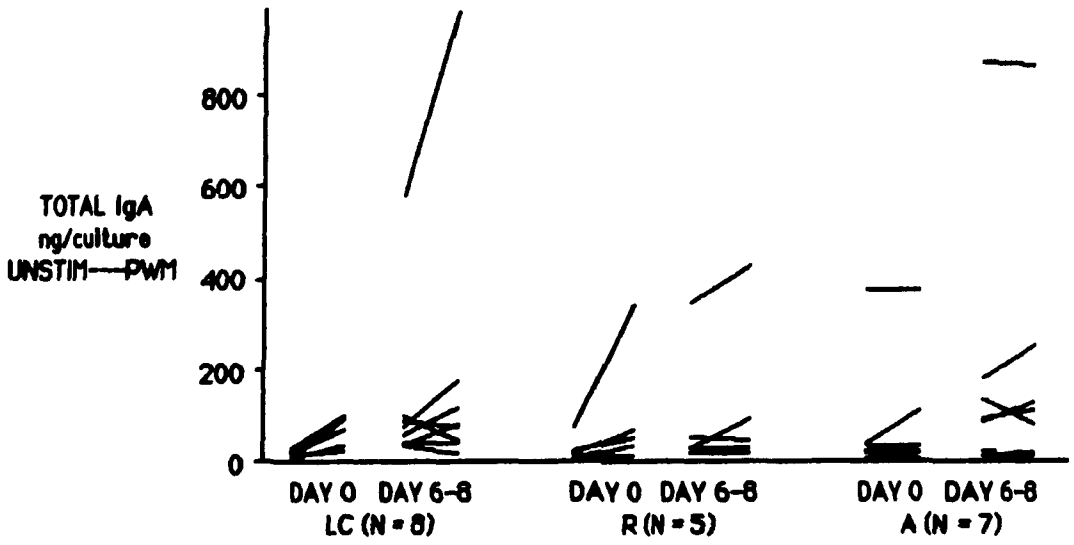
Eight LC, 5 R and 7 A subjects were immunized with PPS on day 0. Blood MNCs were harvested on days 0, 6 and 8, and cultured *in vitro* for 10 days. Culture supernatants were assayed for content of total IgG. Data for either day 6 or 8 was selected on the basis of maximal specific IgG antibody synthesis. The data are shown for each subject as a line connecting the amounts produced either without (UNSTIM) or with PWM stimulation (PWM).

FIGURE 38: PATIENT TOTAL IgM SYNTHESIS AFTER PNEUMOCOCCAL POLYSACCHARIDE VACCINATION *IN VIVO*



Eight LC, 5 R and 7 A subjects were immunized with PPS on day 0. Blood MNCs were harvested on days 0, 6 and 8, and cultured *in vitro* for 10 days. Culture supernatants were assayed for content of total IgM. Data for either day 6 or 8 was selected on the basis of maximal specific IgM antibody synthesis. The data are shown for each subject as a line connecting the amounts produced either without (UNSTIM) or with PWM stimulation (PWM).

FIGURE 39: PATIENT TOTAL IgA SYNTHESIS AFTER PNEUMOCOCCAL POLYSACCHARIDE VACCINATION *IN VIVO*



Eight LC, 5 R and 7 A subjects were immunized with PPS on day 0. Blood MNCs were harvested on days 0, 6 and 8, and cultured *in vitro* for 10 days. Culture supernatants were assayed for content of total IgA. Data for either day 6 or 8 was selected on the basis of maximal IgA antibody synthesis. The data are shown for each subject as a line connecting the amounts produced either without (UNSTIM) or with PWM stimulation (PWM).

TABLE 14: *IN VITRO* TOTAL IMMUNOGLOBULIN SYNTHESIS AFTER PNEUMOCOCCAL POLYSACCHARIDE VACCINATION *IN VIVO*

	ISOTYPE			TOTAL
GROUP (N)	**IgG**	**IgM**	**IgA**	**IgG+IgM+IgA**
UNSTIMULATED				
PRE-VACCINATION				
CONTROL (8)	160 ± 42	15 ± 2	16 ± 3	191 ± 43
REACTIVE (5)	285 ± 84	9 ± 1	27 ± 12	321 ± 91
ANERGIC (7)	308 ± 126	32 ± 13	74 ± 51	414 ± 188
POST-VACCINATION				
CONTROL	854 ± 107	24 ± 5	127 ± 66	1005 ± 106
REACTIVE	784 ± 380	15 ± 3	95 ± 63	894 ± 445
ANERGIC	901 ± 221	36 ± 13	201 ± 114	1138 ± 335
PWM STIMULATED				
PRE-VACCINATION				
CONTROL	690 ± 80	238 ± 77	77 ± 11	1005 ± 138
REACTIVE	612 ± 186	191 ± 73	104 ± 60	907 ± 310
ANERGIC	*#390 ± 157	28 ± 7	86 ± 51	504 ± 200
POST-VACCINATION				
CONTROL	967 ± 149	254 ± 119	197 ± 117	1416 ± 341
REACTIVE	857 ± 400	265 ± 122	121 ± 78	1243 ± 585
ANERGIC	618 ± 153	74 ± 44	210 ± 113	902 ± 278

Blood MNC were harvested from immunized subjects 0, 6 and 8 days after vaccination, and cultured with and without PWM. Eight LC, 5 R and 7 A subjects were studied. For post-vaccination data, either day 6 or 8 was selected for each individual on the basis of maximal spontaneous specific antibody synthesis *in vitro* (see table 13), and all data from that date used. Arithmetic means ± SEM are shown. Total immunoglobulin produced was calculated as the sum of the individual isotypes for each subject. * indicates $p < 0.05$ compared to LC group, and # compared to R group (unpaired t test).

spontaneous synthesis of anti-PPS on either day 6 or 8 were used. In the three groups, there was a quantitative correlation for each isotype between spontaneous *in vitro* synthesis and serum antibody responses (r_s = 0.53, 0.60 and 0.59, p = 0.04, 0.01 and 0.01 for IgG, IgM and IgA).

In PWM stimulated cultures, similar correlations were observed due to the uniform change in specific antibody synthesis. For IgG, IgM and IgA, the correlation coefficients were r_s = 0.47, 0.54 and 0.61 ($p < 0.05$, 0.02 and 0.01). The general reduction in specific antibody synthesis was also reflected in high correlation coefficients for each isotype between spontaneous and PWM stimulated antibody synthesis after vaccination (all $r_s > 0.8$, p = 0.001).

2. TOTAL IMMUNOGLOBULIN PRODUCTION IN VITRO

In order to further define abnormalities in total Ig synthesis *in vitro*, the culture system will be described in terms of cell numbers, optimal PWM and FBS concentrations, kinetics of Ig synthesis, and changes in cultured cell populations. Because patient cell preparations were observed to contain abnormal T cell numbers, an excess of monocytes, and a greater degree of contamination with neutrophils, the roles of these cells on the in vitro system was also determined.

2.1 EFFECT OF CELL NUMBERS, PROTEIN SYNTHESIS INHIBITORS, MEDIUM COMPOSITION, AND CONCENTRATION OF POKEWEED MITOGEN ON IN VITRO IMMUNOGLOBULIN SYNTHESIS

The influence of cell number, medium composition and concentration of PWM on Ig production was examined. The kinetics of Ig production and the effect of protein synthesis blockers were studied to demonstrate new protein synthesis *in vitro*. Cell culture dynamics were described in terms of viability, proliferation and appearance of blast lymphocytes. Culture conditions were designed to minimize the mitogenic effect of FBS on human lymphocytes .

Experiments on the effect of cell numbers showed that, in the range from 1 to 8 $*10^5$ lymphocytes/well, 4 was optimal for IgG synthesis for most individuals (table 15). This was generally optimal for IgM and IgA also.

Cultures without FBS produced very little Ig (table 16). As [FBS] increased, more Ig of all isotypes was produced in unstimulated cultures, likely due to heterologous stimulation. With PWM, most Ig was produced in higher [FBS]. Medium supplementation with ITS increased Ig production, and satisfactory responses could be obtained at 2% [FBS].

In dose response studies on [PWM], maximal production occurred with final dilutions of 1:200 or greater (table 17). For normal MNCs, the stimulatory effect on the synthesis of most isotypes extended several doubling dilutions beyond 1:200.

Replicate culture sets were established, and harvested at intervals to determine the kinetics of Ig production *in vitro* (figure 40). In unstimulated cultures, there was a gradual accumulation of small amounts of Ig. There were minor qualitative differences among isotypes. In several LC subjects, there was a late increase in the rate of spontaneous IgG synthesis (data not shown). This did not occur with IgM and IgA, which increased little over the *in vitro* culture period. In PWM stimulated cultures, the synthesis of all isotypes began to increase sharply day 4. Synthesis was maximal for approximately 4 days, then tapered off.

Changes in cultured cell populations were related to Ig synthesis by determining viability, total cell yields, % blast MNCs, and IgG levels in cultures at intervals after initiation. There was a continuous drop in viable cell yields, similar in both PWM stimulated and unstimulated cultures (figure 41). Blast MNCs began appearing by day 2 in PWM stimulated cultures, had increased maximally by day 4, and remained constant thereafter. Without stimulation, there was a small late peak in blasts. Spontaneous IgG synthesis was low and continuous. Progressively fewer monocytes were seen in cultures from initiation to day 10.

Some Ig may be carried into the culture wells on the surface, or within cells. To minimize this, MNCs were extensively washed, including a serum free incubation at 37° C to release sIg. Protein synthesis inhibitors were used to show that the majority of Ig in culture supernatants was produced *in vitro*. The addition of either 10^{-4}M emetine or 10^{-5}M cycloheximide at the initiation of cultures reduced the amount of Ig in both stimulated and unstimulated cultures to similar low levels (table 18). In unstimulated cultures, however,

TABLE 15: EFFECT OF CELL NUMBER ON TOTAL IMMUNOGLOBULIN SYNTHESIS *IN VITRO*

LYMP/WELL ($*10^5$)	DONOR A		DONOR B		DONOR C	
	UNSTIM	STIM	UNSTIM	STIM	UNSTIM	STIM
1	99	178	12	17	11	14
2	32	978	26	222	32	695
4	160	695	172	116	160	978
8	99	111	203	563	99	178

Blood MNC were harvested from control subjects and cultured at varying lymphocyte (LYMP) concentrations under otherwise standard conditions. Cultures were either unstimulated (UNSTIM), or stimulated with PWM (STIM). Supernatants were assayed for cumulative synthesis of IgG after 10 days. Arithmetic means of triplicate culture wells are shown. $4*10^5$ lymphocytes per well was used as standard conditions.

TABLE 16: EFFECT OF CULTURE MEDIUM CHARACTERISTICS ON TOTAL IMMUNOGLOBULIN SYNTHESIS *IN VITRO*

IgG [FBS]	DONOR A UNSTIM	DONOR A STIM	DONOR B UNSTIM	DONOR B STIM	DONOR C UNSTIM	DONOR C STIM
WITHOUT ITS						
0	14	22	25	15	-	-
2	36	251	34	100	127	570
5	42	397	201	495	187	847
10	125	279	301	879	233	521
WITH ITS						
0	11	29	23	15	-	-
2	**42**	**506**	**53**	**292**	**158**	**1256**
5	51	299	154	483	280	702
10	74	136	500	893	233	521

IgM [FBS]	DONOR A UNSTIM	DONOR A STIM	DONOR B UNSTIM	DONOR B STIM	DONOR C UNSTIM	DONOR C STIM
WITHOUT ITS						
0	78	129	111	126	-	-
2	164	377	137	394	51	203
5	304	728	252	1356	33	520
10	196	332	-	-	38	506
WITH ITS						
0	73	130	99	111	-	-
2	**224**	**641**	**179**	**530**	**23**	**421**
5	242	391	255	1073	31	837
10	265	343	1231	1296	88	621

IgA [FBS]	DONOR A UNSTIM	DONOR A STIM	DONOR B UNSTIM	DONOR B STIM	DONOR C UNSTIM	DONOR C STIM
WITHOUT ITS						
0	14	15	16	8	-	-
2	48	50	39	62	121	163
5	73	106	56	808	219	252
10	138	64	-	-	221	175
WITH ITS						
0	5	9	10	4	-	-
2	**47**	**99**	**22**	**109**	**83**	**118**
5	20	57	80	292	112	109
10	99	37	209	366	152	103

Blood MNC were harvested from control subjects and cultured under standard conditions, except for the concentration of fetal bovine serum, [FBS], %, and the presence or absence of insulin-transferrin-selenium supplement, ITS. Cultures were either unstimulated (UNSTIM), or stimulated with PWM (STIM). Supernatants were assayed for cumulative synthesis of IgG, IgM and IgA after 10 days. Arithmetic means of triplicate culture wells are shown.

TABLE 17: EFFECT OF POKEWEED MITOGEN CONCENTRATION ON TOTAL IMMUNOGLOBULIN SYNTHESIS *IN VITRO*

	DONOR A			DONOR B			DONOR C		
1/[PWM]	IgG	IgM	IgA	IgG	IgM	IgA	IgG	IgM	IgA
0	71	27	90	54	52	15	223	5	126
100	329	457	142	630	693	90	303	230	115
200	**449**	**633**	**178**	**604**	**505**	**50**	**849**	**230**	**187**
400	467	608	136	606	881	79	371	244	157
800	380	446	127	485	631	40	290	200	58
1600	371	492	150	298	214	19	261	214	98
3200	313	431	130	242	234	22	124	153	62
6400	208	431	95	166	148	16	133	173	87

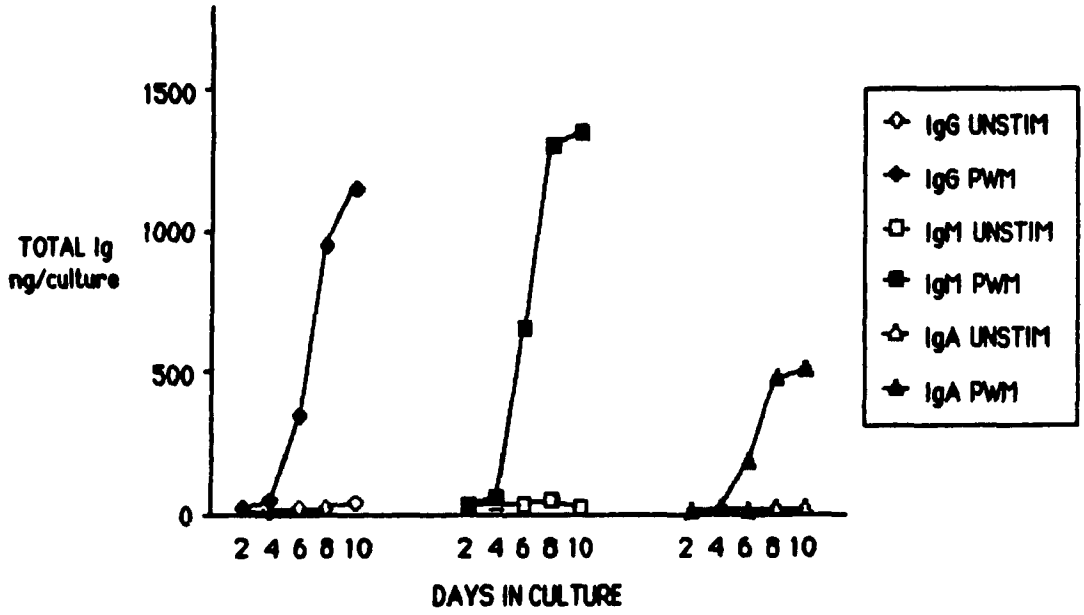
Blood MNC were harvested from control subjects and cultured under standard conditions. Cultures were either unstimulated, or stimulated with PWM at a range of final dilutions from 1:100 to 1:6400. Supernatants were assayed for cumulative synthesis of IgG, IgM and IgA after 10 days. Arithmetic means of triplicate culture wells are shown. A final concentration of 1:200 was selected for standard conditions.

TABLE 18: EFFECT OF PROTEIN SYNTHESIS INHIBITORS ON TOTAL IMMUNOGLOBULIN SYNTHESIS *IN VITRO*

	DONOR A			DONOR B			DONOR C		
	IgG	IgM	IgA	IgG	IgM	IgA	IgG	IgM	IgA
UNSTIM	284	27	25	71	17	8	82	16	13
+CYCLOHEX	29	23	3	19	23	5	12	16	4
+EMETINE	42	22	7	31	25	8	18	21	6
PWM STIM	961	173	73	776	699	97	880	364	102
+CYCLOHEX	22	31	5	24	28	5	14	34	5
+EMETINE	69	32	8	29	34	5	17	24	5

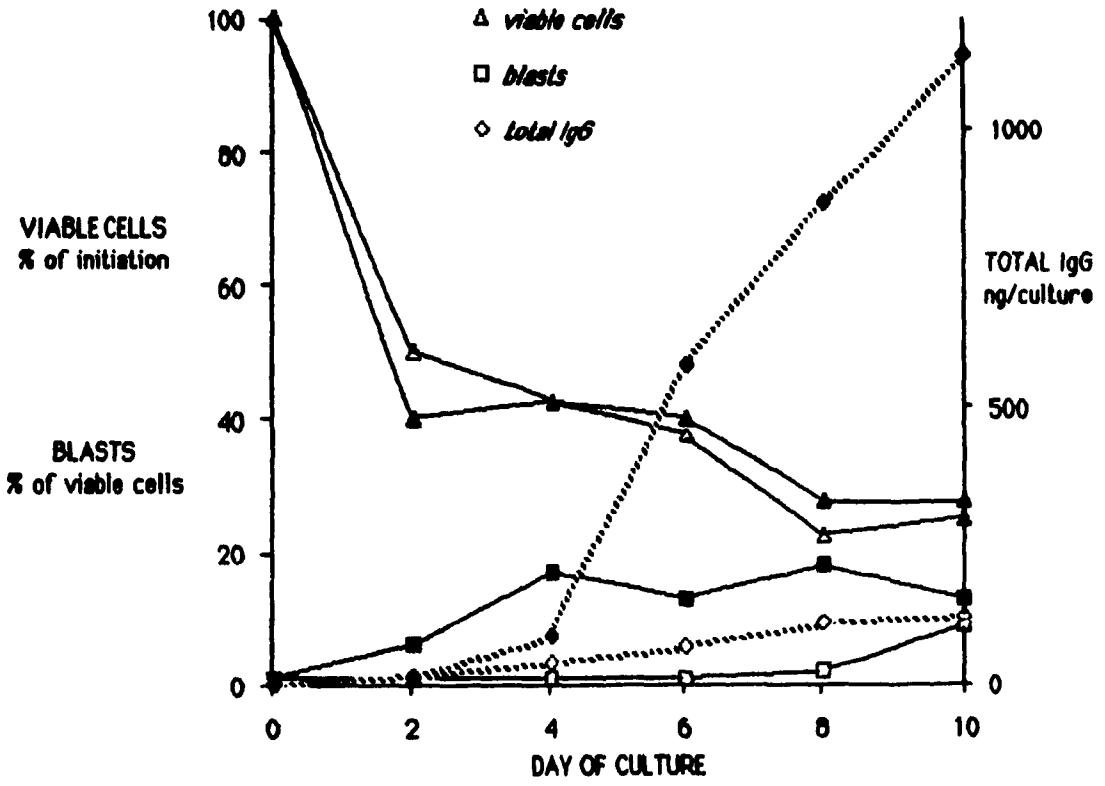
Blood MNC were harvested from control subjects and cultured under standard conditions. Cultures were either unstimulated (UNSTIM), or stimulated with PWM at a final dilution of 1:200 (PWM STIM). Either cycloheximide at a final concentration of 10^{-5}M, or emetine at a final concentration of 10^{-4}M, was added at the beginning of cultures from the same donors. Supernatants were assayed for cumulative synthesis of IgG, IgM and IgA after 10 days. Arithmetic means of triplicate culture wells are shown.

FIGURE 40. NORMAL TIME COURSE OF TOTAL IMMUNOGLOBULIN SYNTHESIS *IN VITRO*



Mononuclear cells from an LC subject were cultured *in vitro* under standard conditions, either without (UNSTIM) or with PWM (PWM). Supernatants were harvested from a set of cultures every 2 days,* and assayed for cumulative content of total IgG, IgM and IgA.

FIGURE 41: CHANGES IN THE COMPOSITION OF CELL CULTURES DURING INCUBATION WITH AND WITHOUT POKEWEED MITOGEN



During a time course study of the synthesis of Ig *in vitro* by LC MNCs (figure 40), the cells were harvested at 2 day intervals from replicate wells. Cultures were either unstimulated (open markers) or stimulated with PWM (solid markers). Viability was determined by trypan blue exclusion, and blast cells were counted by morphology. Viable cell data are shown as the % of the number of cells at initiation, and blast cell data as the percentage of viable cells at each time.

there was no difference in IgM between cultures with and without protein synthesis inhibitors. This indicated that little was spontaneously produced, and that the amounts detected were carried into the culture either on or in cells. This was in contrast to the large quantities of IgA and especially IgG synthesized in uninhibited cultures (table 18). These data parallel the observations of time course studies, where there was a persistent increase in IgG, and relatively little change in IgM in unstimulated cultures.

In summary, the effect of changes in the standard culture system on Ig synthesis were described. Changes in cultured cells suggest that PWM induces a population of Ig producing cells by day 4, and that the synthesis rate remained the same until day 10. The beginning of stimulated IgG synthesis was temporally associated with the appearence of blast cells, the level of which remained constant thereafter.

2.2 ROLE OF T CELLS IN IMMUNOGLOBULIN PRODUCTION IN VITRO

To demonstrate that T cells were necessary for PWM stimulation in this culture system, cell separation and recombination experiments were done.

Whole MNCs were cultured using standard conditions, with $4*10^5$ lymphocytes/well. E^+ and E^- fractions were cultured separately or combined with $1*10^5$ lymphocytes from each fraction per well. All combinations were autologous. Supernatants were assayed after 10 days of culture. Percentages of E^+, sIg^+ and L^+ in the cell fractions are shown in table 19. Monocytes, characterized as L^+, co-purify with sIg^+ cells. The recombination of $1*10^5$ lymphocytes from each E^+ and E^- fraction allowed approximately the same numbers of sIg^+ cells per recombination well as was present in MNC wells. For experiments 1 and 2 respectively, the calculated final $\%E^+$ in the recombination wells was 60% and 41%, compared to 85% and 60% in whole MNCs.

The Ig synthesis data are shown in table 20, and demonstrate the T cell dependant PWM responses. Synthesis in E^- fractions was likely due to contamination by E^+ cells.

TABLE 19: CHARACTERISTICS OF MONONUCLEAR CELL PREPARATIONS IN CELL SEPARATION EXPERIMENTS USING SRBC ROSETTES

EXPERIMENT 1

CELL PREPARATION	LYM/MONO	% ER^+	% sIg^+	% $LATEX^+$
WHOLE	10	85	13	7
ER^+ FRACTION	95	89	3	4
ER^- FRACTION	2	20	43	28

EXPERIMENT 2

CELL PREPARATION	LYM/MONO	% ER^+	% sIg^+	% $LATEX^+$
WHOLE	10	60	9	13
ER^+ FRACTION	90	75	2	2
ER^- FRACTION	5	6	47	18

Blood MNCs were harvested from control subjects in two experiments. Unseparated preparations of MNCs (WHOLE) were separated into SRBC receptor positive (ER^+) and negative (ER^-) fractions using SRBC rosette technique. Each cell preparation was analyzed for the ratio of lymphocytes to monocytes by visual count (LYM/MONO), ER^+ cells by rosetting (ER^+, % of non-phagocytic MNCs only), sIg^+ cells by Immunobead technique (sIg^+, % of non-phagocytic MNCs only), and percentage of total MNCs that were phagocytic using latex bead ingestion.

TABLE 20: CELL SEPARATION EXPERIMENTS ON TOTAL IMMUNOGLOBULIN SYNTHESIS *IN VITRO* USING SRBC ROSETTES

	EXPERIMENT 1		*EXPERIMENT 2*					
CELLS	**IgG SPON**	**IgG STIM**	**IgG SPON**	**IgG STIM**	**IgM SPON**	**IgM STIM**	**IgA SPON**	**IgA STIM**
WHOLE	520	1489	57	729	32	880	18	237
ER^+	9	11	3	4	37	35	5	5
ER^-	10	705	21	142	32	69	16	35
ER^+ER^-	76	2001	19	257	39	174	15	64
ER^+ER^- + CYCLO	-	-	12	16	43	46	7	5

Blood MNC were harvested from control subjects in two experiments. Unseparated preparations of PBMC (WHOLE) were separated into SRBC receptor positive (ER^+) and negative (ER^-) fractions using SRBC rosette technique. Cultures were established with whole cell preparations, ER^- and ER^+ fractions, and recombinations of ER^+ and ER^- fractions (ER^+ER^-). A recombined cell preparation was also cultured with cycloheximide at a final concentration of 10^{-5}M, added at the initiation of culture. Cultures were either unstimulated (SPON) or stimulated with PWM (STIM). After 10 days of culture, supernatants were assayed for cumulative content of IgG in experiment 1, and IgG, IgM and IgA in experiment 2. Data are shown as arithmetic means of triplicate cultures.

2.3 ROLE OF MACROPHAGES IN IMMUNOGLOBULIN PRODUCTION IN VITRO

Monocytes co-purify with lymphocytes, and are present in variable numbers in MNC preparations from different individuals. To assess the role of monocytes in total Ig synthesis *in vitro*, cell separation and recombination experiments using adherence were done.

Adherent (ADH^+) cells were removed and recovered from a control MNC preparation. Non-adherent cells (ADH^-) were recombined with variable numbers of adherent cells, and results of culture supernatant assay after 10 days incubation compared to whole MNC cultures.

Prior to separation, 8% of MNCs were monocytes by differential counting. The percentage of L^+ cells was 12, and PMN contamination 1%. There were 9% sIg^+ cells. The ADH^- population contained 4% residual monocytes by morphology, 3% L^+ cells, and 9% sIg^+ cells. The recovered ADH^+ fraction contained 81% monocytes by morphology and 77% L^+ cells. Nearly all lymphocytes present were sIg^+.

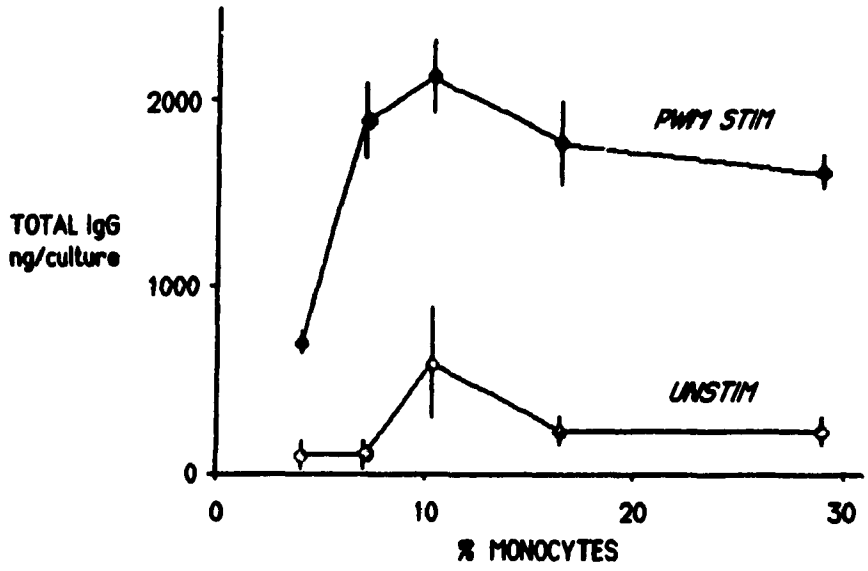
The range of monocytes as a % of total MNCs was from 4 to 29%. There was a dose response relationship between number of monocytes present and IgG synthesis. The least amount of IgG was produced with 4%, and most with 10% monocytes in both PWM stimulated and unstimulated cultures (figure 42). Increased monocyte numbers decreased IgG synthesis. A lymphocyte:monocyte ratio of 10:1 was optimal.

2.4 EFFECT OF NEUTROPHILS ON IMMUNOGLOBULIN PRODUCTION IN VITRO

Density gradient centrifugation with Ficoll-Hypaque allowed a variable degree of contamination with PMNs. This was not different among LC and R subjects (table 21). In MNCs from A subjects, there was a significantly greater degree of contamination.

Neutrophils may carry surface bound Ig. The amount was measured to determine if heavy contamination could falsely elevate the amount of supernatant Ig in MNC cultures. Because the *in vitro* life span of PMNs is short, the Ig content of isolated PMNs incubated 3 days was measured. Using a number of LC PMNs equivalent to 100% contamination in triplicate standard culture volumes, 8 ± 1, 38 ± 5 and 6 ± 1 ng of IgG, IgM and IgA respectively were found in culture supernatants.

FIGURE 42: EFFECT OF MONOCYTES ON *IN VITRO* SYNTHESIS OF IMMUNOGLOBULIN



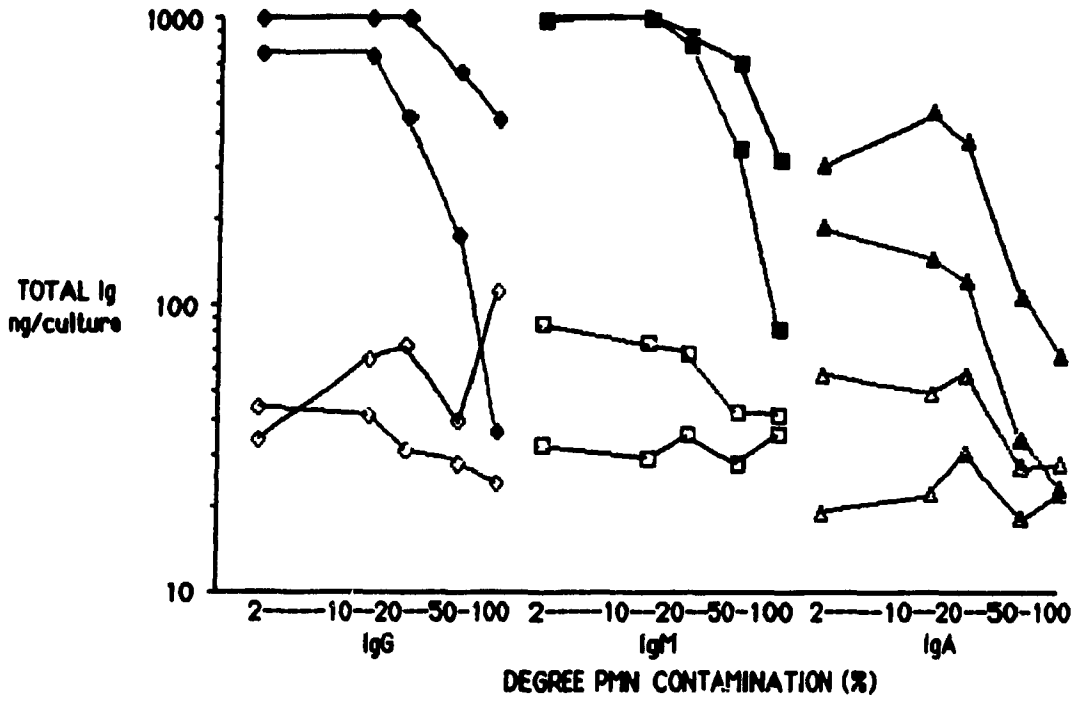
Monocytes were isolated from a preparation of normal MNCs using adherance techniques. They were recombined with autologous lymphocytes in varying numbers, shown as the % monocytes of the total cells present in each culture. After a 10 day incubation period, with or without PWM (PWM STIM) stimulation (UNSTIM), supernatants were assayed for total IgG. Arithmetic means $\pm$ SEM of triplicate wells are shown.

TABLE 21: CHARACTERISTICS OF ISOLATED BLOOD MONONUCLEAR CELL PREPARATIONS

GROUP	N	LYMPH YIELD	MONO:LYMPH	% PMN
LC	**43**	**1.2 ± 0.1**	**0.15 ± 0.01**	**3 ± 1**
WR	**29**	**1.0 ± 0.1**	**0.22 ± 0.02**	**7 ± 2**
HR	**15**	**0.7 ± 0.1**	**0.27 ± 0.05**	**9 ± 4**
ALL R	**44**	***0.9 ± 0.1**	***0.24 ± 0.02**	**7 ± 2**
WA	**10**	**0.6 ± 0.1**	**0.39 ± 0.06**	**27 ± 13**
HA	**10**	**0.5 ± 0.1**	**0.27 ± 0.03**	**15 ± 5**
IA	**35**	**0.7 ± 0.1**	**0.41 ± 0.04**	**19 ± 4**
ALL A	**55**	***#0.6 ± 0.1**	***#0.38 ± 0.03**	***#20 ± 4**

Blood mononuclear cells were harvested from LC subjects and patients, stratified according to DTH responses. The lymphocyte yield (LYMPH YIELD) is shown in cells*10^6/ml blood. The ratio of monocytes to lymphocytes (MONO:LYMPH), and the % of contaminating PMNs was calculated on the basis of morphological counts during cell isolation procedures. Arithmetic means ± SEM are shown. * indicates $p < 0.05$ compared to LC, and # compared to ALL R (unpaired t tests).

FIGURE 43: EFFECT OF NEUTROPHIL CONTAMINATION ON *IN VITRO* SYNTHESIS OF IMMUNOGLOBULIN



Using autologous PMNs, varying degrees of contamination of MNC cell cultures from 2 LC subjects were produced at the beginning of culture, from 2 to 100%. Cultures were either unstimulated (open markers) or stimulated with PWM (solid markers) After 10 days, supernatants were assayed for content of total IgG, IgM, and IgA.

Experiments were done to measure the influence of PMN contamination on *in vitro* Ig synthesis. The effect of deliberate contamination with autologous PMNs at the initiation of standard cultures is shown in figure 43. At 20% or less, there was minimal interference with the synthesis of any isotype. At 50% or greater, there was a dose dependent reduction of PWM induced production of all isotypes, with a lesser or no effect on spontaneous synthesis.

In summary, although neutrophil contamination carries little Ig into culture wells, the presence of > 50% PMNs decreased PWM dependent Ig synthesis.

2.5 NORMAL TOTAL IMMUNOGLOBULIN PRODUCTION IN VITRO

The normal pattern of Ig synthesis *in vitro* was established using standard cultures of MNCs from 46, 32 and 23 LC subjects for IgG, IgM and IgA respectively. The age range and mean $\pm$ SEM were 20 to 72 and 40 $\pm$ 3. The ratio of M:F was 1:1.

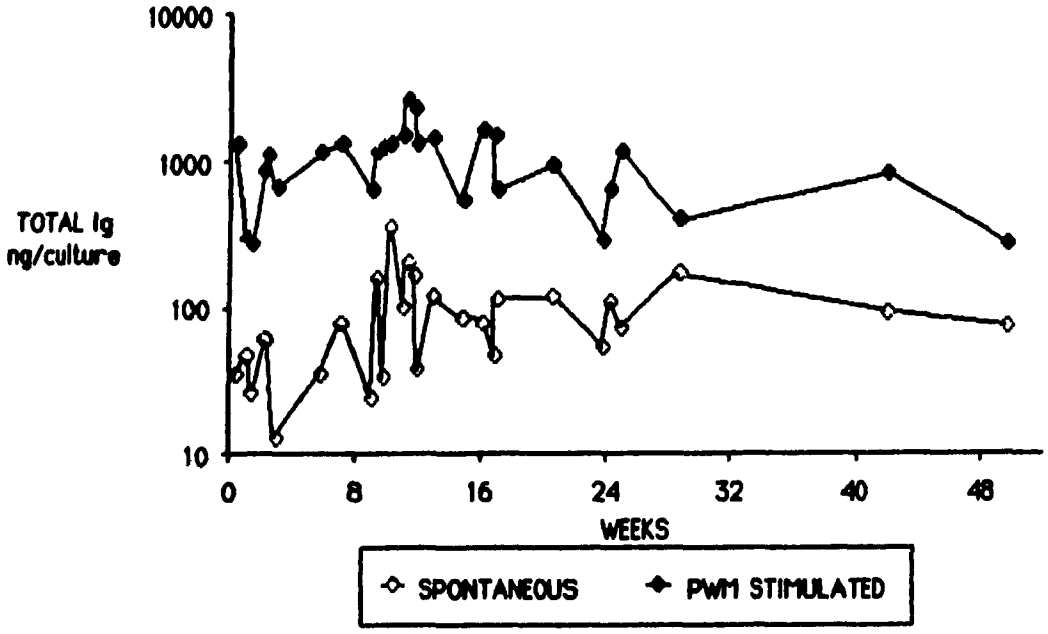
In both spontaneous and PWM stimulated cultures, IgG was produced in the largest quantities, followed by IgM and IgA (tables 22, 23 and 24). Most subjects showed a statistically significant increase with PWM stimulation in all isotypes, considered a normal or positive response (tables 25 and 26).

The relationship between age and *in vitro* Ig synthesis was examined. For IgG, there was a modest and statistically insignificant indirect correlation between age and spontaneous Ig synthesis, such that older individuals tended to produce less IgG (r_s = -0.264, p = 0.08). There was no relationship between age and PWM induced synthesis (r_s = -0.07, p > 0.8). For IgM and IgA, relationships between age and either spontaneous or PWM stimulated Ig synthesis were weak (r_s from -0.15 to 0.17, all p > 0.4).

There was reasonable consistency over time for IgG synthesis. Cells from the same LC donor were cultured repeatedly over a year. Comparable amounts of IgG was produced on most occasions (figure 44).

In summary, quantities of total IgG, IgM and IgA produced *in vitro* were determined. Most normal subjects show a significant positive response to PWM stimulation in all isotypes.

FIGURE 44: REPEATED STUDY OF IgG PRODUCTION *IN VITRO* BY MONONUCLEAR CELLS FROM A SINGLE NORMAL SUBJECT



Using standard culture conditions, MNCs from an LC subject were repetitively cultured over 48 weeks. Quantities of total IgG produced in unstimulated and PWM stimulated cultures on these occasions are is shown.

2.6 PATIENT TOTAL IMMUNOGLOBULIN PRODUCTION IN VITRO

Blood MNCs were harvested and cultured *in vitro* using the standard methods described above. Except for time course studies, all supernatants were assayed after 10 days of culture. Each isotype is analyzed separately. Although some patients were studied more than once, data from only one occasion for each subject in a given classification were included in the following analysis. Trauma patients were analyzed separately.

2.6.1 PATIENT CELL PREPARATION CHARACTERISTICS

There were differences among study groups in some relevant aspects of the MNC preparations. The yield of lymphocytes was significantly decreased among all patients, especially those with decreased DTH (table 21). In a separate patient group, there were similar differences in blood lymphocyte count (table 5). For all classes of subjects, the yield of lymphocytes was approximately 50% of the circulating counts. There were more monocytes in cultures from patients compared to LC. The ratio of monocyte:lymphocyte in isolated MNC, based on differential counting, was significantly increased in both groups of patients compared to LC (table 21, $p = 0.001$ for both comparisons, unpaired t-tests). Ratios were higher in the A ($p = 0.001$, unpaired t-test). A variable number of monocytes therefore accompanied the fixed number of lymphocytes used in culture studies. Compared to LC, there were approximately 20% more monocytes in R subjects, and 100% more in A.

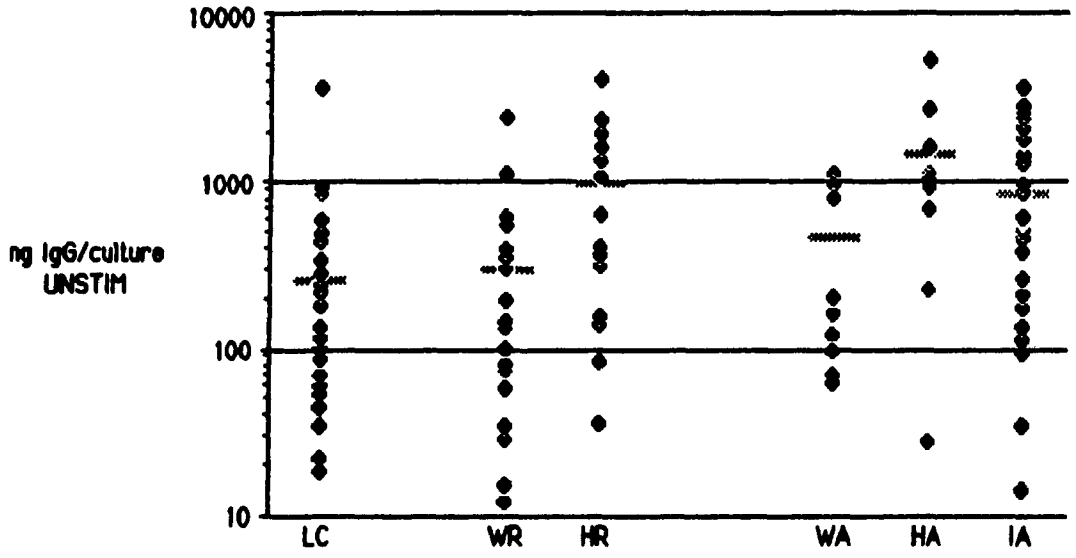
In summary, lymphocyte yield from patients is reduced in parallel with circulating cell counts. Significantly more monocytes were present in cultures from patients, especially those with reduced DTH reactivity.

2.6.2 PATIENT SPONTANEOUS IMMUNOGLOBULIN PRODUCTION IN VITRO

Blood MNCs from classified patients were cultured *in vitro* under standard conditions for 10 days. No lectin or antigen was added. Culture supernatants were assayed for cumulative content of isotype specific Ig.

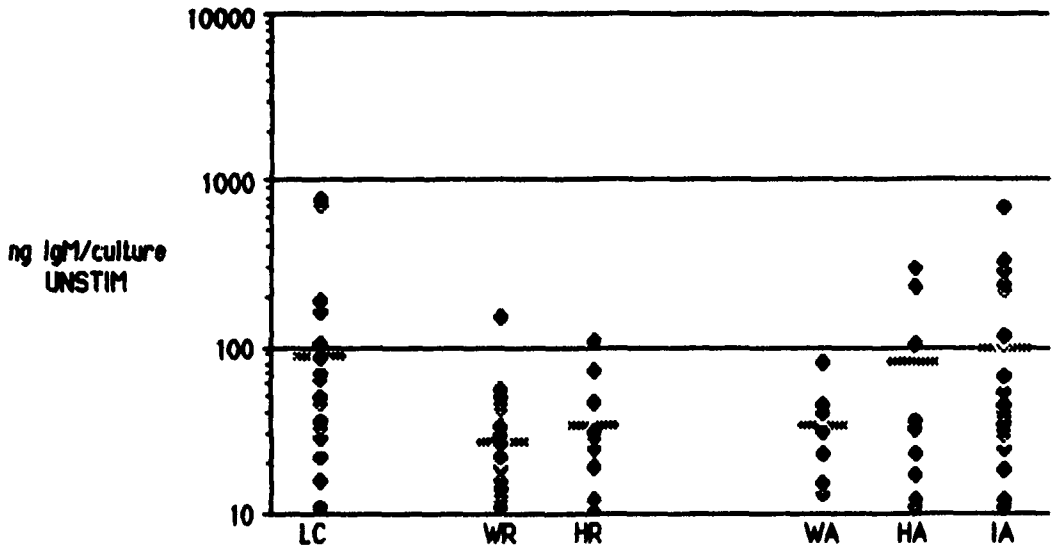
The average ng of IgG, IgM and IgA produced in unstimulated cultures are shown figures 45, 46 and 47, and in the first columns of tables 22, 23 and 24 respectively. More IgG was

FIGURE 45: SPONTANEOUS TOTAL IgG SYNTHESIS *IN VITRO* BY PATIENT MONONUCLEAR CELLS



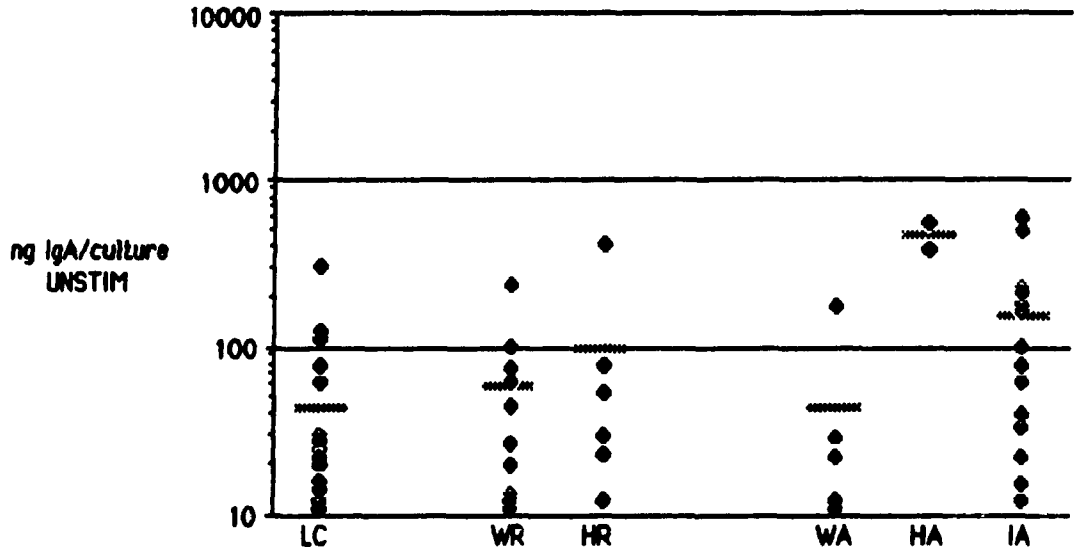
Whole MNC preparations from LC and patients stratified according to DTH responses were cultured *in vitro* without polyclonal stimulation. After 10 days, supernatants were assayed for total IgG. The arithmetic mean of triplicate wells is shown for each subject, and the geometric means for each group indicated by the horizontal bar.

FIGURE 46: SPONTANEOUS TOTAL IgM SYNTHESIS *IN VITRO* BY PATIENT MONONUCLEAR CELLS



Whole MNC preparations from LC and patients stratified according to DTH responses were cultured *in vitro* without polyclonal stimulation. After 10 days, supernatants were assayed for total IgM. The arithmetic mean of triplicate wells is shown for each subject, and the geometric means for each group indicated by the horizontal bar.

FIGURE 47: SPONTANEOUS TOTAL IgA SYNTHESIS *IN VITRO* BY PATIENT MONONUCLEAR CELLS



Whole MNC preparations from LC and patients stratified according to DTH responses were cultured *in vitro* without polyclonal stimulation. After 10 days, supernatants were assayed for total IgA. The arithmetic mean of triplicate wells is shown for each subject, and the geometric means for each group indicated by the horizontal bar.

TABLE 22: SPONTANEOUS AND POKEWEED MITOGEN STIMULATED TOTAL IgG PRODUCTION *IN VITRO*

GROUP	N	TOTAL IgG SPONTANEOUS	TOTAL IgG PWM STIMULATED
LC	**46**	**259 ± 89**	**1470 ± 335**
WR	29	295 ± 88	1017 ± 248
HR	15	977 ± 297	1712 ± 397
ALL R	**44**	**527 ± 125**	**1254 ± 215**
WA	10	461 ± 142	401 ± 155
HA	10	1450 ± 481	1420 ± 332
IA	35	850 ± 159	714 ± 116
ALL A	**55**	***888 ± 139**	***#785 ± 107**

Blood mononuclear cells were harvested from control and patient subjects and cultured in triplicate under standard conditions. Cultures were unstimulated (SPONTANEOUS) or stimulated with 1:200 PWM (PWM STIMULATED). Supernatants were assayed in duplicate for cumulative synthesis of IgG after 10 days. Arithmetic means ± sem are shown. * indicates $p < 0.05$ compared to LC, and # compared to ALL R (unpaired t tests).

TABLE 23: SPONTANEOUS AND POKEWEED MITOGEN STIMULATED TOTAL IgM PRODUCTION *IN VITRO*

GROUP	N	TOTAL IgM UNSTIMULATED	TOTAL IgM PWM STIMULATED
LC	**32**	**90 ± 29**	**553 ± 89**
WR	26	27 ± 6	238 ± 46
HR	12	34 ± 8	319 ± 78
ALL R	**38**	***30 ± 4**	***264 ± 40**
WA	9	34 ± 7	47 ± 9
HA	9	83 ± 34	189 ± 60
IA	30	99 ± 24	194 ± 55
ALL A	**48**	**84 ± 17**	***166 ± 37**

Blood mononuclear cells were harvested from control and patient subjects and cultured in triplicate under standard conditions. Cultures were unstimulated (SPONTANEOUS) or stimulated with 1:200 PWM (PWM STIMULATED). Supernatants were assayed in duplicate for cumulative synthesis of IgM after 10 days. Arithmetic means ± sem are shown. * indicates $p < 0.05$ compared to LC (unpaired t tests).

TABLE 24. SPONTANEOUS AND POKEWEED MITOGEN STIMULATED TOTAL IgA PRODUCTION *IN VITRO*

GROUP	N	TOTAL IgA UNSTIMULATED	TOTAL IgA PWM STIMULATED
LC	**23**	**43 ± 11**	**109 ± 17**
WR	**10**	**58 ± 13**	**156 ± 35**
HR	**6**	**100 ± 39**	**170 ± 36**
ALL R	**16**	**74 ± 16**	**161 ± 25**
WA	**6**	**43 ± 20**	**34 ± 17**
HA	**2**	**461 ± 38**	**404 ± 10**
IA	**16**	**153 ± 29**	**273 ± 62**
ALL A	**24**	***151 ± 24**	**224 ± 42**

Blood mononuclear cells were harvested from control and patient subjects and cultured in triplicate under standard conditions. Cultures were unstimulated (SPONTANEOUS) or stimulated with 1:200 PWM (PWM STIMULATED). Supernatants were assayed in duplicate for cumulative synthesis of IgA after 10 days. Arithmetic means ± sem are shown. * indicates $p < 0.05$ compared to LC (unpaired t tests).

produced by MNC from all patients, most in the A group (figure 45, table 22, p = 0.001, unpaired t-test). Considering sub-groups, there was large variability. Significantly more was produced in HR compared to WR. Among the A patients, most was produced by HA, followed by IA and WA, but the differences were not significant.

Less IgM was produced spontaneously by patients compared to LC (figure 46, table 23). The lowest producers were all the R and WA patients. Quantities similar to LC subjects were produced by IA subjects.

The pattern of spontaneous IgA synthesis was similar to IgG (figure 47, table 24). Patients produced more, especially those with reduced DTH skin tests (p = 0.01, unpaired t-test).

To summarize, compared to LC, patient MNC spontaneously produced similar or smaller amounts of IgM, and more IgG and IgA, especially those with reduced DTH.

2.6.3 PATIENT POKEWEED-MITOGEN STIMULATED IMMUNOGLOBULIN PRODUCTION IN VITRO

Standard culture conditions were used with a final [PWM] of 1:200. Results were analyzed as total Ig produced, as well as the difference and ratio between unstimulated and stimulated cultures. Also, the data for each individual were examined to determine if PWM caused a significant increase (positive response) or decrease (negative response) for each isotype in each subject.

The absolute amounts of Ig produced by patient MNCs in PWM stimulated cultures are shown in the second columns of tables 22, 23 and 24. Less IgG was produced by all patients, least in those with reduced DTH (p = 0.03 for LC versus A, and 0.04 for R versus A, unpaired t-tests). This trend was the opposite of spontaneous synthesis of this isotype.

The difference between PWM stimulated and unstimulated Ig synthesis was calculated for each subject. For both IgG and IgM, the differences were smaller in the patient groups, least in the A subjects (table 25). The mean difference in IgG synthesis among A patients was negative, a reversal of the normal pattern of PWM stimulation (p = 0.001 compared to both LC and R, unpaired t-tests). The differences in IgM synthesis were also greater in LC, and less among patients, especially the A group. Although the A group differences were significantly less than either LC (p = 0.001, unpaired t-test) or R (p = 0.01, unpaired t-test),

TABLE 25: COMPARISONS BETWEEN SPONTANEOUS AND PWM STIMULATED TOTAL IgG, IgM and IgA PRODUCTION *IN VITRO*

DIFFERENCES BETWEEN SPONTANEOUS AND PWM STIMULATED CULTURES

GROUP	N	IgG	IgM	IgA
LC	46	1211 ± 260	463 ± 75	65 ± 17
ALL R	44	727 ± 190	*234 ± 39	87 ± 18
ALL A	55	*#-103 ± 114	*#82 ± 36	73 ± 32

RATIOS BETWEEN SPONTANEOUS AND PWM STIMULATED CULTURES

GROUP	N	IgG	IgM	IgA
LC	46	11.9 ± 2.2	13.7 ± 2.1	4.6 ± 0.8
ALL R	44	8.6 ± 2.7	16.2 ± 3.5	4.4 ± 0.7
ALL A	55	*#1.9 ± 0.7	*#3.3 ± 0.8	*#1.5 ± 0.2

Blood mononuclear cells were harvested from control and patient subjects and cultured in triplicate under standard conditions. Cultures were unstimulated (SPONTANEOUS) or stimulated with 1:200 PWM (PWM STIMULATED). Supernatants were assayed in duplicate for cumulative synthesis of IgG, IgM and IgA after 10 days. The differences (PWM STIMULATED minus SPONTANEOUS) and ratios (PWM STIMULATED divided by SPONTANEOUS) were calculated for the cultures from each individual. Arithmetic means ± sem for each isotype in each group are shown. * indicates $p < 0.05$ compared to LC, and # compared to ALL R for each isotype (unpaired t tests). Other comparisons between LC, ALL R and ALL A are not statistically significant.

the average was still positive. The differences in IgA synthesis were comparable for all groups.

When analyzed as ratio of PWM stimulated to spontaneous synthesis, trends comparable to the analysis of differences were evident (table 25). In this case, there is a difference for IgA in the A group ($p = 0.02$, unpaired t-test).

The statistical significance between PWM stimulated and unstimulated cultures was determined for each isotype in each individual using unpaired t-tests on the mean and SD of the absolute quantities produced in triplicate cultures. The response to PWM stimulation was thus determined to be either positive, not significantly different, or negative. The normal response to PWM is positive, i.e. a statistically significant increase in Ig synthesis. In a negative response, significantly less Ig was produced in response to PWM stimulation, a reversal of the normal response.

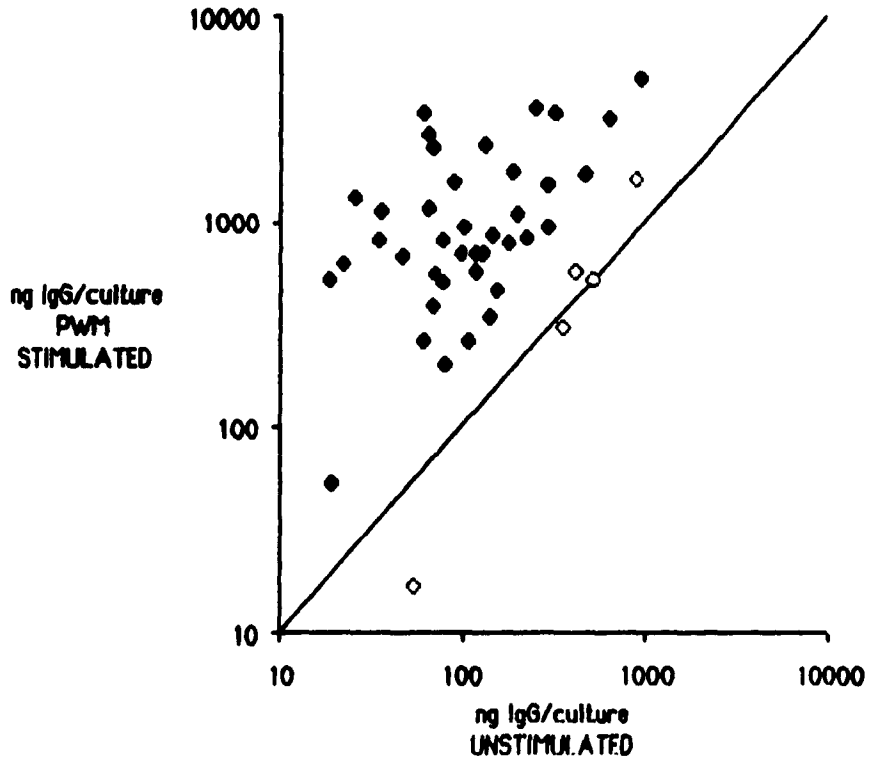
The data are shown in figures 48 to 56 as individuals in each of LC, R and A classes giving positive, not significant or negative responses in the synthesis of IgG, IgM and IgA isotypes. There is wide variability in quantities of Ig produced both spontaneously and with stimulation. Nearly all LC subjects gave a significant positive response in all isotypes. While many R subjects showed positive responses, some gave insignificant responses, and a few significant decreases with PWM. Among the A class, most subjects showed no significant response to PWM, while some gave negative or positive responses.

There was a statistically significantly different distribution of responses among the classes for each isotype. The data are summarized in table 26 as numbers of individuals showing a given response to PWM in the synthesis of IgG, IgM and IgA respectively. For IgG synthesis, no LC and occasional R subjects showed negative responses to stimulation. More A subjects (27%) gave significant negative reponses than positive (20%). For IgM and IgA, there were frequent insignificant, and occasional negative responses among patients.

In comparisons of patient subgroups, no differences were found in response distribution for any isotype among WR and HR , and WA, HA and IA (all X^2_1 and $X^2_2 < 4.59$, all $p < 0.33$).

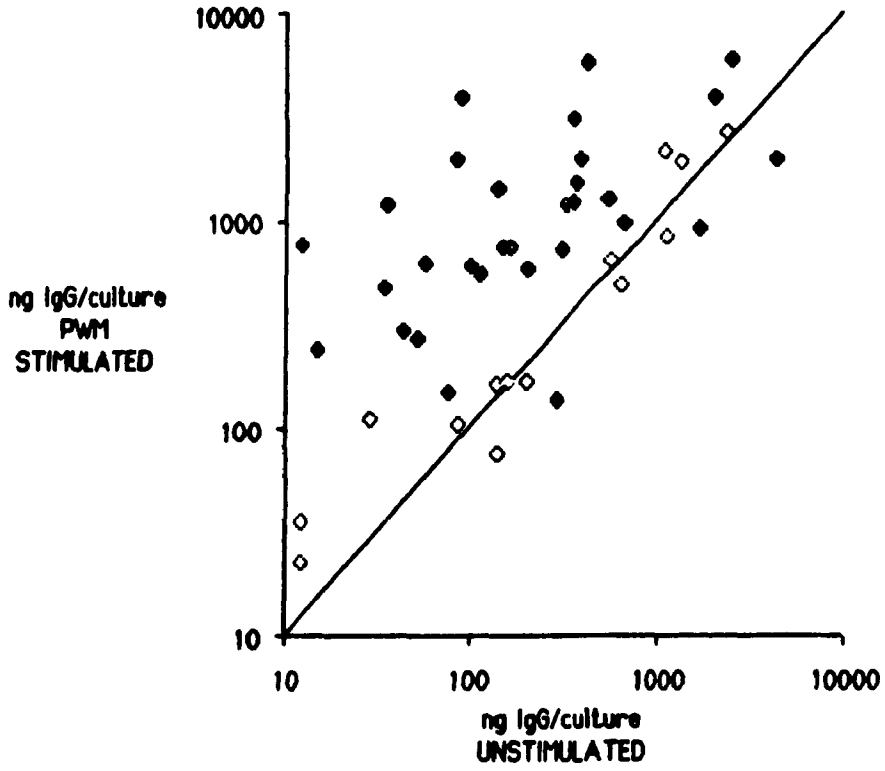
Within each subject class, there was little difference between the type of response to PWM in each isotype. In LC, similar proportions of responses to PWM were found for all isotypes ($X^2_4 = 5.57$, $p = 0.23$). In R patients, there was a shift towards fewer positive, more

FIGURE 48: RELATIONSHIP BETWEEN SPONTANEOUS AND POKEWEED MITOGEN STIMULATED *IN VITRO* IgG SYNTHESIS BY NORMAL MONONUCLEAR CELLS



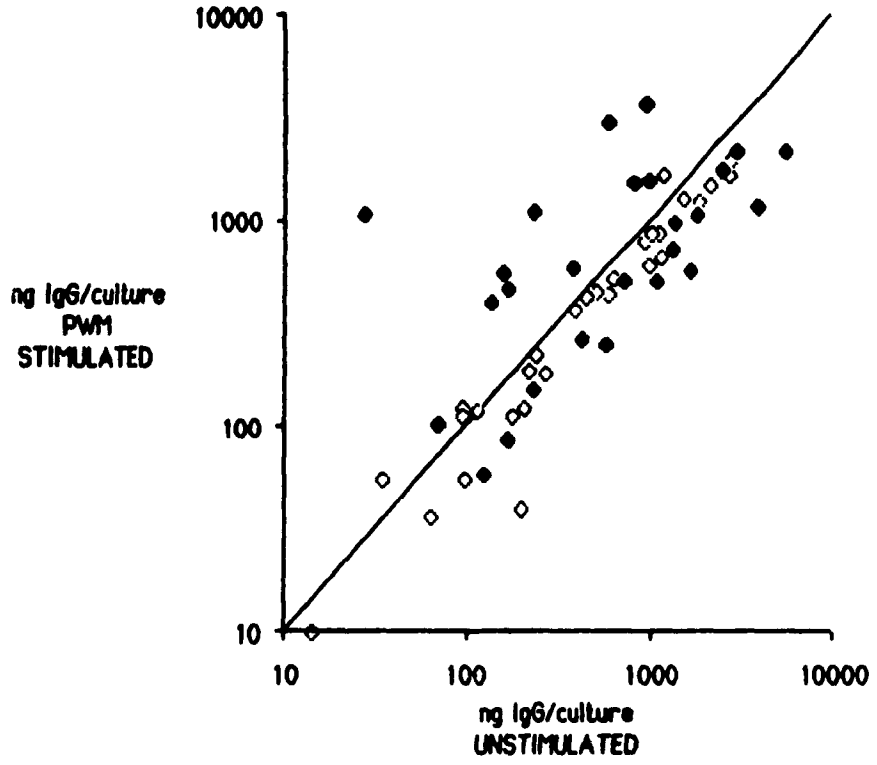
Blood MNCs from LC subjects were cultured *in vitro* under standard conditions, either without (UNSTIMULATED) or with PWM (PWM STIMULATED). After 10 days, supernatants were assayed for total IgG. The statistical significance of the difference between spontaneous and PWM stimulated synthesis was determined for each subject using unpaired t-tests. The data are shown as a solid marker above the line of unity if PWM stimulated cultures produced significantly more than unstimulated cultures, a solid marker below the line if there was a significant reduction in PWM cultures, and an open marker if there was no significant difference between them. Nearly all LC subjects produced a significant positive response to PWM stimulation.

FIGURE 49: RELATIONSHIP BETWEEN SPONTANEOUS AND POKEWEED MITOGEN STIMULATED *IN VITRO* IgG SYNTHESIS BY DTH REACTIVE PATIENT MONONUCLEAR CELLS



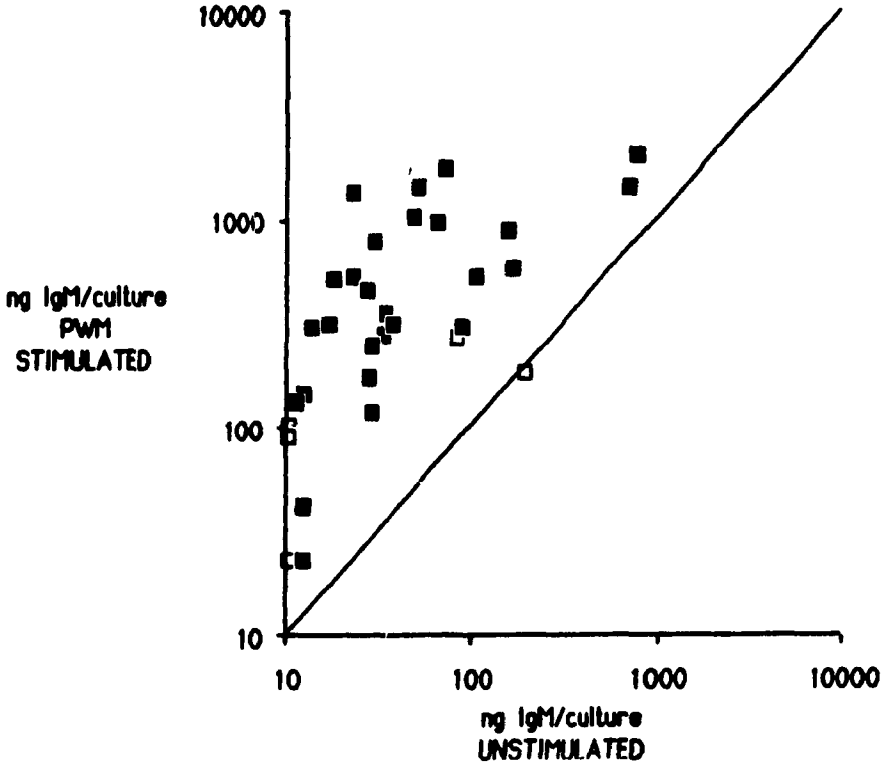
Blood MNCs from DTH R subjects were cultured *in vitro* under standard conditions, either without (UNSTIMULATED) or with PWM (PWM STIMULATED). After 10 days, supernatants were assayed for total IgG. The statistical significance of the difference between spontaneous and PWM stimulated synthesis was determined for each subject using unpaired t-tests. The data are shown as a solid marker above the line of unity if PWM stimulated cultures produced significantly more than unstimulated cultures, a solid marker below the line if there was a significant reduction in PWM cultures, and an open marker if there was no significant difference between them. Most R patients produced a significant positive response to PWM stimulation, although some responses were insignificant or negative..

FIGURE 50: RELATIONSHIP BETWEEN SPONTANEOUS AND POKEWEED MITOGEN STIMULATED *IN VITRO* IgG SYNTHESIS BY DTH ANERGIC PATIENT MONONUCLEAR CELLS



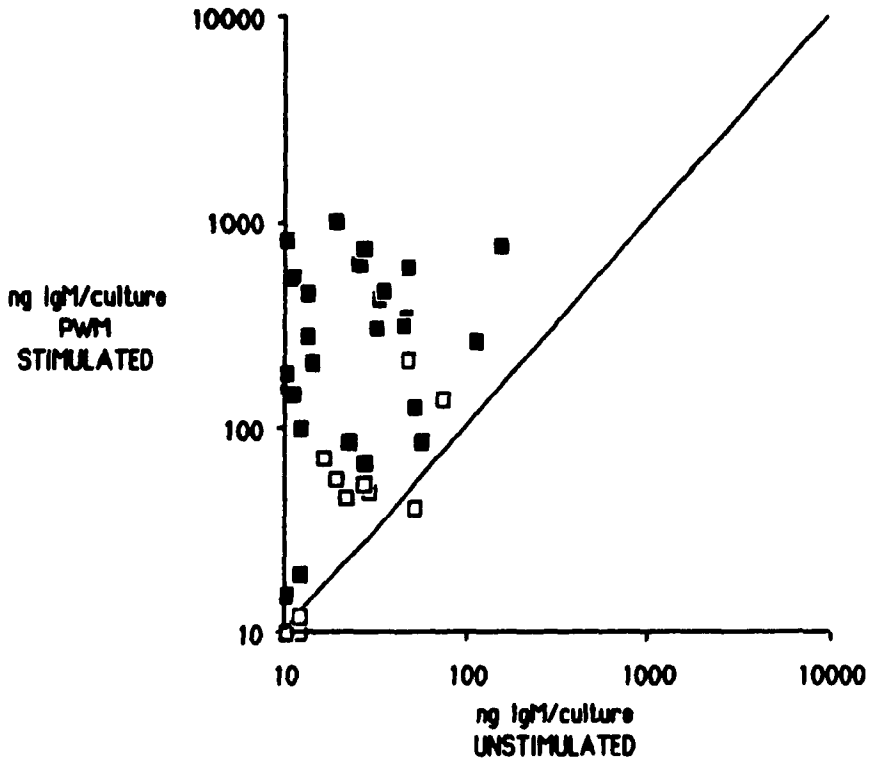
Blood MNCs from DTH A subjects were cultured *in vitro* under standard conditions, either without (UNSTIMULATED) or with PWM (PWM STIMULATED). After 10 days, supernatants were assayed for total IgG. The statistical significance of the difference between spontaneous and PWM stimulated synthesis was determined for each subject using unpaired t-tests. The data are shown as a solid marker above the line of unity if PWM stimulated cultures produced significantly more than unstimulated cultures, a solid marker below the line if there was a significant reduction in PWM cultures, and an open marker if there was no significant difference between them. Many A patients produced either negative or insignificant responses to PWM stimulation, although some responses were positive..

FIGURE 51: RELATIONSHIP BETWEEN SPONTANEOUS AND POKEWEED MITOGEN STIMULATED *IN VITRO* IgM SYNTHESIS BY NORMAL MONONUCLEAR CELLS



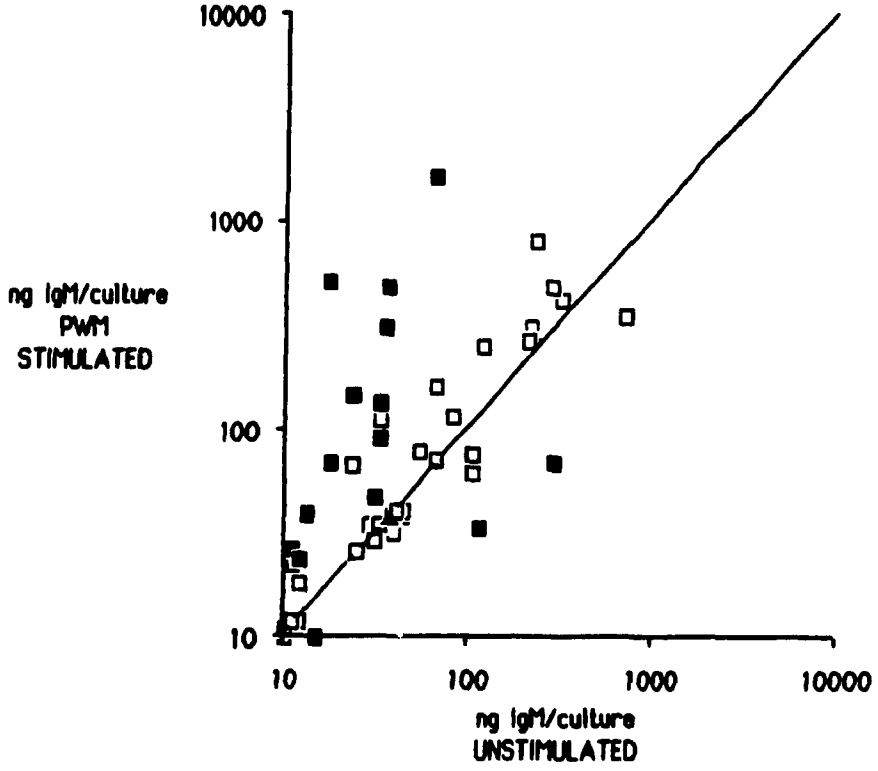
Blood MNCs from LC subjects were cultured *in vitro* under standard conditions, either without (UNSTIMULATED) or with PWM (PWM STIMULATED). After 10 days, supernatants were assayed for total IgM. The statistical significance of the difference between spontaneous and PWM stimulated synthesis was determined for each subject using unpaired t-tests. The data are shown as a solid marker above the line of unity if PWM stimulated cultures produced significantly more than unstimulated cultures, a solid marker below the line if there was a significant reduction in PWM cultures, and an open marker if there was no significant difference between them. Nearly all LC subjects produced a significant positive response to PWM stimulation.

FIGURE 52: RELATIONSHIP BETWEEN SPONTANEOUS AND POKEWEED MITOGEN STIMULATED *IN VITRO* IgM SYNTHESIS BY DTH REACTIVE PATIENT MONONUCLEAR CELLS



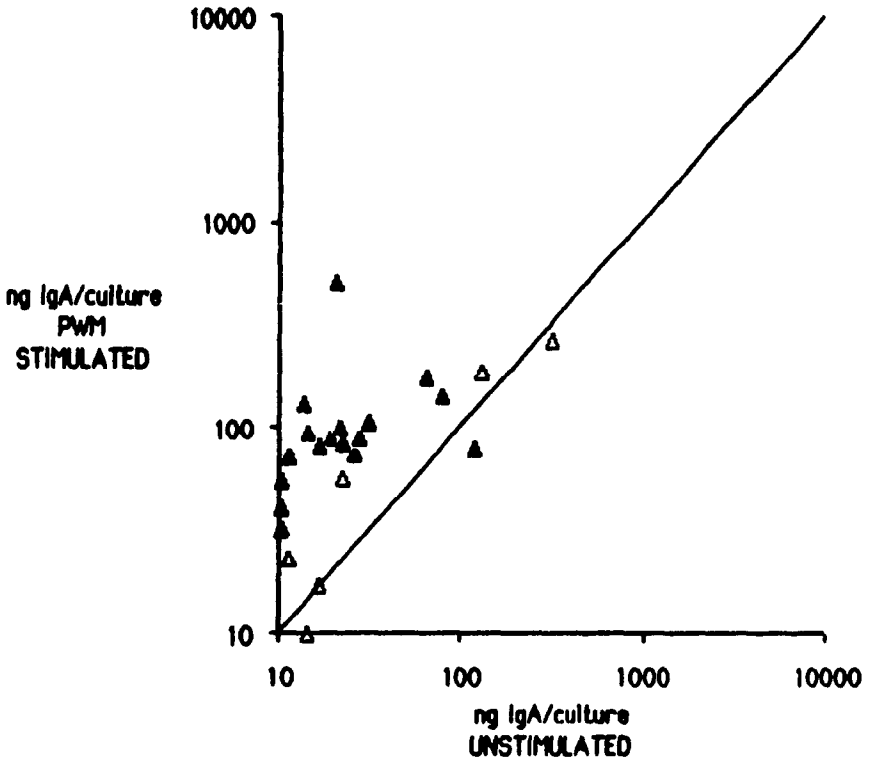
Blood MNCs from DTH R subjects were cultured *in vitro* under standard conditions, either without (UNSTIMULATED) or with PWM (PWM STIMULATED). After 10 days, supernatants were assayed for total IgM. The statistical significance of the difference between spontaneous and PWM stimulated synthesis was determined for each subject using unpaired t-tests. The data are shown as a solid marker above the line of unity if PWM stimulated cultures produced significantly more than unstimulated cultures, a solid marker below the line if there was a significant reduction in PWM cultures, and an open marker if there was no significant difference between them. Most R patients produced a significant positive response to PWM stimulation, although some showed no significance. There were no negative responses.

FIGURE 53: RELATIONSHIP BETWEEN SPONTANEOUS AND POKEWEED MITOGEN STIMULATED *IN VITRO* IgM SYNTHESIS BY DTH ANERGIC PATIENT MONONUCLEAR CELLS



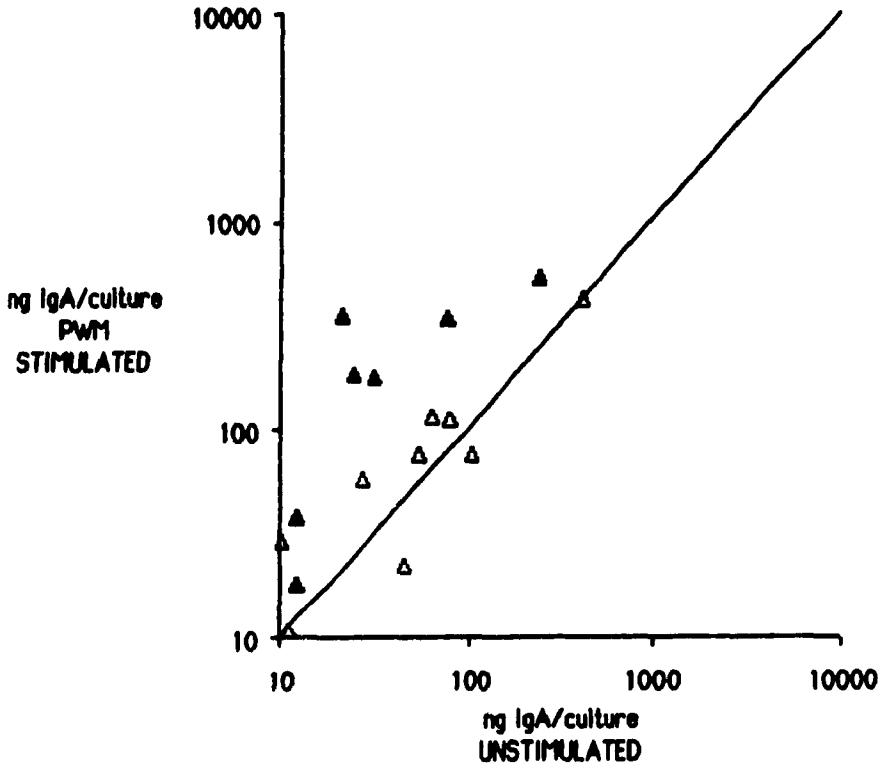
Blood MNCs from DTH A subjects were cultured *in vitro* under standard conditions, either without (UNSTIMULATED) or with PWM (PWM STIMULATED). After 10 days, supernatants were assayed for total IgM. The statistical significance of the difference between spontaneous and PWM stimulated synthesis was determined for each subject using unpaired t-tests. The data are shown as a solid marker above the line of unity if PWM stimulated cultures produced significantly more than unstimulated cultures, a solid marker below the line if there was a significant reduction in PWM cultures, and an open marker if there was no significant difference between them. Most A patients produced insignificant responses to PWM stimulation. Some showed positive responses, and the only negative IgM responses were found in this class.

FIGURE 54: RELATIONSHIP BETWEEN SPONTANEOUS AND POKEWEED MITOGEN STIMULATED *IN VITRO* IgA SYNTHESIS BY NORMAL MONONUCLEAR CELLS



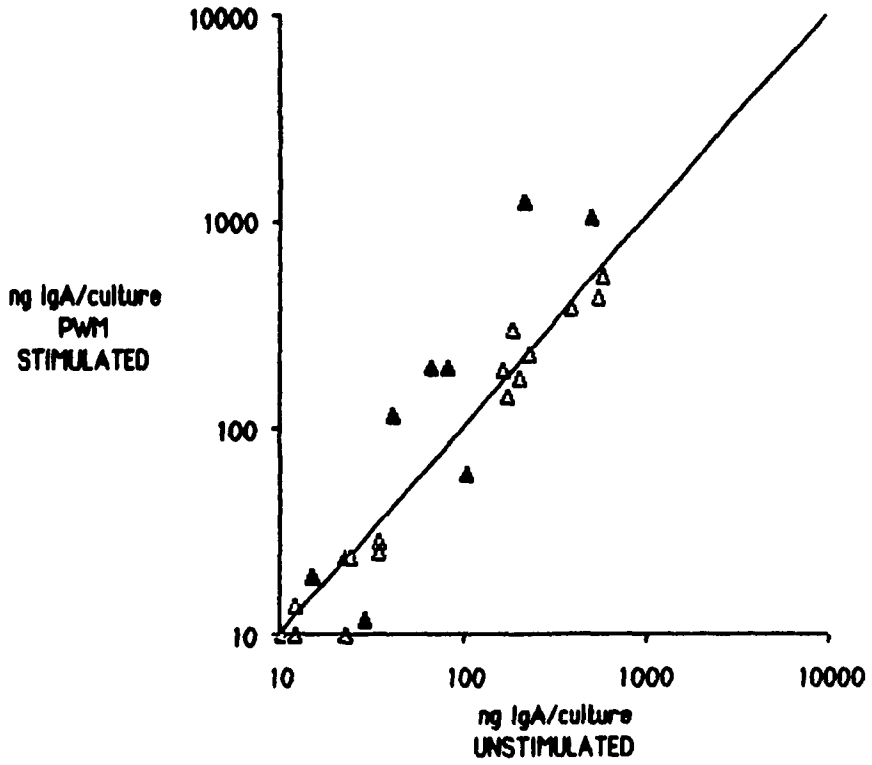
Blood MNCs from LC subjects were cultured *in vitro* under standard conditions, either without (UNSTIMULATED) or with PWM (PWM STIMULATED). After 10 days, supernatants were assayed for total IgA. The statistical significance of the difference between spontaneous and PWM stimulated synthesis was determined for each subject using unpaired t-tests. The data are shown as a solid marker above the line of unity if PWM stimulated cultures produced significantly more than unstimulated cultures, a solid marker below the line if there was a significant reduction in PWM cultures, and an open marker if there was no significant difference between them. Most LC subjects produced a significant positive response to PWM stimulation.

FIGURE 55: RELATIONSHIP BETWEEN SPONTANEOUS AND POKEWEED MITOGEN STIMULATED *IN VITRO* IgA SYNTHESIS BY DTH REACTIVE PATIENT MONONUCLEAR CELLS



Blood MNCs from DTH R subjects were cultured *in vitro* under standard conditions, either without (UNSTIMULATED) or with PWM (PWM STIMULATED). After 10 days, supernatants were assayed for total IgA. The statistical significance of the difference between spontaneous and PWM stimulated synthesis was determined for each subject using unpaired t-tests. The data are shown as a solid marker above the line of unity if PWM stimulated cultures produced significantly more than unstimulated cultures, a solid marker below the line if there was a significant reduction in PWM cultures, and an open marker if there was no significant difference between them. Reponses were either positive or insignificant.

FIGURE 56: RELATIONSHIP BETWEEN SPONTANEOUS AND POKEWEED MITOGEN STIMULATED *IN VITRO* IgA SYNTHESIS BY DTH ANERGIC PATIENT MONONUCLEAR CELLS



Blood MNCs from DTH A subjects were cultured *in vitro* under standard conditions, either without (UNSTIMULATED) or with PWM (PWM STIMULATED). After 10 days, supernatants were assayed for total IgA. The statistical significance of the difference between spontaneous and PWM stimulated synthesis was determined for each subject using unpaired t-tests. The data are shown as a solid marker above the line of unity if PWM stimulated cultures produced significantly more than unstimulated cultures, a solid marker below the line if there was a significant reduction in PWM cultures, and an open marker if there was no significant difference between them. Most A patients produced insignificant responses to PWM stimulation. Some showed positive responses, and the only negative IgA responses were found in this class.

TABLE 26: PROPORTION OF SUBJECTS SHOWING POSITIVE, INSIGNIFICANT, OR NEGATIVE RESPONSES TO POKEWEED MITOGEN STIMULATION OF *IN VITRO* TOTAL IMMUNOGLOBULIN SYNTHESIS

IgG

GROUP	N	POS (%)	NIL (%)	NEG (%)	
LC	46	40 (87)	6 (13)	0 (0)	
ALL R	44	27 (61)	14 (32)	3 (7)	$X^2_4 = 49.9$
ALL A	55	11 (20)	29 (53)	15 (27)	$p = 0.0001$

IgM

GROUP	N	POS (%)	NIL (%)	NEG (%)	
LC	32	27 (84)	5 (16)	0 (0)	
ALL R	38	27 (71)	11 (29)	0 (0)	$X^2_4 = 34.0$
ALL A	48	12 (25)	33 (69)	3 (6)	$p = 0.0001$

IgA

GROUP	N	POS (%)	NIL (%)	NEG (%)	
LC	23	16 (70)	6 (26)	1 (4)	
ALL R	16	7 (44)	9 (56)	0 (0)	$X^2_4 = 10.7$
ALL A	24	6 (25)	16 (67)	2 (8)	$p = 0.03$

Blood mononuclear cells were harvested from control and patient subjects and cultured in triplicate under standard conditions. Cultures were unstimulated or stimulated with 1:200 PWM. Supernatants were assayed in duplicate for cumulative synthesis of IgG, IgM and IgA after 10 days. For each subject, the statistical significance of the response to PWM stimulation was determined by unpaired t-test on the means and SD of the triplicate cultures. Responses were thus characterized as either significantly postive (POS, PWM stimulated > unstimulated, $p < 0.05$), negative (NEG, PWM stimulated < unstimulated, $p < 0.05$), or not significant (NIL, $p > 0.05$). Subject numbers, percentages and the X^2 value are shown for each isotype.

TABLE 27: PATTERN OF ABNORMAL RESPONSES TO POKEWEED MITOGEN STIMULATION OF *IN VITRO* TOTAL IMMUNOGLOBULIN SYNTHESIS ACCORDING TO ISOTYPE

	G	M	A		G	M	A		G	M	A
LC	0	0	0	HR	0	0	0	HA	-	0	0
LC	0	0	0	WR	0	0	0	IA	-	0	0
LC	+	0	0	WR	0	0	0	IA	-	0	0
LC	+	0	+	HR	0	+	0	WA	-	0	-
LC	+	0	+	WR	0	+	0	WA	-	0	0
LC	+	+	-	WR	0	+	+	WA	-	0	0
LC	+	+	0	WR	+	0	0	IA	-	+	0
LC	+	+	0	WR	+	0	0	HA	0	0	0
LC	+	+	0	HR	+	+	0	IA	0	0	-
LC	+	+	+	HR	+	+	+	IA	0	0	0
LC	+	+	+	HR	+	+	+	IA	0	0	0
LC	+	+	+	HR	+	+	+	IA	0	0	0
LC	+	+	+	WR	+	+	0	IA	0	0	0
LC	+	+	+	WR	+	+	+	IA	0	0	0
LC	+	+	+	WR	+	+	+	IA	0	0	0
LC	+	+	+	WR	+	+	+	IA	0	0	+
LC	+	+	+					WA	0	0	0
LC	+	+	+					WA	0	0	0
LC	+	+	+					IA	+	0	+
LC	+	+	+					IA	+	0	+
LC	+	+	+					WA	+	0	0
LC	+	+	+					IA	+	+	+
LC	+	+	+					IA	+	+	+
								IA	+	+	+

Blood mononuclear cells were harvested from control and patient subjects and cultured in triplicate under standard conditions. Cultures were unstimulated or stimulated with 1:200 PWM. Supernatants were assayed in duplicate for cumulative synthesis of IgG (G), IgM (M) and IgA (A) after 10 days. For each subject, the statistical significance of the response to PWM stimulation was determined by unpaired t-test on the means and SD of the triplicate cultures. Responses were thus characterized as either significantly positive (PWM stimulated > unstimulated, $p < .05$, indicated by +), negative (PWM stimulated < unstimulated, $p < .05$, indicated by -), or not significant ($p > 0.05$, indicated by 0).

TABLE 28: SUMMARY OF ABNORMALITIES IN SPONTANEOUS AND POKEWEED MITOGEN STIMULATED TOTAL IMMUNOGLOBULIN SYNTHESIS IN PATIENT GROUPS

GROUP	SPONTANEOUS Ig SYNTHESIS IgG	IgM	IgA	RESPONSE TO PWM STIMULATION (IgG and IgA) INCREASE	SAME	DECREASE
LAI CONTROL	N	N	N	all	few	none
DTH REACTIVE	>N	<N	>N	most	some	few
DTH ANERGIC	>>N	N	>>N	some	most	some

The table summarizes the abnormalities in spontaneous and PWM stimulated total Ig synthesis *in vitro*. The normal response is abbreviated as N, and > indicates greater than N.

insignificant and occasional negative responses compared to LC, but no significant differences among the isotypes within the class (X^2_4 = 7.65, p = 0.11). In the A group, there was a significant difference in distribution of responses among the isotypes (X^2_4 = 9.77, p = 0.04). The greatest contribution to the statistical significance was from the high number of negative responses in the synthesis of IgG.

There was a consistent pattern of responses across isotypes within a given individual. The statistical pattern of PWM responses from 63 subjects with complete data for all 3 isotypes was examined. Groups were formed indexed on the significance of the IgG response. As shown in table 27, subjects who showed a negative IgG response to PWM, tended to have insignificant responses in the other isotypes. Those with insignificant or positive responses for IgG tended to have similar responses in the other isotypes. These abnormalities were found with cells from all subclasses of A patients, and were thus not restricted to the most ill IA group.

In summary (table 28), more IgG and IgA was produced spontaneously across the biological continuum of subjects from LC to R to A. In PWM stimulated cultures, less IgG and IgM, and more IgA was produced. For all isotypes, PWM stimulation caused fewer positive, and more insignificant and negative responses, especially those with reduced DTH reactivity. The patterns of response among the isotypes within a class were similar.

2.6.4 EFFECT OF MEDIUM COMPOSITION, PROTEIN SYNTHESIS INHIBITORS, AND CONCENTRATION OF POKEWEED MITOGEN ON PATIENT IMMUNOGLOBULIN PRODUCTION IN VITRO

The influence of [PWM], [FBS], protein synthesis inhibitors, duration of culture, and change in clinical status on Ig synthesis *in vitro* were studied. Because patients with reduced DTH showed the greatest abnormalities in Ig production, with increased spontaneous synthesis and abnormal responses to PWM stimulation, attention is focused on the A group.

The amount of Ig produced in response to various concentrations of PWM in 3 A subjects is shown in table 29. The first subject showed a significant negative IgG response to PWM stimulation at the standard [PWM]. Although not uniform, statistically significant negative responses occurred with most other concentations, including 1:100 and 1:6400. The other two donors were also A, but showed significant positive IgG responses to PWM. These

TABLE 29: DOSE RESPONSE OF POKEWEED MITOGEN CONCENTRATION ON CELLS FROM ANERGIC SUBJECTS WITH DIFFERENT PATTERNS OF RESPONSIVENESS

	SUBJECT A POSITIVE			SUBJECT B INSIGNIFICANT			SUBJECT C NEGATIVE		
[PWM]	IgG	IgM	IgA	IgG	IgM	IgA	IgG	IgM	IgA
0	301	50	44	375	117	500	121	81	28
100	356	16	98	298	207	583	59	119	31
200	729	40	22	586	249	1026	58	115	12
400	397	29	113	591	229	855	73	249	19
800	210	24	16	217	146	592	53	87	26
1600	202	16	20	486	150	759	77	181	15
3200	117	28	26	226	140	559	49	74	36
6400	131	26	24	276	97	473	57	203	18

Blood MNC were harvested from three anergic subjects and cultured in triplicate under standard conditions, except for PWM dilution. Cultures were either unstimulated (UNSTIM), or stimulated with PWM at a variable final dilution (1:100 to 1:6400). Supernatants were assayed in duplicate for cumulative synthesis of IgG, IgM and IgA after 10 days. For each subject, the statistical significance of the IgG response to PWM stimulation (using data from the standard final dilution of PWM, 1:200) was determined by unpaired t-test on the means and standard deviations of the triplicate cultures. Subject A showed a significantly positive response (PWM stimulated > unstimulated, $p < 0.05$), subject B an insignificant response ($p > 0.05$), and subject C a significantly negative response (PWM stimulated < unstimulated, $p < 0.05$). Subjects A and B were from the IA group, and C was an HA patient. Arithmetic means of triplicate culture wells are shown.

increases were either less or absent at concentrations other than 1:200. The other isotypes showed similar patterns to IgG. In summary, the optimal [PWM] was 1:200 for both positive and negative responses, although higher dilutions were also effective in the latter.

The concentration of FBS, from 2 to 10%, had a variable and minor effect on spontaneous and stimulated IgG synthesis by patient MNC (data not shown).

Protein synthesis inhibition with 10^{-5}M cycloheximide at culture initiation did not completely eliminate the appearance of supernatant Ig. Culture supernatants from A subjects had somewhat more non-inhibitable IgG and IgA than LC in cycloheximide-treated cultures (table 31, compare with table 18). Whether this Ig was present on the surface of cells, or within cells actively producing Ig at the time of bleeding, and subsequently released during culture, is unknown. Significant quantities of IgG and IgA are nonetheless spontaneously produced by *de novo* protein synthesis in culture. Quantities of IgM were comparable to LC. In PWM stimulated cultures, cycloheximide-treated cultures contained Ig quantities similar to unstimulated wells.

Figure 57 shows that in cultures from a subject whose MNCs produced large amounts of IgG spontaneously, most of the Ig was synthesized early. Figure 58 shows data from an A subject with a negative response to PWM. When PWM produced a negative IgG response in standard cultures, there was an early and continuous spontaneous production of IgG. In PWM stimulated wells, less was produced continuously, suggesting an early action of PWM to suppress immediate spontaneous synthesis. The time course of Ig synthesis over the 10 day culture period was normal for patients showing similar responses to LC MNCs.

Some subjects were studied sequentially. Excluding immunized patients, 12 subjects were studied on 2 to 6 occasions. Those whose skin test classification remained the same (5 R and 4 A) produced similar quantities of Ig, and gave similar responses to PWM stimulation Three patients had improved skin tests. They were initially studied as IA patients, and from 3 to 24 weeks later as HR. For IgG, 2 gave insignificant, and 1 a negative response to PWM stimulation when A. When R, 1 response was insignificant, and 2 positive. For IgM, 2 and 1 of the subjects respectively produced positive and insignificant responses on both occasions. IgA responses were also the same on both occasions.

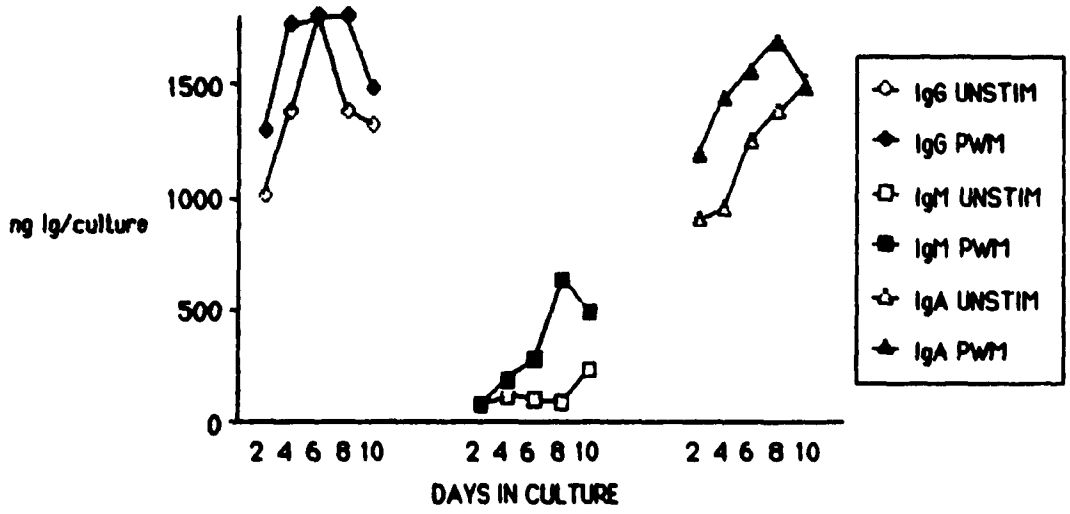
In summary, varying concentrations of PWM and FBS had little impact on patient Ig synthesis. There was some carry-over of preformed IgA and IgG in some cases. High

TABLE 30: EFFECT OF PROTEIN SYNTHESIS INHIBITORS ON CULTURES FROM ANERGIC SUBJECTS WITH HIGH SPONTANEOUS *IN VITRO* SYNTHESIS OF IMMUNOGLOBULIN

	SUBJECT A			SUBJECT B			SUBJECT C		
CULTURE	**IgG**	**IgM**	**IgA**	**IgG**	**IgM**	**IgA**	**IgG**	**IgM**	**IgA**
UNSTIM	164	32	63	1454	323	-	506	44	225
+CYCLO	108	33	42	77	37	-	170	41	54
PWM STIM	458	91	194	1269	408	-	449	38	226
+CYCLO	128	27	24	251	35	-	183	48	64

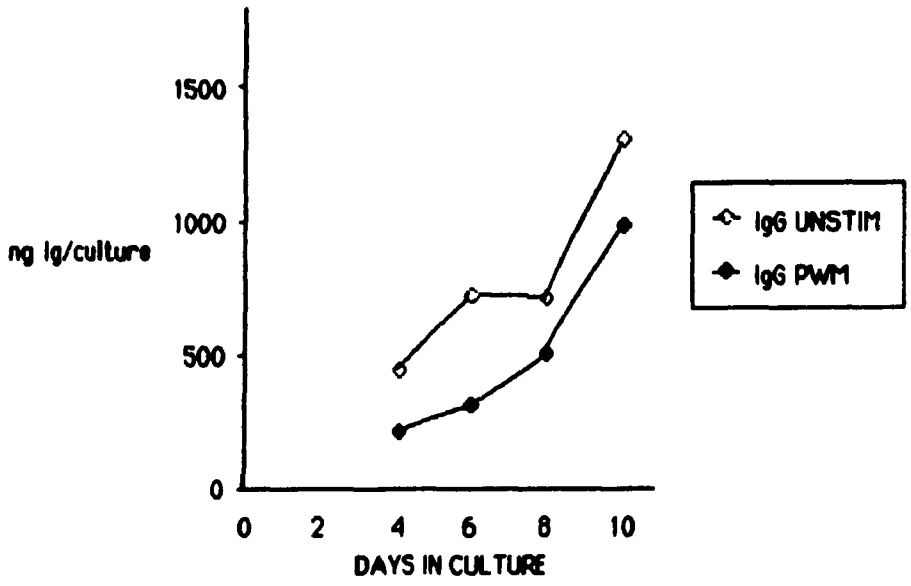
Blood MNC were harvested from three anergic subjects (all IA) and cultured in triplicate under standard conditions. Cultures were either unstimulated (UNSTIM), or stimulated with PWM at a final dilution of 1:200 (PWM STIM). Cycloheximide at a final concentration of 10^{-5}M was added at the beginning of cultures from the same donors. Supernatants were assayed in duplicate for cumulative synthesis of IgG, IgM and IgA after 10 days. Arithmetic means of triplicate culture wells are shown.

FIGURE 57: TIME COURSE OF *IN VITRO* IMMUNOGLOBULIN SYNTHESIS BY MONONUCLEAR CELLS FROM AN ANERGIC PATIENT



Mononuclear cells from a DTH A subject were cultured *in vitro* under standard conditions, either without (UNSTIM) or with PWM (PWM). Supernatants were harvested from a set of cultures every 2 days, and assayed for cumulative content of total IgG, IgM and IgA. The addition of 10^{-5}M cycloheximide to the cultures at the initiation decreased the amount of IgG detectable at 10 days to 77 ng/culture.

FIGURE 58: TIME COURSE OF *IN VITRO* IgG SYNTHESIS BY MONONUCLEAR CELLS FROM A PATIENT WITH A SIGNIFICANT NEGATIVE RESPONSE TO POKEWEED MITOGEN



A time course was done with MNCs from a patient that produced statistically significantly less IgG in PWM stimulated cultures than spontaneously. Supernatants were harvested from a set of cultures every 2 days, and assayed for cumulative content of total IgG. The amount of IgG in the PWM cultures was reduced on every day tested.

spontaneous synthesis of Ig occurred early in culture. When PWM stimulation reduced IgG synthesis, the lectin acted early *in vitro*. The pattern of Ig responses *in vitro* followed the clinical condition and DTH reactivity of patients studied sequentially. As skin tests improved, the pattern of Ig synthesis *in vitro* returned to normal.

2.7 EFFECT OF HYDROCORTISONE ON IMMUNOGLOBULIN PRODUCTION IN VITRO

The effect of hydrocortisone (HC) on Ig synthesis was studied. The drug was added at the initiation of standard cultures. The [HC] in FBS was unknown, but likely minimal at 2% FBS. Supernatants were assayed for total IgG, and for anti-TT or anti-PPS in recently immunized individuals. Unpaired t-tests were used to determine the statistical significance of the influence of HC.

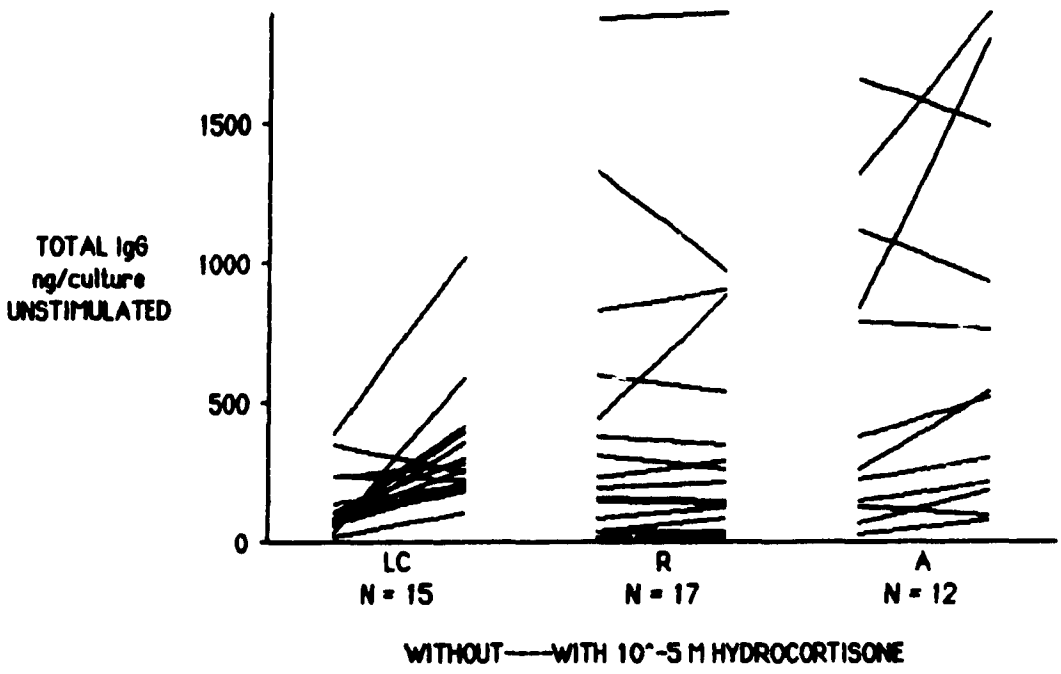
Preliminary experiments with LC cells showed maximal effect in both unstimulated and PWM stimulated cultures with 10^{-5} to 10^{-7}M HC (data not shown). Further experiments were done with 10^{-5} and 10^{-7}M. The latter is approximately equal to maximal endogenous cortisol levels in highly stressed patients, and the former was chosen for maximal pharmacologic effect.

Spontaneous synthesis of total IgG was significantly increased by 10^{-5}M HC in 10 of 15 LC subjects (figure 59). In both R and A patients, in whom the average production of IgG without HC was more than LC, few subjects showed an increase with this [HC] (1 of 17 R, 3 of 12 A, $X^2_4 = 14.0$, $p = 0.008$). At [HC] of 10^{-7}M (figure 60), a greater number of patients, mostly A, showed a significant increase (9 of 15 LC, 2 of 17 R, and 9 of 12 A, $X^2_4 = 14.5$, $p = 0.006$). Occasional subjects also produced less with HC (2 R and 1 A).

In PWM stimulated cultures, the same pattern as in spontaneous cultures was present with [HC] of 10^{-5}M (figure 61, $X^2_4 = 9.5$, $p = 0.05$). At 10^{-7}M, HC had little effect. Cells from most patients continued to show insignificant responses to PWM, as in cultures without HC (figure 62, $X^2_4 = 3.9$, $p = 0.42$).

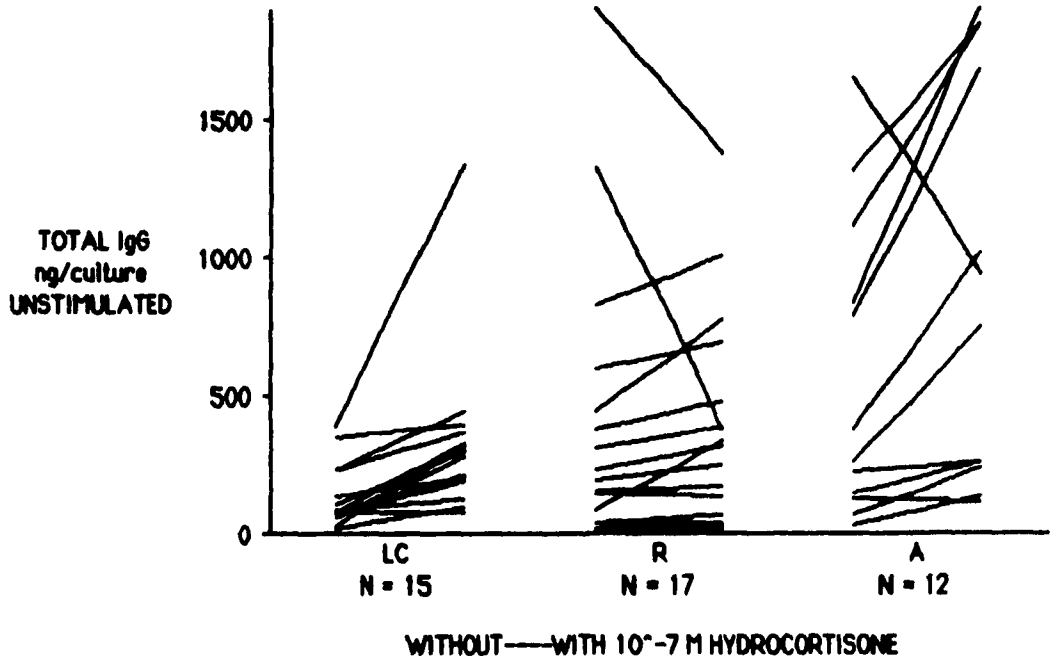
Exogenous hydrocortisone had no impact on specific antibody synthesis *in vitro*. In studies of 2 LC, 2 R and 2 A subjects immunized with TT, neither spontaneous nor PWM stimulated cultures of MNCs harvested on day 6 post-immunization showed any difference in anti-TT

FIGURE 59: EFFECT OF 10^{-5}M HYDROCORTISONE ON SPONTANEOUS IgG SYNTHESIS *IN VITRO*



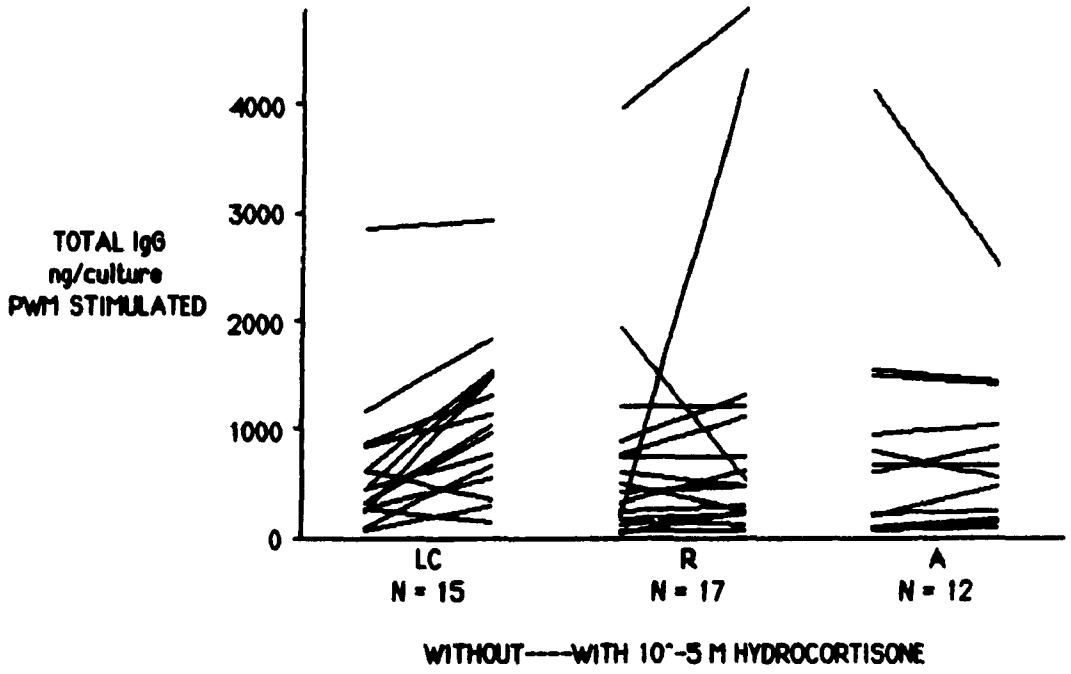
Blood MNCs from LC, R and A subjects were cultured under standard unstimulated conditions. Hydrocortisone (HC) was added at the beginning of the cultures in a final concentration of 10^{-5}M. After 10 days, supernatants were assayed for quantity of IgG. The data are shown as a line between the amounts of IgG produced with and without HC.

FIGURE 60: EFFECT OF 10^{-7}M HYDROCORTISONE ON SPONTANEOUS IgG SYNTHESIS *IN VITRO*



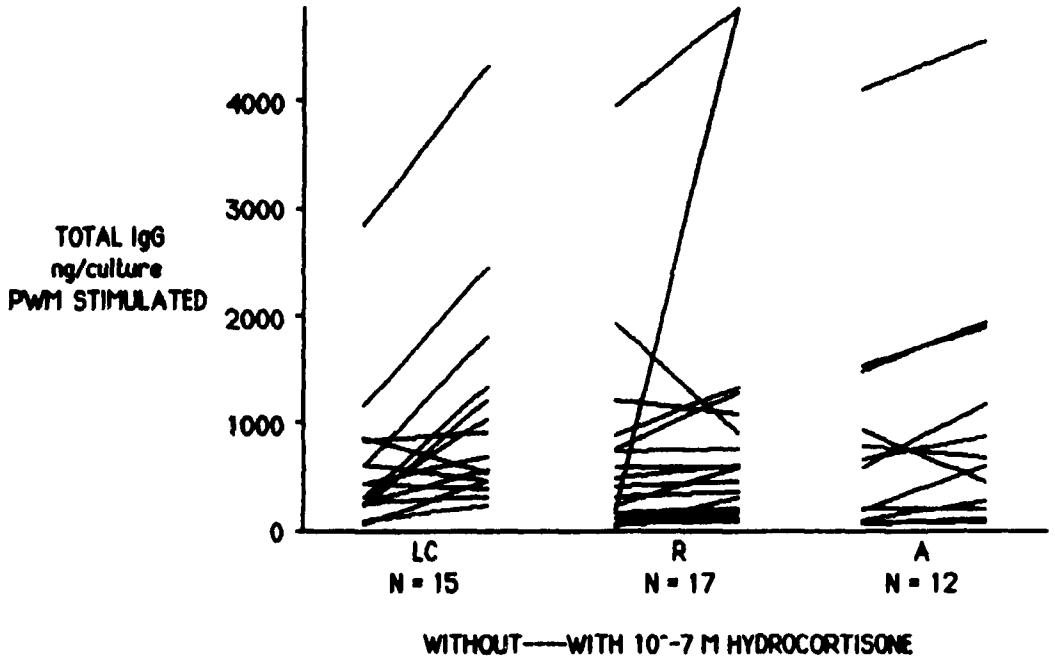
Blood MNCs from LC, R and A subjects were cultured under standard unstimulated conditions. Hydrocortisone (HC) was added at the beginning of the cultures in a final concentration of 10^{-7}M. After 10 days, supernatants were assayed for quantity of IgG. The data are shown as a line between the amounts of IgG produced with and without HC.

FIGURE 61: EFFECT OF 10^{-5}M HYDROCORTISONE ON POKEWEED MITOGEN STIMULATED IgG SYNTHESIS *IN VITRO*



Blood MNCs from LC, R and A subjects were cultured under standard conditions with PWM stimulation. Hydrocortisone (HC) was added at the beginning of the cultures in a final concentration of 10^{-5}M. After 10 days, supernatants were assayed for quantity of IgG. The data are shown as a line between the amounts of IgG produced with and without HC.

FIGURE 62: EFFECT OF 10^{-7}M HYDROCORTISONE ON POKEWEED MITOGEN STIMULATED IgG SYNTHESIS *IN VITRO*



Blood MNCs from LC, R and A subjects were cultured under standard conditions with PWM stimulation. Hydrocortisone (HC) was added at the beginning of the cultures in a final concentration of 10^{-7}M. After 10 days, supernatants were assayed for quantity of IgG. The data are shown as a line between the amounts of IgG produced with and without HC.

synthesis *in vitro* with and without 10^{-5}M or 10^{-7}M HC (data not shown). Similarly, the synthesis of anti-PPS by MNCs harvested 6 to 8, and 14 days after vaccination of 2 LC, 4 R and 6 A subjects with PPS was not changed by HC at either concentration (data not shown).

In summary, spontaneous total IgG synthesis was normally increased by levels of exogenous corticosteroid comparable to, and 100 fold greater than, maximal *in vivo* levels. Patient MNCs, already producing more IgG than controls, produced even greater amounts spontaneously with a [HC] comparable to levels achievable *in vivo*. Stimulated synthesis was not affected. The combined effect of exogenous corticosteroid thus exaggerated the pattern of total IgG synthesis seen in patients. Exogenous corticosteroid did not affect specific antibody synthesis *in vitro* after recent vaccination *in vivo*.

3. CELL PHENOTYPES

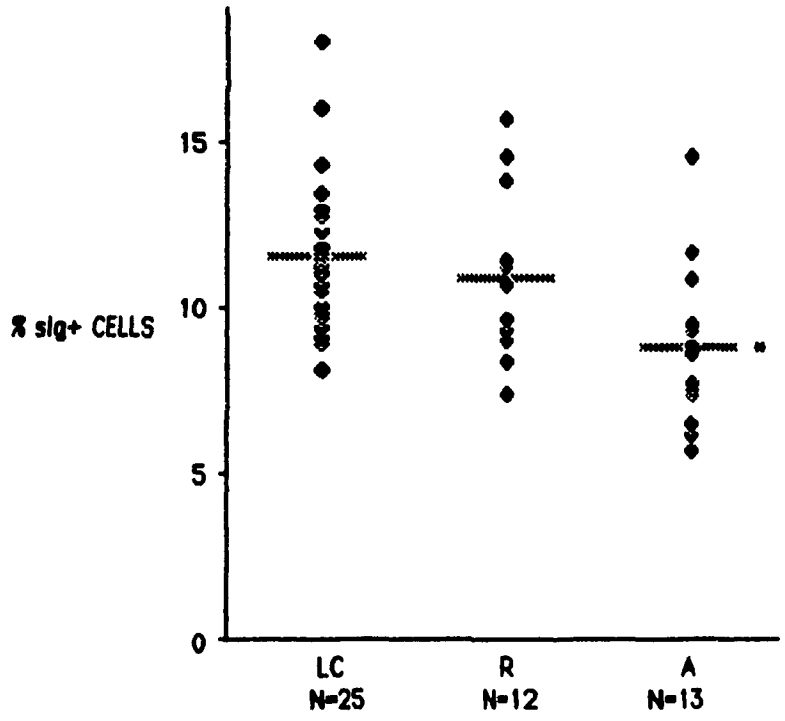
Phenotypes of circulating B and T cells were studied by analysis of cell surface markers. Monocytes were studied by cell surface marker and phagocytosis. All tests were done on blood MNCs isolated in standard fashion, including serum free incubation to remove cytophilic Ig.

3.1 B CELL ENUMERATION

The percentage of sIg^+ cells was determined with anti-Ig coated polyacrylamide beads and latex particles, as described. On the basis of morphology, latex particle ingestion and binding of anti-Ig coated polyacrylamide beads to the cell surface, cells were counted as sIg^+ or sIg^- lymphocytes, monocytes or PMNs. Twenty five LC, 12 R and 13 A subjects were studied. The mean $\pm$ SEM sIg^+ cells was 11.5 $\pm$ 0.5, 10.8 $\pm$ 0.8 and 8.7 $\pm$ 0.7 for LC, R and A subjects respectively (figure 63). Only the difference between A and LC was statistically significant ($p = 0.005$, unpaired t-test).

In summary, the detectable % of circulating sIg^+ cells was decreased modestly among patients, especially those with reduced DTH reactivity.

FIGURE 63: PERCENTAGE OF sIg^+ BLOOD MONONUCLEAR CELLS



Surface Ig^+ MNCs were counted in blood cell preparations fom LC, R and A patients using the polyacrylamide bead technique. The data are expressed as the % of sIg^+ MNCs. Arithmetic means are shown by the horizontal bars.

3.2 T CELL ENUMERATION AND SUBCLASS ANALYSIS

The study population for FACS analysis of circulating MNCs consisted of 10 LC, 12 R and 12 A subjects. Of the R patients, 7 were WR, and 5 HR. Of the A patients, 3 were WA, 2 HA and 7 IA. The mean $\pm$ SEM ages were 58 $\pm$ 5, 56 $\pm$ 3 and 68 $\pm$ 3, respectively. The mean $\pm$ SEM STSs were 29 $\pm$ 5, 31 $\pm$ 4 and 0 $\pm$ 0, respectively. The mean $\pm$ SEM APACHE II scores were 3 $\pm$ 1, 4 $\pm$ 1 and 13 $\pm$ 2 ($p < 0.01$ compared to both LC and R, unpaired t-test), respectively.

The cell preparations were relatively pure. Contamination with PMNs was less than 10% for all subjects, and averaged 3, 4 and 6% for LC, R and A respectively. Some patients showed a large cell population on the right of the log-vol plots (figure 1).

For each stain, data were examined in two ways. The first was as the % positive of the FACS cell preparation. The second method was as the absolute number of positive cells/mm^3 of whole blood, calculated as the % positive * (total lymphocyte count + total monocyte count). The total cell counts were obtained from differential cell counts performed in the clinical laboratory on specimens taken the same time as the blood for FACS analysis.

There are T cell abnormalities in patients. The % positive data are shown in table 31. Total T cells were significantly decreased among all patients, helper and suppressor subsets were decreased among R patients, and NK cells cells were increased in the A group, although not significantly so. The helper:suppressor ratio was decreased among all patients, lowest in those with reduced DTH reactivity The differences among the 3 groups, however, were not statistically significant ($p = 0.21$ for LC versus R, 0.15 for LC vs A, unpaired t-tests). The greatest difference was between LC and all patients combined, 1.59 $\pm$ 0.37 compared to 1.10 $\pm$ 0.07 ($p = 0.06$, unpaired t-test).

The data comparing the absolute numbers of cells present per volume blood are listed in table 32. Total WBC, PMN and monocyte counts were all elevated in A patients. Lymphocyte counts were low, although not signficantly so in this group. Again, Leu-4^+ cells were decreased among all the patients. There was no trend in the T cell subclasses with this method of analysis.

In A patients, there were cells that were positive for T cell subclass markers, but negative for the global T cell antigen, Leu-4. Adding the percentages of Leu-2a$^+$, Leu-3a$^+$ and Leu-7$^+$ cells, and dividing by % Leu-4$^+$, shows that these subclass-marker positive populations account for 103, 108 and 151% of Leu-4 cells in the LC, R and A classes respectively (table 31). Similarly, subtracting the sum of the absolute numbers of subclass marker positive cells from Leu-4 shows that there are substantial numbers of cells staining with subclass markers that are Leu-4$^-$ (table 32).

In summary, there are alterations in the T cell phenotypic composition of blood in patients. The abnormalities were generally worse among all patients, but in some instances were worst among those with reduced DTH. Total T cells and the helper:suppressor ratio were reduced and natural killer cells elevated. There were cells that are positive for T cell subclass antigens, but negative for a global T cell surface marker.

3.3 MONOCYTES AND HLA-DR ANTIGEN

Blood monocytes were elevated among patients, especially those with reduced DTH. There was a statistically significant difference among A compared to both LC and R in the number of blood monocytes in clinical laboratory differential counts, and by FACS staining (tables 31 and 32).

There were more HLA-DR$^+$ cells in patients with reduced DTH (table 31). There was no difference between LC and R. This may be a manifestation of changes in monocytes, or activated T or B cells, because all these cell types can be HLA-DR$^+$. There was a correlation between the % of Leu-M3$^+$ and HLA-DR$^+$ cells ($N = 34$, $r_s = 0.711$, $p = 0.0005$), suggesting these cells may be the major contributor.

In summary, there were several indications of elevated monocyte numbers in patients with reduced DTH. HLA-DR positive cells were also elevated.

TABLE 31: PERCENTAGES OF MONONUCLEAR CELL PHENOTYPES BY FACS ANALYSIS

MARKER	LAB CONTROL	REACTIVE	ANERGIC
N	10	12	12
Leu-4 (CD3)	55 ± 3	*37 ± 3	*37 ± 4
Leu-3a (CD4)	25 ± 2	*16 ± 3	19 ± 3
Leu-2a (CD8)	19 ± 2	*14 ± 2	#19 ± 2
Leu-7	11 ± 1	11 ± 2	14 ± 1
Leu-M3	5 ± 1	6 ± 1	*#12 ± 1
HLA-DR	11 ± 1	10 ± 2	*#17 ± 2
sIg^+	16 ± 2	15 ± 2	*#24 ± 2
Leu 3a/2a	1.59 ± 0.37	1.15 ± 0.10	1.06 ± 0.11
Leu 2a+3a+7/Leu 4	1.03 ± 0.06	1.08 ± 0.01	*#1.51 ± 0.01

After harvesting PBMNCs and staining, 10,000 cells were counted using a Becton Dickinson FACS III analyzer, with gates set as described in Materials and Methods. Arithmetic means ± sem of the percentage positive are shown. The ratio of cells positive for Leu-3a and Leu-2a (Leu3a/Leu-2a) were calculated for each subject, and averaged. To show the relationship between the sum of T cell subclass markers and total T cells, the ratio of the sum of Leu 2a + 3a + 7 to Leu 4 was calculated for each subject, and averaged. * indicates $p < 0.05$ compared to LC group, and # compared to R group (unpaired t tests).

TABLE 32: ABSOLUTE NUMBERS OF MONONUCLEAR CELL PHENOTYPES BY FACS ANALYSIS

	LAB CONTROL	REACTIVE	ANERGIC
N	10	12	12
CELL COUNTS			
WBC	5770 ± 503	7608 ± 848	*#13483 ± 2482
PMNL	3460 ± 331	4983 ± 876	*#10583 ± 2351
LYMP	1780 ± 168	1708 ± 133	1333 ± 261
MONO	330 ± 37	333 ± 45	*#642 ± 114
CELL MARKERS			
Leu-4 (CD3)	1144 ± 119	*778 ± 105	*706 ± 129
Leu-3a (CD4)	521 ± 64	340 ± 65	413 ± 104
Leu-2a (CD8)	415 ± 59	281 ± 42	383 ± 79
Leu-7	239 ± 46	235 ± 47	265 ± 35
Leu-M3	108 ± 28	112 ± 27	*#238 ± 42
HLA-DR	241 ± 41	195 ± 39	358 ± 90
sIg^+	325 ± 43	301 ± 44	509 ± 121
Leu 4 - (Leu 2a + 3a + 7)	-79 ± 80	-31 ± 62	*#-355 ± 90

The absolute number of white blood cells (WBC), polymorphonuclear leukocytes (PMNL), lymphocytes (LYMP) and monocytes (MONO) were determined by automated cell counters in the Clinical Hematology Laboratory of the Royal Victoria Hospital. Data for cell counts are shown as arithmetic means ± sem of number of cells per mm^3. After harvesting MNCs from simultaneously obtained specimens and staining, 10,000 cells were counted using a Becton Dickinson FACS III analyzer, with gates set as described in Materials and Methods. Absolute numbers of cells positive for a given marker were calculated per mm^3 of blood. To be consistent with the FACS methodology, the following formula was used: absolute number = % positive * (LYMP+MONO). Arithmetic means ± sem for the groups are shown. To show the relationship between the sum of T cell subclass markers and total T cells, the difference between the sum of Leu 2a+3a+7 and Leu 4 was calculated for each subject, and averaged. * indicates $p < 0.05$ compared to LC group, and # compared to R group (unpaired t tests).

3.4 RELATIONSHIP OF CELL PHENOTYPES TO IN VITRO IMMUNOGLOBULIN PRODUCTION

Blood MNCs from the same cell preparations used for FACS analysis were cultured *in vitro* under standard conditions. Data were examined for relationships between cell phenotypes and *in vitro* Ig synthesis.

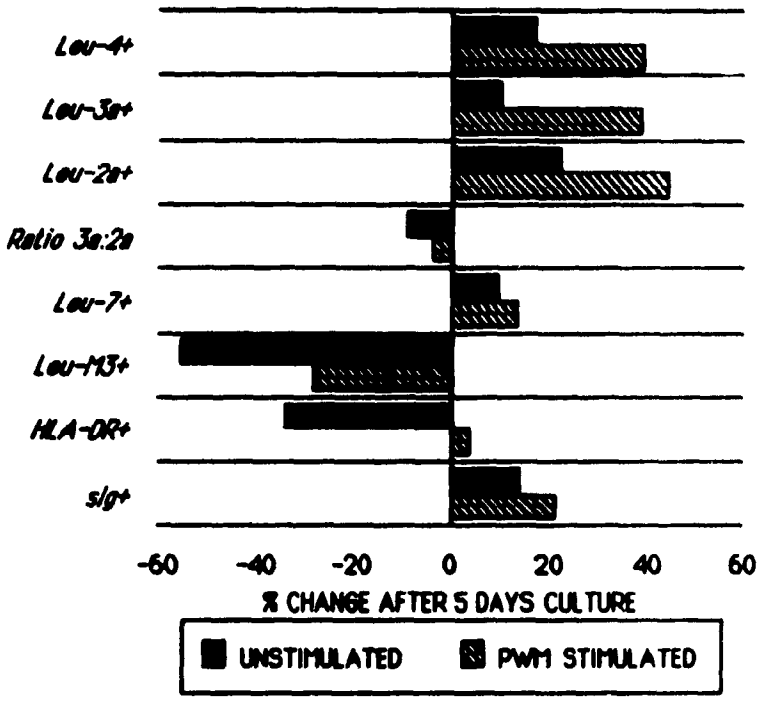
The pattern of Ig synthesis *in vitro* was similar to other patient groups. Spontaneous synthesis of IgG was significantly greater in A (572 $\pm$ 156) compared to both LC (115 $\pm$ 22) and R (206 $\pm$ 59) ($p = 0.04$, unpaired t-tests). For LC, R and A groups, the following numbers of subjects gave signficantly positive, insignificant, or negative reponses to PWM stimulation: 8-2-0, 7-5-0, and 2-4-6 ($X^2_4 = 16.43$, $p = 0.003$). For IgM and IgA, the spontaneously produced quantities were not different, and the patterns of PWM responses were not significant by contingency table analysis.

No significant relationships or trends were present between total or subset T cell numbers and PWM stimulated or unstimulated Ig synthesis *in vitro*. There was, however, a trend with helper:suppressor ratios. The average ratio of Leu-3a$^+$:Leu-2a$^+$ cells was greater in all subjects showing a positive IgG response to PWM (N = 17, mean Leu-3a$^+$:Leu-2a$^+$ ratio = 1.43) compared to subjects giving a negative response (N = 6, mean ratio = 0.98). The difference in ratios, however, was not significant ($p = 0.28$, unpaired t-test). Subjects whose cells responded insignificantly to PWM had an intermediate helper:suppressor ratio (N = 11, mean ratio = 1 10)

Cell preparations with greater monocyte numbers produced more IgG spontaneously. There was a significant direct correlation between Leu-M3$^+$ and spontaneous IgG synthesis ($r_s = 0.42$, $p = 0.01$), but not with HLA-DR$^+$ ($r_s = 0.20$, $p = 0.25$). Increased monocytes was associated with decreased PWM stimulated synthesis. There were negative correlations between Leu-M3$^+$ and both the ratio and difference between unstimulated and PWM stimulated IgG production ($r_s = -0.39$, $p = 0.02$, $r_s = -0.52$, $p = 0.002$).

The effect of *in vitro* culture with and without PWM on surface phenotypes patterns was studied with LC cells. FACS analysis was performed on cell preparations before, and after 5 days of culture. At this time, significant PWM stimulated Ig synthesis had begun (357 ng/culture compared to 31 in unstimulated wells). The overall viable cell yield after culture was comparable with PWM ($2.1*10^5$/well) and without ($2.0*10^5$/well). There was a greater

FIGURE 64: CHANGES IN THE PHENOTYPIC COMPOSITION OF CELL CULTURES DURING INCUBATION WITH AND WITHOUT POKEWEED MITOGEN



A whole MNC preparation from an LC subject was cultured with and without PWM. The cells were analyzed for prevalence of surface markers using the FACS methods described, at the beginning of the culture, and after 5 days. The data are expressed as the % change in the prevalence of each stain. Ratios of Leu-3a:2a were calculated, and the % change in the ratio shown.

% of blasts in stimulated cultures, seen both with differential counting (15 compared to 1% of total cells), and as a second peak on the right side of a FACS log-vol plot (figure 1). Analysis of % change in surface phenotypes showed that all T cell subsets and sIg^+ cells were increased after culture, more with stimulation (figure 64). The Leu-$3a^+$:Leu-$2a^+$ ratio was marginally altered. Monocytes were decreased. Cells positive for HLA-DR were reduced without PWM, but increased with stimulation.

In summary, cell preparations with more monocytes showed increased spontaneous IgG production, and decreased PWM stimulated production. There was a trend for lower helper:suppressor T cell ratios as responses to PWM diminished or became negative, but this was not significant. The pattern of surface phenotypes after culture suggests that PWM stimulates T cells of all subclasses, with little change in the helper:suppressor ratio.

4. HUMORAL IMMUNITY AFTER TRAUMA

Trauma patients were analyzed separately from non-trauma patients, but with standard demographic data collection and outcome definitions. Tetanus toxoid vaccination is a routine part of trauma care, and the antibody response to this antigen was selected for study. *In vitro* studies of Ig and antibody synthesis were performed in standard fashion.

Booster doses of 0.5 Lf of aluminum phosphate adsorbed TT were given in the emergency room. Human hyperimmune globulin, nearly all IgG by electrophoresis (data not shown), was also given at a separate site if there is insufficient evidence of primary immunization, or very contaminated wounds. Although this method of passive immunization does not interfere with the normal host response to toxoid administration[383], it could alter the measured antibody response. Eight subjects received 250 - 500 IU of hyperimmune globulin. Only one showed a significant increase in serum antibody on day 3. The level achieved, 30 IU/ml, was much higher than could be accounted for by passive means, and remained approximately the same for 2 weeks. All these subjects were therefore included in the study.

The administration of plasma is frequently an important part of early trauma resuscitation. The timing and volume of plasma transfusions were recorded, and additional serum samples taken at intervals after vaccination to allow determination of false positive responses due to passive immunization. Seventeen of 35 patients received plasma during their hospitalization, nearly all given within the first 3 days. Only one subject showed a substantial

increase in anti-TT level at day 3, from .8 to 2.1 U/ml serum. At day 14, used for the calculation of the antibody response, there was a further increase to 21.6 U/ml, so that the passively administered antibody constituted little of the eventual response. All these subjects were therefore included in the study.

A final consideration is that some pre-immunization samples were not taken until after the administration of some plasma. If large amounts of anti-TT were transfused, this would produce a false diminution in response calculated at day 14. Because most patients showed good responses regardless, this possibile artifact was discounted.

4.1 PATIENT CLASSIFICATION AND CLINICAL DATA

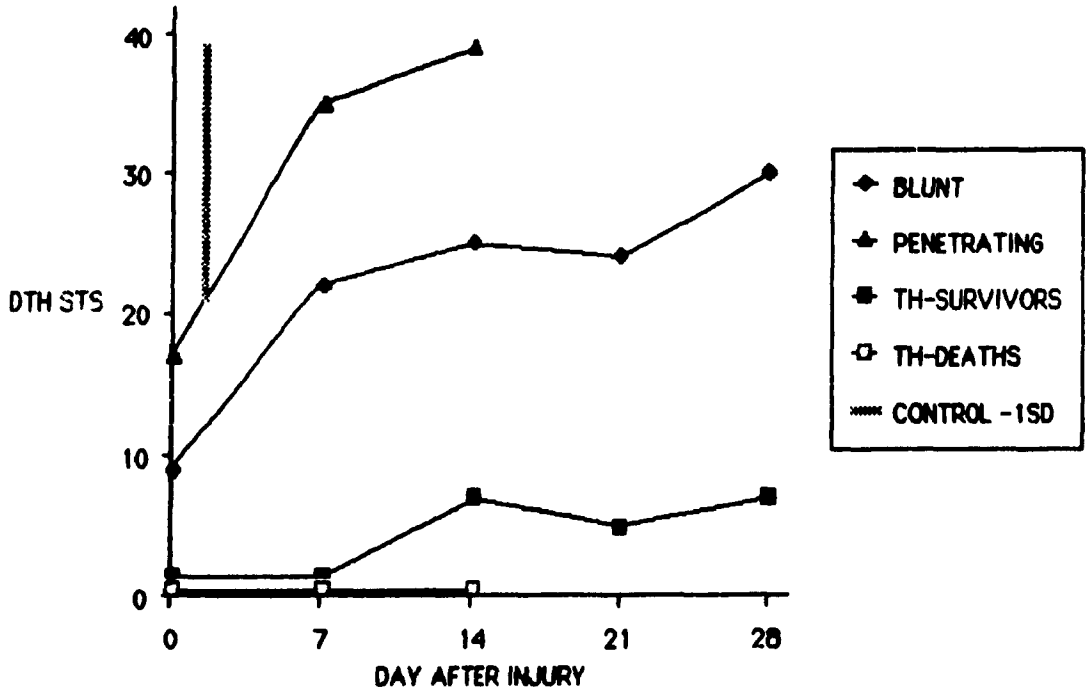
Subjects were classified according to type of injury as blunt, penetrating or thermal. Injury severity was stratified according to the MISS, blood transfusions and duration of ICU and hospital stays. Thermally injured patients are described by percentage of 2nd and 3rd degree burns. Only patients surviving at least 14 days after injury were included.

Thirty-five subjects were studied; 24 blunt, 7 penetrating and 4 thermal injuries. For some analyses, blunt and penetrating trauma are combined. Thermal injury is discussed separately.

Twenty-four patients suffered blunt injury. The age range, median and mean $\pm$ SEM were 18-68, 35, 34 $\pm$ 2. The sex ratio was 19:5 M:F. The MISS ranged from 10 to 50, with a median of 26. In each of the anatomic regions, the number of subjects with scores indicating severe injury, i.e. 3 or greater, was 1, 6, 13, 11 and 19 respectively for neurologic, head and face, chest, abdomen and pelvis & extremities. The majority of severe injuries were thus orthopaedic, chest and abdominal. An average of 9.5 units of red blood cells (PC) were given per person, nearly all within the first 3 days. The range, median and mean $\pm$ SEM for duration of stay in ICU were 1-27, 6, and 9 $\pm$ 1, and for duration of stay in hospital were 11-113, 47, and 53 $\pm$ 6.

Skin test scores were depressed in some subjects, but increased rapidly after injury (figure 65). Nine of 24 patients had an initial STS of 0. All patients were reactive before discharge from hospital, except two, who were relatively anergic. One of these subjects was re-

FIGURE 65: DELAYED TYPE HYPERSENSITIVITY AFTER TRAUMA



In patients after blunt (N = 24), penetrating (N = 7), or thermal trauma (TH, N = 4), DTH STS were obtained within 48 hours of admission, and weekly thereafter. Data are shown as the arithmetic means in mm. The data are shown separately for survivors (N = 2) and non-survivors (N = 2) after thermal injury. The mean - 1SD of STSs from LC subjects is shown as a vertical bar (39 ± 18).

admitted with an abdominal abscess. The other, although defined as RA, had a satisfactory STS of 18 mm at discharge.

APACHE II scores were calculated in the emergency room, and on days 1, 3, 6, 14, 21 and 28 after admission. Figure 66 shows the kinetics of acute physiologic alterations after injury. After blunt trauma, abnormalities in vital signs and respiratory parameters constituted the major portion of disturbed physiology, but improved rapidly in an exponential fashion. Abnormalities in temperature, heart rate, respiratory rate, serum creatinine and neurologic function all increased after evaluation in the emergency room. After 28 days, there were residual abnormalities in blood pressure and heart rate.

Clinical outcome was determined at discharge. Major sepsis occurred in 3 subjects. Twenty-nine episodes of minor sepsis occurred in 17 subjects. There were no mortalities.

Seven patients suffered penetrating injury. The age range, median and mean $\pm$ SEM were 33-62, 48, and 45 $\pm$ 4. The sex ratio was 6:1 M:F Penetrating injuries were classified using MISS, which ranged from 1 to 43, with a median of 20. In each of the anatomic regions, the number of subjects with scores indicating severe injury, i.e. 3 or greater, was 1, 0, 3, 4 and 1 respectively for neurologic, head and face, chest, abdomen and pelvis & extremities Chest and abdominal injuries predominated. An average of 3 units PC were given per person, most within the first 3 days. The range, median and mean $\pm$ SEM for duration of stay in ICU were 1-5, 3, and 3 $\pm$ 1, and for duration of stay in hospital were 3-32, 11, and 14 $\pm$ 9.

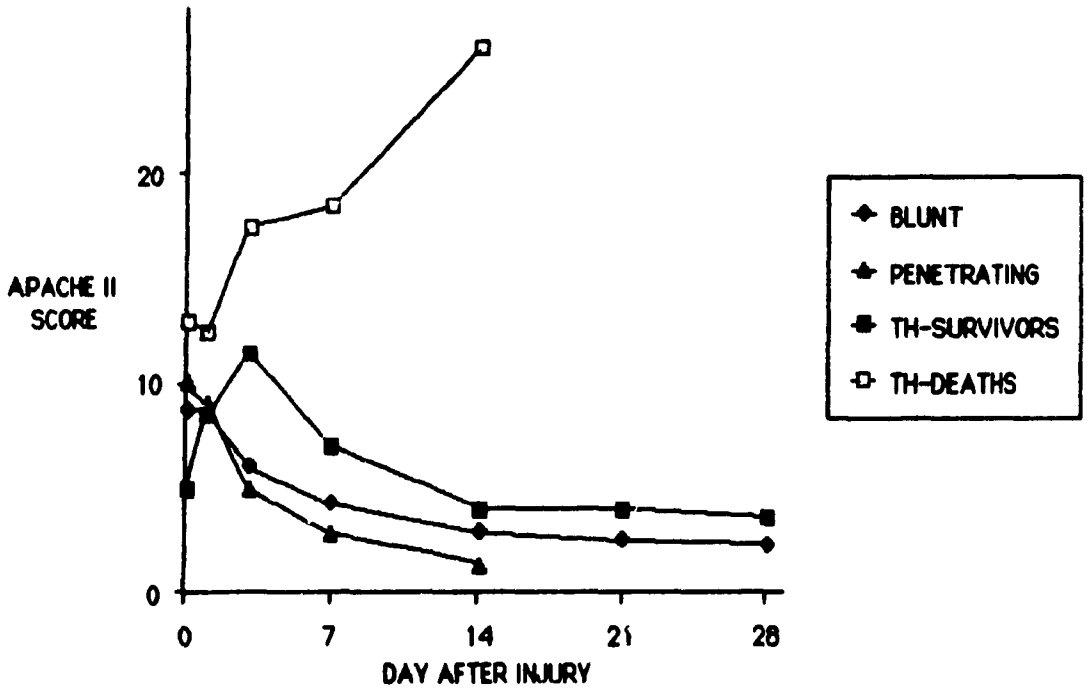
ALthough 2 of 7 patients had admission STS of 0, average DTH skin testing was less depressed than after blunt trauma, and increased rapidly (figure 65). All patients were reactive before discharge from hospital.

Physiologic derangement was equal to blunt trauma cases in the emergency room, but improved slightly more rapidly in an exponential fashion (figure 66).

Clinical outcome was determined at discharge. Major sepsis occurred in 1 subject. Four episodes of minor sepsis occurred in 2 subjects. There were no mortalities.

Four patients suffered thermal injury. All were males, with age range of 18-69. Three subjects received 2nd and 3rd degree flame burns of 25% (age 18), 45% (age 64) and 60% (age 56) of body surface area. One patient received electrical injury requiring amputation (age 69). There were two mortalities due to major sepsis, the patient with 60% flame burn

FIGURE 66: PHYSIOLOGICAL ABNORMALITIES AFTER TRAUMA



In patients after blunt (N = 24), penetrating (N = 7), or thermal trauma (TH, N = 4), APACHE II scores were calculated from data obtained in the emergency room, and on days 3, 7, 14, 21 and 28 thereafter. Data are shown as the arithmetic means. The data are shown separately for survivors (N = 2) and non-survivors (N = 2) after thermal injury.

injury, and the electrical injury victim. These patients died after 16 and 18 days in the ICU, while the survivors were in ICU for 4 and 8 days.

DTH scores were 0 on the day after admission for all thermal injury subjects. and remained so for at least 1 week (figures 65 and 67). On day 14, STSs were again 0 for the patients who eventually died, but had improved in the survivors (figure 65). Even a 25% thermal burn in an 18 year old subject, which is associated with minimal mortality, thus produced anergy on admission, and persistent hypo-reactivity for 4 weeks.

There was a consistent pattern in physiologic abnormalities and DTH skin testing in survivors and non-survivors. APACHE II scores were elevated on admission in relation to degree of injury, and increased in the first 2 to 3 days (figure 66). In survivors, these subsequently declined, while in non-survivors, scores continually increased. A reduction in physiologic derangement was accompanied by an increase in DTH reactivity.

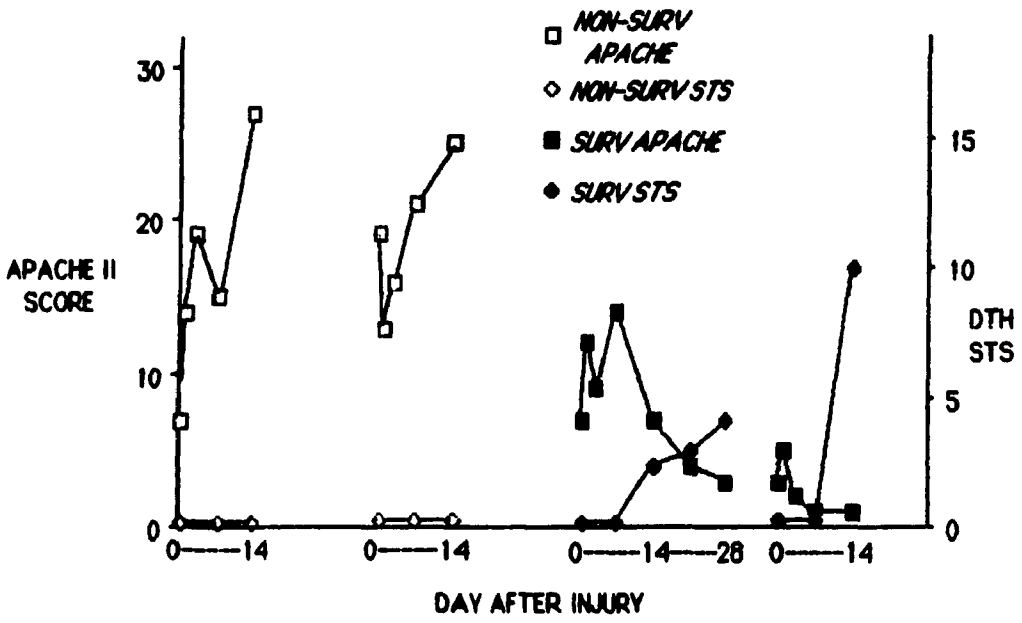
In summary, all forms of trauma depress DTH reactivity, though not equally. In survivors, DTH scores began to increase in parallel with improvement in physiologic homeostasis. This occurred soon after injury in blunt and penetrating trauma, later in survivors of thermal injury, and not at all in non-survivors. Blunt trauma caused greater depression of DTH reactivity, slower recovery of normal physiology, and longer ICU and hospital stays than penetrating injuries.

4.2 IN VIVO ANTIBODY RESPONSES TO TETANUS TOXOID

Serum samples were assayed for antibody content at intervals after admission to day 28. Peak antibody levels occurred on day 14 for most subjects (figure 68), indicating kinetics comparable to normal. The usual definitions of antibody responses were therefore used. The data were compared to a control group of 26 healthy subjects, whose age range, median and mean $\pm$ SEM are 21-65, 41, and 41 + 3.

Antibody responses to TT in controls and after blunt and penetrating trauma are shown in figure 69. The mean responses, and rate of positive response, are listed in table 33. The responses were comparable to normal when calculated either as the difference or ratio between pre- and post-immunization samples (table 33, p = 0.38, 0.34, WRST). Using

FIGURE 67: DELAYED TYPE HYPERSENSITIVITY AND PHYSIOLOGICAL ABNORMALITIES AFTER THERMAL INJURY



In 4 patients after thermal trauma, DTH STSs (mm) were obtained within 48 hours of admission, and weekly thereafter. APACHE II scores were calculated from data obtained in the emergency room, and on days 3, 7, 14, 21 and 28 thereafter. Data are shown as the individual values for each subject. From the left, the first 2 patients shown were non-survivors from 60% 2nd and 3rd degree flame burns, and electrical burn requiring amputation, respectively. The two patients shown on the left survived 45%, and 25% 2nd and 3rd degree flame burns.

arbitrary definitions for a positive response, the data are again comparable for blunt trauma patients and controls (X^2_1 = 0.92, 0.55 for differences and ratios respectively, p = 0.34, 0.46).

After day 14, there was a gradual decline in antibody levels (figure 68). Serum samples up to day 28 were available for 14 patients. From a mean peak at day 14 of 6.89 x: 0.17 U/ml serum, there was a gradual decline to 3.53 x: 0.27 at day 21, and 2.68 x: 0.28 at day 28.

The antibody response to TT after penetrating injury was comparable to both control and blunt trauma groups (figure 69). Responses were similar to both control and blunt trauma groups when calculated either as difference or ratio between pre- and post-immunization samples (table 33, p = 0.40, 0.43 compared to LC, p = 0.34, 0.48 compared to blunt trauma, WRST). Using arbitrary definitions for a positive response, the data are again comparable (X^2_1 = 0.89, 0.09, p = 0.45, 0.76 for differences and ratios for LC, X^2_1 = 0.30, 0.04, p = 0.58, 0.85 for blunt trauma group).

The anti-TT responses of the two survivors after thermal injury were normal (39.3 and 2.5 new U/ml serum at day 14). The responses of the non-survivors were reduced (0.02, 0.00 new U/ml serum.)

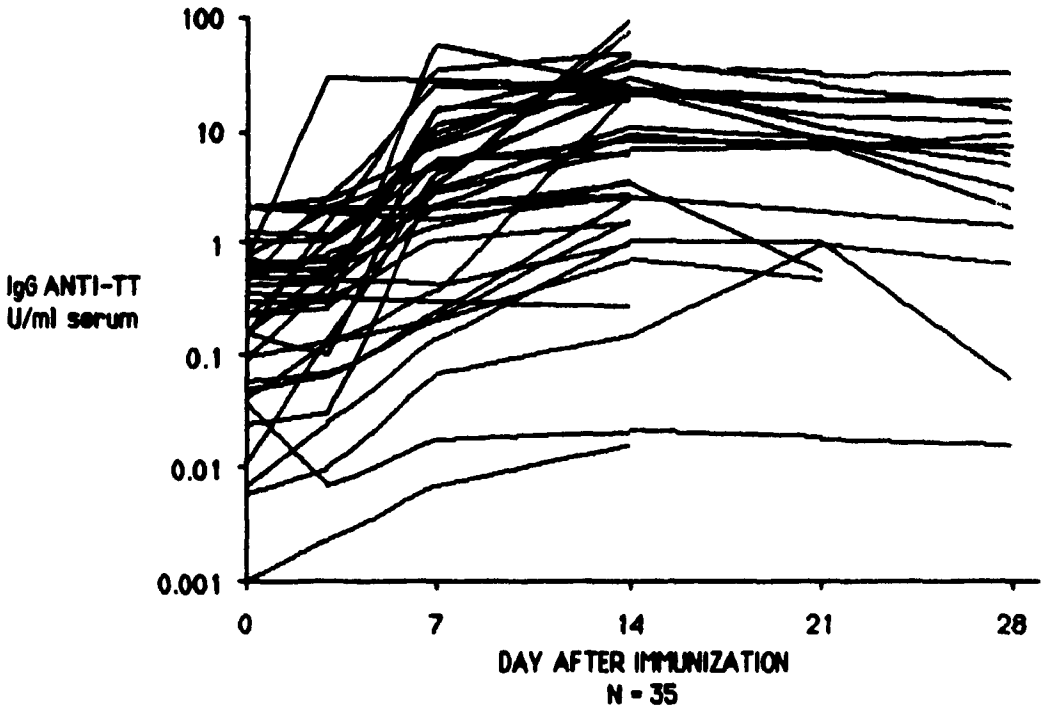
In summary, antibody responses were normal after both blunt and penetrating injury, and in survivors of thermal injury. Responses in non-survivors of burns were markedly reduced.

4.3 RELATIONSHIPS BETWEEN ANTIBODY RESPONSES AND CLINICAL DATA

There were several significant relationships between clinical data and antibody responses in trauma patients. For analysis, blunt and penetrating trauma groups were combined, and thermal injury discussed separately. The difference between pre- and day 14 post-immunization antibody levels was used as the antibody response.

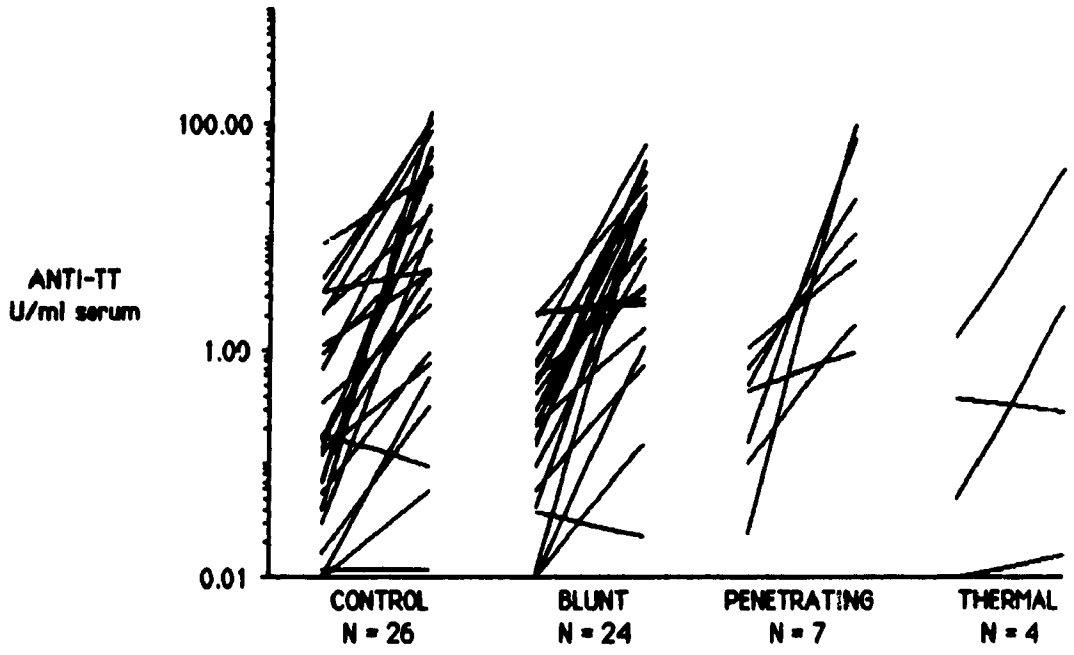
The relationship between physiologic derangement and antibody response was examined with rank correlation coefficients. Although the correlations between APACHE II scores and anti-TT response were weak, the best negative correlation was observed between emergency room APACHE II values (r_s = -0.20), with progressive decline during the following week (-0.13, -0.09, -0.08 on days 1, 3, and 7).

FIGURE 68: TIME COURSE OF *IN VIVO* ANTIBODY RESPONSES TO TETANUS TOXOID AFTER TRAUMA



Serum was collected from patients at intervals after blunt (N = 24) or penetrating (N = 7) trauma. The response to TT vaccination in the emergency room is shown for each subject as a curve of serum IgG anti-TT levels over time.

FIGURE 69: *IN VIVO* ANTIBODY RESPONSES TO TETANUS TOXOID AFTER TRAUMA



The IgG anti-TT content of sera from TT immunized LC and trauma patients was measured in sera obtained just before, or within 48 hours after vaccination. The data are shown for each subject as a line connecting pre-, and day 14 post-immunization anti-TT levels.

TABLE 33: SERUM ANTIBODY RESPONSES TO TETANUS TOXOID AFTER VACCINATION *IN VIVO* OF TRAUMA PATIENTS

		DIFFERENCE			RATIO			POSITIVE RESPONSES DIFFERENCE		RATIO	
CLASS	N	mean	x÷	sem	mean	x÷	sem	N	(%)	N	(%)
CONTROL	26	4.39	x÷	0.27	28.35	x÷	0.19	23	(88)	17	(65)
BLU	24	5.66	x÷	0.19	31.83	x÷	0.17	23	(96)	18	(75)
PEN	7	6.81	x÷	0.41	44.15	x÷	0.71	7	(100)	5	(71)
BLU+PEN	31	6.38	x÷	0.23	34.27	x÷	0.16	30	(97)	23	(74)
THERMAL	4	0.35	x÷	0.88	11.73	x÷	0.41	2	(50)	3	(75)

Patients were vaccinated with TT after blunt (BLU), penetrating (PEN) or thermal injury (THERMAL). *In vivo* antibody responses to TT vaccination were calculated as either the difference or the ratio between pre-, and day 14 post-immunization serum anti-TT levels. The geometric means x÷ sem (log base 10) are shown. A positive response was defined as either > = 0.1 new units/ml serum (DIFFERENCE) or > = 10-fold increase in antibody (RATIO).

The relationship between DTH and antibody responses was similar. There was a statistically significant direct correlation between admission STS and anti-TT responses (r_s = +0.39, p = 0.03). The correlations with STS at day 7 and 14 decreased to +0.28 and +0.19, neither statistically significant. Although overall normal, the antibody responses of patients who were anergic on admission were thus lower than those who were reactive (table 33).

There were no significant relationships between antibody responses and MISS, age, blood transfusion or days in ICU or hospital.

There was a significant relationship between altered host physiology and DTH responses early after injury. Considering the combined blunt and penetrating groups, the APACHE II score in the emergency room was 12.5 $\pm$ 2.6 (N = 11) for patients that were anergic on the first skin test, compared to 7.2 $\pm$ 1.1 (N = 20) for those who were skin test reactive ($p < 0.05$, unpaired t-test). Furthermore, there was a significant negative correlation between emergency room APACHE II scores and STS on admission with r_s = -0.34 (p = 0.05), so that more ill patients showed reduced DTH reactivity. The differences in APACHE II scores between A and R patients was found mostly in vital signs (data not shown).

There were other interesting relationships. A simple factor such as volume of blood transfused in the first 3 days after injury correlated significantly with emergency room APACHE II scores, and days in ICU and in hospital (r_s = +0.45, p = 0.01, r_s = +0.61, p = 0.001, r_s = +0.49, p = 0.006 respectively). The MISS value correlated with emergency room APACHE II score, and with days in ICU (r_s = +0.40, p = 0.02, r_s = +0.47, p = 0.008 respectively). Although there was a negative relationship between MISS and admission STS, it was not statistically significant (r_s = -0.32, p = 0.07).

The relationship between septic outcome and antibody responses, DTH and APACHE II scores was examined. The antibody responses of those patients who developed major sepsis in the blunt and penetrating groups were normal. The early STS and APACHE II scores tended to be low and high respectively in patients eventually developing sepsis, but no clear pattern emerged due to the low incidence of major infection. Two of 20 (10%) DTH reactive patients developed sepsis, compared to 3 of 11 (27%) anergic patients (X^2_1 with Yates = 1.57, p = 0.46).

After thermal injury, there was a one-to-one relationship between reduced antibody responses, reduced DTH, and increased degree of injury and physiologic derangement associated with the occurrence of major sepsis and mortality.

In summary, subjects with greater physiologic abnormalities early after injury tended to have more depressed DTH, and lower antibody responses. Burn patients showed a striking correspondence between deranged physiology, reduced DTH and antibody responses, and mortality.

4.4 COMPARISON BETWEEN ANTIBODY RESPONSES IN TRAUMA AND NON-TRAUMA PATIENTS

Antibody responses and clinical data of trauma patients were compared to non-trauma patients with equivalent DTH reactivity. Blunt and penetrating trauma patients were classified according to admission skin test, and compared to non-trauma patients with the same skin test classification at the time of vaccination (tables 34 and 35).

There were differences in the evolution of DTH skin test reactivity, APACHE II scores, and outcome. The STS for the DTH reactive trauma patients were significantly less than in non-trauma patients at the time of immunization. Two weeks after immunization, only 3 of 36 non-trauma patients had become either R or RA, compared to 10 of 11 trauma subjects. The APACHE II scores, calculated on the day of immunization, were higher in both trauma groups than the corresponding non-trauma patients, but the differences were not statistically significant ($p = 0.07, 0.17$ for A and R comparisons respectively, unpaired t-tests). Major sepsis and mortality rates were low in both trauma and non-trauma patients with good DTH reactivity. Sepsis most most frequent in the non-trauma A group. The mortality rate in this group was significantly greater than in the trauma A group ($X^2_1 = 4.9, p = 0.03$).

Antibody responses were better in trauma patients. The anti-TT responses, both as ratios and differences, were highly significantly better among the trauma patients in both DTH classes ($p = 0.0005, 0.01$ for difference and ratio between R groups, $p = 0.001, 0.001$ between A groups, WRST). Positive response rates were also better among trauma than DTH comparable non-trauma subjects ($X^2_1 = 14.2, 3.1$, $p = 0.0002, 0.08$ for difference and ratio between R groups, $X^2_1 = 15.2, 14.6$, $p = 0.0001, 0.0001$ between A groups, WRST).

TABLE 34: DEMOGRAPHIC DATA AND OUTCOME IN TRAUMA COMPARED TO NON-TRAUMA PATIENTS STRATIFIED ACCORDING TO DTH SKIN TESTING

	DTH REACTIVE		DTH ANERGIC	
	TRAUMA	NON-TRAUMA	TRAUMA	NON-TRAUMA
N	20	19	11	36
AGE (years)	36 ± 2	*62 ± 3	37 ± 5	*65 ± 2
APACHE II	7.2 ± 1	5.4 ± 0.7	12.5 ± 2.6	8.9 ± 0.8
STS (mm)	17.0 ± 2.0	*30.7 ± 3.4	0.0 ± 0.0	0.8 ± 0.3
SEPSIS N (%)	2 (10)	1 (5)	3 (27)	15 (42)
DEATH N (%)	0 (0)	1 (5)	0 (0)	12 (33)

Data for blunt plus penetrating trauma patients were compared to non-trauma patients. APACHE II scores were calculated on the day of vaccination, which was on admission to hospital for the trauma patients. STS shown were done within 48 hours of admission. Age, STS and APACHE II scores are shown as arithmetic means ± SEM (* $p < 0.05$ between trauma and non-trauma patient groups with same DTH reactivity, unpaired t-tests). There was no significant difference in sepsis, but death was more frequent in non-trauma anergic patients compared to the trauma anergic group ($X^2_1 = 4.9, p = 0.03$).

TABLE 35: ANTIBODY RESPONSES TO TETANUS TOXOID IN TRAUMA COMPARED TO NON-TRAUMA PATIENTS STRATIFIED ACCORDING TO DELAYED TYPE HYPERSENSITIVITY

	DTH REACTIVE		DTH ANERGIC	
	TRAUMA	NON-TRAUMA	TRAUMA	NON-TRAUMA
N	20	19	11	36
ANTI-TT (U/ml)				
PRE-VACC	*0.25 x÷ 0.14	0.02 x÷ 0.16	*0.18 x÷ 0.26	0.02 x÷ 0.19
RESPONSE				
DIFFERENCE	*10.02 x÷ 0.16	0.09 x÷ 0.23	*2.81 x÷ 0.35	0.01 x÷ 0.26
RATIO	*46.90 x÷ 0.21	9.40 x÷ 0.20	*19.24 x÷ 0.24	2.26 x÷ 0.12
POSITIVE RESPONSE				
DIFF N (%)	*20 (100)	9 (47)	*10 (91)	9 (25)
RATIO N (%)	15 (75)	9 (47)	*8 (73)	7 (19)

Data for blunt plus penetrating trauma patients were compared to non-trauma patients. *In vivo* antibody responses to TT vaccination were calculated as either the difference (DIFF) or the ratio (RATIO) between pre-, and day 14 post-immunization serum anti-TT levels. The geometric means x÷ sem (log base 10) are shown for antibody levels before vaccination (PRE-VACC), and the calculated responses (* indicates $p < 0.05$ between trauma and non-trauma patient groups with same DTH reactivity, WRST). A POSITIVE RESPONSE to TT vaccination was defined as either > = 0.1 new units/ml serum (DIFF) or > = 10-fold increase in antibody (RATIO). The numbers of subjects showing a positive response in each group are shown. Comparing trauma and non-trauma patient groups with same DTH reactivity, there were significant differences in response rates, except for the ratio responses of DTH reactive trauma versus non-trauma groups (* indicates $X^2_1 > 14.2$, $p < 0.002$).

In summary, with similar DTH reactivity at the time of vaccination, trauma patients produced significantly better antibody responses than non-trauma patients. Although higher in all subjects with decreased DTH, sepsis and mortality rates were lower in trauma than in non-trauma groups.

4.5 IN VITRO SPECIFIC AND TOTAL IMMUNOGLOBULIN SYNTHESIS AFTER TRAUMA

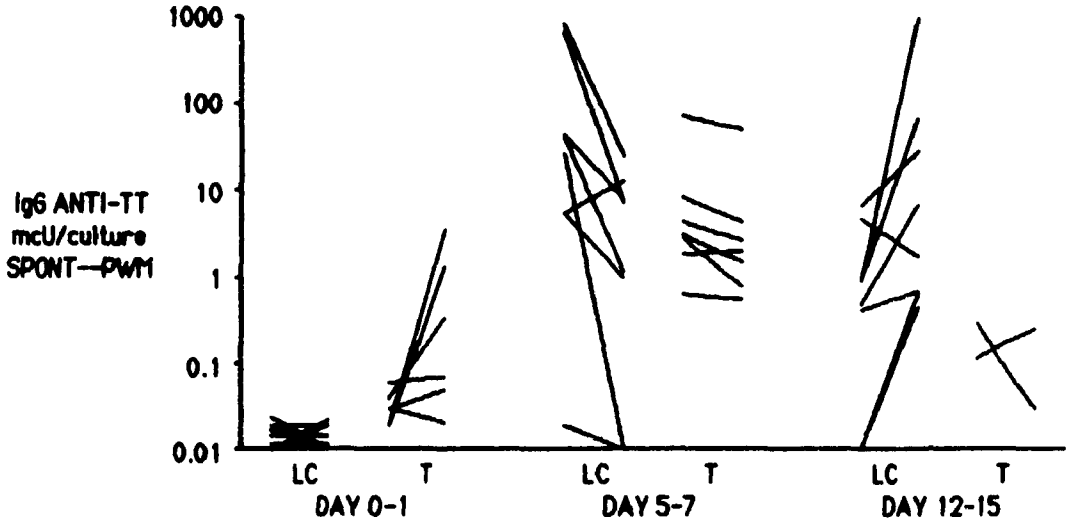
The *in vitro* synthesis of anti-TT specific IgG, and total IgG, IgM and IgA was studied at intervals after trauma in 11 subjects. Blunt and penetrating trauma cases were combined. No burn patients were studied. No subjects received hyperimmune globulin. Initial MNC cultures were established on day 1 or 2 after admission, that is within 24 to 48 hours of injury. The supernatants were assayed for content of total and anti-TT specific immunoglobulin.

The control group for comparisons consists of 9 LC subjects also immunized with TT. To account for the differences in specific and total Ig synthesis after vaccination, data after injury and TT injection were compared to data from the control group at the same interval after vaccination. There was no difference in the *in vivo* anti-TT response between the LC and trauma patients (4.23 x̄ 0 45 compared to 4.36 x̄ 0.25 new units/ml at day 14, p = 0.40, WRST).

Similar to other patients, cell preparations from trauma patients contained a greater number of monocytes. The average $\pm$ SEM monocyte:lymphocyte ratios for 9 LC subjects 1, 6 and 14 days after vaccination were 0.19 $\pm$ 0.03, 0.12 $\pm$ 0.01 and 0.17 $\pm$ 0.03. These were compared (unpaired t-tests) to 10, 11 and 5 patients at 1, 5-7 and 12-15 days after injury, with average ratios of 0.35 $\pm$ 0.06 (p = 0.05), 0.36 $\pm$ 0.07 (p = 0.0005) and 0.28 $\pm$ 0.06 (p = 0.12). There was a modest increase in PMN contamination in the trauma patients, from an average of 11%, compared to 4% in the LC group.

In unstimulated cultures, little specific antibody was produced soon after admission (figure 70, table 36). At the time of peak spontaneous *in vitro* anti-TT IgG synthesis, day 5-7, amounts comparable to LC subjects were produced by the trauma patients (p = 0.13, WRST). After 12-15 days, a small amount of spontaneous synthesis continued.

FIGURE 70: ANTI-TETANUS TOXOID ANTIBODY SYNTHESIS *IN VITRO* AFTER VACCINATION OF TRAUMA PATIENTS *IN VIVO*



After TT vaccination of 9 LC and 7 blunt and penetrating trauma subjects on day 0, blood MNCs were harvested on days 0-1, 5-7, and 12-15. The MNCs were cultured *in vitro* in either unstimulated, or PWM stimulated cultures under standard conditions for 10 days. Culture supernatants were assayed for amounts of IgG anti-TT. The data are shown as lines connecting the amounts of IgG anti-TT produced on different days after vaccination, in unstimulated (SPONT) and PWM stimulated (PWM) cultures. One patient was not cultured on day 0-1, and five patients had been discharged by day 12-15.

TABLE 36: SYNTHESIS OF IgG ANTI-TETANUS TOXOID *IN VITRO* AFTER VACCINATION *IN VIVO* OF TRAUMA PATIENTS

CLASS	N	SPONTANEOUS	PWM STIMULATED
DAY 0-1			
CONTROL	9	0.01 x̄ 0.01	0.01 x̄ 0.01
TRAUMA	6	0.04 x̄ 0.11	0.22 x̄ 0.36
DAY 5-7			
CONTROL	9	*7.93 x̄ 0.58	0.41 x̄ 0.50
TRAUMA	7	*4.33 x̄ 0.24	2.50 x̄ 0.24
DAY 12-15			
CONTROL	9	*0.26 x̄ 0.38	3.56 x̄ 0.51
TRAUMA	2	0.19 x̄ 0.20	0.09 x̄ 0.49

Blood MNC were harvested from TT immunized control and either blunt or penetrating trauma subjects 0-1, 5-7, and 12-15 days after vaccination, and cultured either with or without PWM. After 10 days *in vitro*, cuture supernatants were assayed for content of IgG anti-TT antibody. Geometric means x̄ SEM are shown. There are no statistical differences between CONTROL and TRAUMA groups in comparisons of either UNSTIM or PWM STIM data ($p > 0.05$, WRST). The effect of PWM on synthesis of specific Ig was compared for each temporal interval in each subject group (* indicates $p < 0.05$, WSRT).

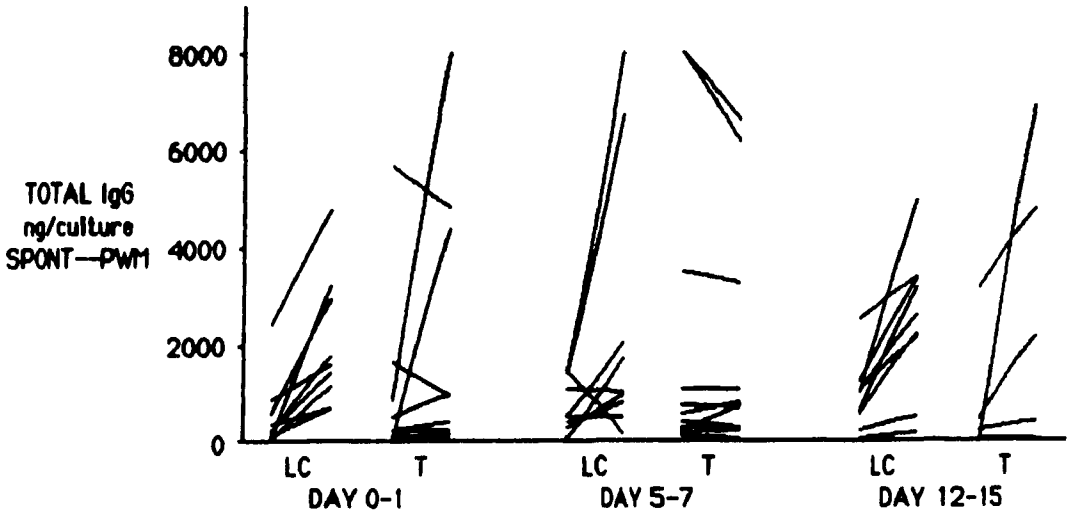
In PWM stimulated cultures established on day 1 or 2 after injury, 3 of 6 patients produced significant amounts of anti-TT antibody. This did not occur in LC subjects (figure 70) On day 5-7, PWM stimulation significantly reduced specific antibody synthesis compared to unstimulated cultures from trauma patients ($p = 0.02$, WRST). This suppressive effect was smaller in the trauma patients than in LC. PWM stimulated cultures of trauma patient MNCs produced approximately one-half as much as spontaneous cultures, while anti-TT synthesis by LC cells was reduced to one-twentieth the amount (table 36). At day 12-15, most LC subjects showed an increase in specific antibody synthesis, while the trauma patients produced comparatively small amounts.

Comparable to non-trauma subjects, a quantitative relationship was sought between *in vitro* synthesis of specific antibody on days 5 to 7 after vaccination, and serum antibody responses measured as number of new units anti-TT/ml at day 14. In trauma patients ($N = 7$), despite the modest reduction in anti-TT synthesis *in vitro* with PWM stimulation, the correlation was slightly better with PWM stimulated cultures ($r_s = +0.86$, $p = 0.01$) than with spontaneous synthesis ($r_s = +0.71$, $p = 0.07$).

In summary, spontaneous synthesis of anti-TT was similar in trauma patients and controls. There were two major differences between the groups in PWM stimulated cultures. First, there was a PWM inducible response early in some patients. Second, during peak *in vitro* spontaneous synthesis, PWM caused a smaller reduction in anti-TT synthesis A shift to the left in *in vitro* kinetics could produce these differences. Spontaneous antibody synthesis would be detected earlier, possibly as soon as 24-48 hours after injury in some individuals An earlier shift from PWM induced decrease to increase in antibody synthesis that normally occurs from day 5-7 to day 10-14 could produce the observed changes later after vaccination In contrast to LC subjects, where the correlation between *in vivo* and *in vitro* antibody responses was best with unstimulated cultures, a better correlation was found with PWM stimulated responses in trauma patients. Finally, the late PWM stimulatable anti-TT response was not present in trauma patients.

Total immunoglobulin quantities were also determined in the culture supernatants. In unstimulated cultures of MNCs harvested on day 1 after injury compared to LC on day 0 of immunization, there was no significant difference in total IgG synthesis (table 37). On days 5 to 7, more IgG was produced by some trauma patients, but the average was not

FIGURE 71: TOTAL IgG SYNTHESIS *IN VITRO* AFTER TETANUS TOXOID VACCINATION OF TRAUMA PATIENTS *IN VIVO*



After TT vaccination of 9 LC and 7 blunt and penetrating trauma subjects on day 0, blood MNCs were harvested on days 0-1, 5-7, and 12-15. The MNCs were cultured *in vitro* in either unstimulated, or PWM stimulated cultures under standard conditions for 10 days. Culture supernatants were assayed for amounts of total IgG. The data are shown as lines connecting the amounts of total IgG produced on different days after vaccination, in unstimulated (SPONT) and PWM stimulated (PWM) cultures. Two patients were not cultured on day 0-1, and six patients had been discharged by day 12-15.

TABLE 37: SYNTHESIS OF TOTAL IgG *IN VITRO* IN TRAUMA AND CONTROL SUBJECTS

	N	UNSTIM	PWM STIM	DIFFERENCE	POS	NIL
DAY 0-1						
CONTROL	9	547 ± 248	2038 ± 449	*1491 ± 314	7	2
TRAUMA	10	923 ± 550	2261 ± 1104	*1339 ± 1059	6	4
DAY 5-7						
CONTROL	9	800 ± 182	2549 ± 1036	*1749 ± 943	6	3
TRAUMA	11	2286 ± 1043	1839 ± 722	*# -447 ± 335	2	9
DAY 12-15						
CONTROL	9	938 ± 243	2497 ± 499	*1558 ± 398	8	1
TRAUMA	5	783 ± 592	2857 ± 1300	*2074 ± 1242	3	2

Blood mononuclear cells were harvested from TT vaccinated LC and patients and cultured in triplicate under standard conditions. Cultures were unstimulated (UNSTIM) or stimulated with 1:200 PWM (STIM). Supernatants were assayed in duplicate for cumulative synthesis of IgG after 10 days. Data are shown as arithmetic means ± SEM. * indicates a statistically significant difference between UNSTIM and STIM cultures, $p < 0.05$, paired t tests; # indicates a statistically significant difference between CONTROL and TRAUMA groups at the same time interval, under the same culture conditions. For each subject, the statistical significance of the response to PWM stimulation was determined by unpaired t-test on the means and SD of the triplicate cultures. Responses were thus characterized as either significantly positive (POS, PWM stimulated > unstimulated, $p < 0.05$), or not significant (NIL, $p > 0.05$). No instances of signficantly negative responses were found in these subjects. Comparing the distribution of POS and NIL responses to PWM STIM at each time interval for CONTROL and TRAUMA subjects, only day 5-7 was significant ($X^2_1 = 4.85$, $p = 0.03$; $X^2_1 = 0.69$, $p = 0.41$ for day 0-1, and $X^2_1 = 1.59$, $p = 0.21$ for day 12-15.

significantly different from LC (figure 71, p = 0.28, unpaired t-test). Late after injury, from days 12 to 15, comparable quantities were produced.

There were significant differences in PWM induced responses (figure 71). Early after injury, several patients showed no significant response to PWM stimulation. This pattern was more prevalent on days 5-7, when a statistically significantly greater proportion of trauma patients than immunized LC subjects showed no increase with PWM (X^2_1 = 4.85, p = 0.03). The average difference between spontaneous and stimulated synthesis was also negative at this time, and significantly different from LC subjects (p = 0.04, unpaired t-test). By day 12 to 15, normal responses had reappeared.

Unlike non-trauma patients, there was no relationship between *in vitro* IgG synthesis and DTH reactivity. Similar quantities of total IgG were produced spontaneously by patients with admission STSs of 0 (N = 4) and > 0 (N = 6). Three of the 4 patients initially anergic, and 3 of the 6 initially reactive, gave significant positive responses to PWM.

Although not involved in the immune response to TT, the *in vitro* synthesis of other isotypes was measured in some subjects (table 38). Absolute quantities of IgM produced spontaneously soon after trauma were greater than before vaccination of LC subjects (p = 0.01, unpaired t-test). On days 5 to 7, there was an increase in spontaneous synthesis in both groups, that decreased by day 12 to 15. The pattern of IgA synthesis in 4 and 6 trauma patients studied on days 1 and 5 to 7 respectively followed that of IgG (data not shown).

In summary, traumatic injury produced substantial spontaneous synthesis of IgG in some subjects. The major differences betwen trauma patients and LC subjects, however, were seen in PWM stimulated cultures. Abnormal responsiveness to PWM in total Ig synthesis began early, was worst after 5 to 7 days, and was improving towards normal by 2 weeks.

5. SERUM IMMUNOGLOBULIN LEVELS

To determine whether the observed increase in spontaneous synthesis of Ig in vitro was associated with elevated serum Ig levels, total protein, serum albumin, gamma globulin, and IgG, IgM and IgA levels were measured in 25 control subjects and in 140 patients with a variety of DTH results.

TABLE 38: SYNTHESIS OF TOTAL IgM *IN VITRO* IN TRAUMA AND CONTROL SUBJECTS

	N	UNSTIM	PWM STIM	DIFFERENCE	POS	NIL
DAY 0-1						
CONTROL	7	13 ± 2	*282 ± 148	270 ± 149	4	3
TRAUMA	10	#55 ± 14	*454 ± 160	399 ± 102	6	4
DAY 5-7						
CONTROL	7	156 ± 97	*490 ± 121	334 ± 150	5	2
TRAUMA	11	158 ± 57	*244 ± 82	87 ± 34	5	6
DAY 12-15						
CONTROL	7	40 ± 11	*721 ± 213	681 ± 222	6	1
TRAUMA	5	67 ± 32	*568 ± 115	501 ± 116	5	0

Blood mononuclear cells were harvested from LC and trauma patients, and cultured in triplicate under standard conditions. Cultures were unstimulated (UNSTIM) or stimulated with 1:200 PWM (STIM). Supernatants were assayed in duplicate for cumulative synthesis of IgM after 10 days. Data are shown as arithmetic means ± SEM. * indicates a statistically significant difference between UNSTIM and STIM cultures, $p < 0.05$, paired t tests; # indicates a statistically significant difference between CONTROL and TRAUMA groups at the same time interval, under the same culture conditions. For each subject, the statistical significance of the response to PWM stimulation was determined by unpaired t-test on the means and SD of the triplicate cultures. Responses were thus characterized as either significantly positive (POS, PWM stimulated > unstimulated, $p < 0.05$), or not significant (NIL, $p > 0.05$). No instances of signficantly negative responses were found in these subjects. Comparing the distribution of POS and NIL responses to PWM STIM at each time interval for CONTROL and TRAUMA subjects, there were no significant differences ($X^2_1 = 0.01$, $p = 0.91$ for day 0-1; $X^2_1 = 1.17$, $p = 0.28$ for day 5-7; $X^2_1 = 0.78$, $p = 0.38$ for day 12-15).

TABLE 39: SERUM PROTEIN LEVELS

	CONTROL	PATIENT	NON-SEPTIC	SEPTIC	ALIVE	DEAD
N	25	140	113	27	114	26
TP	74.0 ± 1.5	*61.3 ± 1.3	61.8 ± 1.1	58.8 ± 1.8	61.5 ± 1.1	60.3 ± 2.1
ALB	44 0 ± 1.0	*31.0 ± 0.8	32.0 ± 0.7	*26.8 ± 1.4	31.7 ± 0.7	*28.1 ± 1.5
G GLOB	11.5 ± 1.0	10.7 ± 0.5	10.1 ± 0.3	*13.0 ± 1.3	10.0 ± 0.1	*13.5 ± 0.1
IgG	8.5 ± 0.4	7.9 ± 0.5	7.5 ± 0.3	*9.6 ± 1.4	6.8 ± 0.4	*7.3 ± 1.4
IgM	1 3 ± 0.1	1.1 ± 0 1	1.0 ± 0.1	1.1 ± 0.2	0 9 ± 0.1	0.9 ± 0 2
IgA	1.4 ± 0.2	2.1 ± 0 2	2.0 ± 0.1	*2 6 ± 0.3	1.8 ± 0.1	*2 0 ± 0.4

Data were collected from a group of non-immunized, diagnostically heterogenous surgical patients, stratified acxcording to the outcome of hospitalization. All data are in g/l, and are shown as arithmetic means ± sem. * indicates $p < 0.05$, unpaired t-tests, for comparisons between controls and all patients, or between corresponding outcome groups.

Compared to controls (table 39), the whole patient group had significantly lower total protein and serum albumin values ($p = 0.001$ for both, unpaired t-tests). Gamma globulin and isotype levels were comparable.

When stratified according to skin testing, differences in Ig levels between R and A patients were also not seen. However, differences were apparent when the patients were stratified according to the presence or absence of major sepsis during the hospitalization. Septic subjects had significantly lower serum albumin levels ($p = 0.001$, unpaired t-test), but gamma globulin levels, due to significant differences in IgG ($p = 0.02$, unpaired t-test) and IgA ($p = 0.05$, unpaired t-test) concentrations, were elevated in septic compared to non-septic patients ($p = 0.003$, unpaired t-test). There were similar differences between survivors and non-survivors. Serum albumin was lower in non-survivors ($p = 0.03$, unpaired t-test), but gamma globulins, IgG, and IgA were increased ($p = 0.001$, $p = 0.001$, $p = 0.001$, unpaired t-tests).

In summary, surgical patients had reduced circulating total protein values, due to low serum albumin. Mortality and sepsis were associated with reduced serum albumin, and elevated Ig levels.

DISCUSSION

The following subjects will be discussed: in vivo antibody responses, in vitro antibody synthesis after vaccination in vivo, total Ig synthesis in vitro, cell phenotypes, and humoral immunity in trauma. Finally, the implications of these data for concepts of anti-bacterial immunity, and the role of biological response modifiers in surgical patients, are examined.

1. IN VIVO ANTIBODY RESPONSES

The detection of a specific antibody response to antigenic encounter is the most fundamental and comprehensive test of humoral immune function in an individual. The antibody responses to a protein antigen, TT, and a polysaccharide antigen, PPS, have been tested in surgical patients.

The antibody response to TT was reduced among all patients, especially so in the group with reduced DTH skin test responses. There are several possible causes of this acquired humoral immune deficiency.

First, the possible causes of decreased antibody responses among all patients compared to LC will be considered. This may be partly accounted for by general biological differences between LC and patient groups. These differences include the possibility of a difference in vaccination history, older age, and the presence of some disease process requiring hospital admission.

Differences in tetanus vaccination history may produce some differences in the results between LC and patient groups. It was not possible to document previous TT immunization history further than to exclude subjects recently vaccinated. However, as was shown in the data, the anti-TT levels in the serum of nearly all patients before vaccination were in a range comparable to controls. This indicates that the response to experimental TT immunization can be considered a secondary antibody response in all study subjects.

Age does not account for differences between LC and patient anti-TT responsiveness. In previous studies of the antibody response to TT in aged subjects, responses were reduced in some [162,163], but preserved in other studies [166,167]. In the present study, there was no difference in the anti-TT responses between young and older subjects in either control or patient groups.

All the patients had some illness requiring hospital care. These diseases, and their secondary effects, include many of the processes known to affect immunity. These included malnutrition, malignancy, anesthesia, blood transfusion and elevated corticosteroid levels. Many surgical patients, regardless of underlying primary diagnosis, are secondarily malnourished. Malnutrition clearly has an adverse effect on immunity, especially T cell dependant processes, in which the antibody response to TT may be included. Many patients had malignant disease, and underwent surgery involving the administration of an anesthetic and blood transfusions. Finally, all the patients have a common susceptibility to the neurohormonal alterations of acute illness, worse in those with reduced DTH responses. Some aspects of these acute physiological changes, for example elevated corticosteroid levels, may further alter immunity. These alterations in the state of homeostasis, a concept originally developed by Cannon in 1939 [384], may produce changes in antibody responsiveness. These changes need not necessarily proceed according to the same

pathophysiologic processes in all patients, but the common final expression is depressed antibody synthesis in response to *in vivo* vaccination with a protein antigen in the majority of surgical patients.

The same concepts are relevant in understanding the differences in responsiveness among the different patient groups. Surgical patients are a diagnostically heterogeneous group. The immune system of each individual may thus be variably affected by various illnesses known to affect humoral immune function. The global effect of these processes is the production of a state of increased severity of illness, associated with altered DTH responses and decreased antibody responsiveness. Even among relatively well, skin test reactive patients, anti-TT responses were reduced. Humoral immunity to a protein antigen was thus abnormal in a group of subjects with a low risk of sepsis, and may thus be more sensitive than DTH testing to acquired deficiencies in immune function.

There is a three-way relationship between reduced DTH, increased severity of illness, and depressed antibody responses to TT. Just as anergic patients produce less antibody than DTH reactives, more ill patients produce less antibody. Similarly, more ill patients have greater depressions in DTH. These conclusions are based on the observed statistically significant correlations between STS and APACHE II scores, antibody responses and STS , and between antibody responses and APACHE II scores. Whether severity of illness or depressed DTH is more closely associated with abnormalities in humoral immunity is difficult to resolve. However, the walk-in DTH reactive and anergic patients, two relatively well patient groups distinguishable only on the basis of different DTH responses, had comparable severity of illness measurements. These two patient groups also had comparable antibody responses. While there was therefore a similarity between severity of illness and antibody responses in these two groups, there was no relationship to skin testing. It is thus evident that although reduced DTH is associated with reduced anti-TT immunity, abnormal humoral immunity is probably more closely related to severity of illness than to anergy.

The diseases known to cause abnormal immunity may be more prevalent, or more severe in many patients with reduced DTH, and increased severity of illness. Reduced DTH has been used as a marker of clinical malnutrition [385], and it may be that more severe malnutrition in anergic patients contibuted significantly to the observed reduction in anti-TT responsiveness. The neurohormonal alterations associated with acute illness are also probably worse in patients with reduced DTH, especially those requiring ICU care. Conceptually, several

underlying disease processes thus contribute to the immunodepression in any given surgical patient, and the net effect is an expression of the magnitude and interaction of these processes.

These data support the concept that the response to acute illness has a measurable impact not only on commonly measured variables of host physiology, but on certain immune processes as well. The degree of impact can be quantified by measuring multiple physiologic variables, and correlated with measurements of host defence capability. There is thus a biological continuum of abnormalities in host physiology and immunity, which, although not entirely interdependent, are parallel throughout a spectrum of severity of illness---acquired immunodeficiency. Severity of illness must therefore be included not only as a determinant of clinical outcome, but also as a determinant of immune function testing.

All measurements of physiologic derangement were taken at the time of immunization. It is therefore likely that there is a decisive period in the first few days after antigen encounterfor the development of a humoral immune response, similar to that known for bacterial infections [386]. The data from trauma patients support this concept, in that the best correlations of APACHE II scores and DTH responses to antibody responses were found soon after injury.

Following the demonstration of a humoral immunodeficiency to a protein antigen, the antibody response to a polysaccharide antigen was studied. This is of interest, because the surface antigens on common gram-negative bacterial pathogens are not proteins, but predominantly polysaccharides. Pneumococcal polysaccharide was used as an experimental antigen to measure patient humoral reactivity to bacterial polysaccharides.

The *in vivo* antibody response to PPS vaccination was found to be normal. Surgical patients, regardless of DTH scores, or clinical physiologic status, produced anti-PPS of all three isotypes after vaccination in normal amounts.

The explanation of the difference in antibody responses to protein and polysaccharide antigens may be related to T lymphocyte function. The antibody response to various antigens is differentially dependent on T cells [70,71]. Based on rodent studies, the response to protein antigens, which includes TT, is classically considered to require the participation of T cells. Anti-polysaccharide antibody responses are generally believed to be relatively T cell independent [387]. Most of the disease processes known to affect humoral immunity, for

example malnutrition and malignancy, have a greater effect on tests of T cell function, and thus may be expected to have a greater impact on the antibody response to protein rather than polysaccharide antigens.

A possible mediator of the T cell-related differential effect of acute illness on humoral immunity to different antigens has been suggested by the recent demonstration of the effect of serum amyloid P-component (SAP) on humoral immunity in mice [388]. SAP is the structural analog of human C-reactive protein (CRP) [389], one of several acute phase reactants that rapidly increase in the blood in systemic illness. In mice, SAP suppressed the antibody response to a T-cell dependant antigen, but not to an independant antigen. Thus, elevated levels of soluble mediators in ill man, possibly CRP, could be associated with the observed decrease in antibody responses to TT, with preservation of responses to PPS.

Normal humoral immunity to PPS implies that anti-polysaccharide responses are independent of the biological continuum of acquired immunodeficiency in surgical patients, and may thus be considered to have no relationship to outcome in terms of sepsis or mortality. Indeed, in this study, the single non-responder to PPS survived a serious septic challenge, while a subject showing an excellent response succumbed to post-operative infection.

In summary, antibody responses to a protein antigen are reduced among all surgical patients, especially those with reduced DTH responses, and increased severity of illness. Humoral immune responses to polysaccharide antigens are normal. Considering the anti-PPS response to be representative of anti-bacterial antibody responses, the data suggest that *in vivo* anti-bacterial antibody responses occur in surgical patients.

2. IN VITRO ANTIBODY SYNTHESIS AFTER VACCINATION IN VIVO

On a cellular and microenvironmental basis, the cause of the diminished antibody response to a protein antigen in patients is not known. The possibilities include any or several steps in the process of an antibody response. Failure of antigen pickup, transfer to lymph nodes, accessory or helper cell malfunction, or failure of clonal proliferation and protein synthesis could singly or in combination produce the observed *in vivo* data.

After antigen encounter *in vivo*, there are changes in the spontaneous and PWM stimulated Ig synthetic activity of blood cells cultured in vitro. These phenomena can be used to advantage in describing abnormalities in humoral immune processes in subjects with altered host defence mechanisms.

The normal response to TT immunization *in vivo*, as detected in *in vitro* cultures, has previously been studied [390]. As soon as five days after immunization, a population of B cells appears in the circulation that spontaneousiy secretes IgG anti-TT *in vitro* [391]. The number of such cells is variable among individuals, but the kinetics are reproducible. As in the serum response [392], the majority of the antibody is IgG_1 [393]. Little antibody of other isotypes is produced [394]. Help from polyclonal B cell stimulants or T cells is not required. The cells have been called lymphoblasts, actively divide to generate more synthetically active cells, and are characterized as HLA-DR^+, sIg^- large lymphocytes [395]. Stimulation with PWM *in vitro* can reduce the synthesis of anti-TT by these cells [396]. This inhibition is mediated by a suppressor cell and/or anti-idiotype antibody [397], and can only be partly reversed by the addition of exogenous corticosteroid [395]. This population rapidly declines, and is undetectable after two weeks [398]. At approximately this time, a second population of B cells appears in the blood. These cells require antigen specific T cells [399], and stimulation with PWM [376], antigen [400] or soluble factors [401] *in vitro* to produce anti-TT. This population may persist in the blood up to two years [402], and probably represents relatively long-lived memory cells. The appearance of these two populations in the blood, namely early spontaneous antibody-producing B cells, and late PWM stimulatable B cells, is dependant on specific activation by *in vivo* vaccination.

The present studies confirm the transient appearance of spontaneous antibody-secreting cells in the blood after TT immunization. The previous observations are extended to show a kinetic relationship between the time of peak *in vitro* spontaneous synthesis, and the time of maximal slope of the increase in serum antibody content. There was also a quantitative correlation between the amount of anti-TT produced *in vitro* on days 5 to 6 after vaccination, and the eventual serum antibody response measured two weeks later. These cells are thus an *in vitro* biologic reflection of the *in vivo* response.

A correlation between *in vivo* antibody responses and production of antibody *in vitro* by blood MNCs does not imply that circulating lymphocytes are the major site of *in vivo* antibody synthesis. Considering the cultured cells to be representative of the circulating

population at any given time, and assuming production of the maximum amount of anti-TT detected in 10 day cultures each day for a week *in vivo*, less than 1% of the total amount of new anti-TT in the serum can be accounted for. Therefore the bulk of antibody production *in vivo* probably occurs in sites other than the blood, most likely the bone marrow . The appearance of spontaneous antibody producing cells in the blood probably represents lymphocyte traffic between these anatomic sites.

In surgical patients who did not show a serum response to TT vaccination, such spontaneous anti-TT secreting lymphoblastoid cells could not be detected. Also, the late appearing PWM stimulatable population of TT-specific B cells could not be detected in the blood of non-responding patients. These data show a failure to produce either population of B cells normally induced by the in vivo response to TT vaccination.

The same culture supernatants were assayed for total as well as anti-TT specific IgG. The production of similar quantities of total IgG by MNC from both antibody responsive and unresponsive subjects shows that failure of anti-TT antibody production was not due to a global failure of IgG synthesis. This suggests that the block occurs in the antigen priming, or sensitization phase after vaccination *in vivo*.

The *in vitro* synthesis of anti-PPS of three isotypes following *in vivo* vaccination was also studied. As was shown for the response to TT, the spontaneous synthesis of anti-PPS *in vitro* after *in vivo* vaccination reflects the kinetics and magnitude of the *in vivo* humoral immune process. The order of appearance of the serum isotype responses is IgM, IgA and IgG. This parallels the *in vitro* findings of peak spontaneous synthesis of IgM, IgA and IgG on days 6, 7 and 8 respectively. There is a quantitative correlation between the magnitude of the early peak of *in vitro* antibody synthesis, and the later peak of *in vivo* levels at two weeks, for each isotype. In contrast to the findings with TT, however, the late appearing specific B cells that require PWM stimulation to produce antibody *in vitro* were not found in normal blood after PPS vaccination.

The pattern of spontaneous and PWM stimulated synthesis of anti-PPS was different for IgG and IgA, compared to IgM. The very low synthesis of IgM anti-PPS *in vitro*, previously reported in normal man [404], despite substantial rises in serum antibody content, suggests differential traffic patterns for cells secreting antibody of different isotypes. In contrast to IgM, the synthesis of IgG and IgA anti-PPS early after vaccination could be suppressed by

PWM, similar to the effect of PWM on anti-TT synthesis. This suggests T cell regulation of at least this aspect of the humoral immune response to PPS.

Just as serum responses to PPS vaccination were normal in patients, the quantities of anti-PPS produced *in vitro*, the isotype pattern, and the effect of PWM were normal in patients.

In summary, the *in vitro* synthesis of specific antibody after *in vivo* vaccination is a biologic reflection of the *in vivo* antibody response to both protein and polysaccharide antigens. Depressed humoral immunity to TT in surgical patients is likely a failure of either sensitization to protein antigens, or lack of specific B cell clonal proliferation. Failure thus probably occurs in the antigen priming or proliferative phase of the humoral immune response. Just as the serum response to a polysaccharide antigen was normal in surgical patients, so was the *in vitro* synthesis of anti-polysaccharide antibody.

3. TOTAL IMMUNOGLOBULIN SYNTHESIS IN VITRO

The synthesis of Ig is a functional expression of B cell activity. This aspect of B cell function is frequently studied using mitogen stimulation *in vitro*. However, because an *in vitro* culture remains an abnormal situation, where regulatory processes unique to the *in vitro* environment may be operative [405], determination of the unstimulated, or spontaneous, degree of B cell activity may provide more accurate information about the activity of the humoral immune system *in vivo*. Mitogen stimulation may then be interpreted as an *in vitro*-induced change in ongoing humoral immune processes.

In the present study, MNCs from surgical patients spontaneously produced more IgG and IgA than healthy subjects. This spontaneous activity was more pronounced in ill patients with reduced DTH responses. IgM synthesis was not increased.

Some aspects of the normal spontaneous *in vitro* synthesis of Ig by MNCs have been examined previously. In normal individuals, there is a small and highly variable number of spontaneous Ig producing cells in the circulation. Most of the Ig is IgG or IgA, with lesser amounts of IgM [121,126]. In culture, such cells can be detected from the initiation [406], with a progressive accumulation of Ig in the culture supernatants for approximately a week [407]. Although these cells are mostly present from the beginning of culture, T cells and monocytes are necessary *in vitro* for full expression of this unstimulated activity [408]. This may be

because these spontaneous Ig secreting cells are actively dividing lymphoblasts [409], that likely require the support of these accessory cells. Spontaneous Ig secreting B cells are also found in the bone marrow [403,410] in quantities sufficient to account for >95% of total Ig synthesis in the body, and gut-associated lymph nodes [411,412]. The origin and role of the circulating cells *in vivo* is not known, but the hypothesis that they represent a traffic phenomenon is supported by the available data [413].

Although the presence of spontaneous Ig secreting cells in the circulation is a product of *in vivo* activation [414], there are two *in vitro* processes that may spuriously produce spontaneous antibody secreting cells without PWM stimulation. These are a mitogenic response to FBS, and an autologous mixed lymphocyte reaction (AMLR). It is known that heterologous proteins can induce mitogenesis in human MNC cultures [356]. In the present studies, cell culture conditions were designed to minimize this effect, by using a concentration of FBS that was demonstrated to have the smallest possible impact on Ig synthesis, while supporting the culture. The same conditions were used for all subjects, and it is therefore unlikely that this effect could produce the observed results.

An AMLR, which as a process of T cell proliferation stimulated by autologous non-T cells, does cause mitogenesis *in vitro* [415], and can generate Ig-secreting cells [416]. The basis of the AMLR is believed to be a T cell response to HLA-DR antigens, which are increased in cell preparations from patients in this study with reduced DTH responses, the same group with increased spontaneous Ig synthesis *in vitro*. However, this effect occurs late in the course of cell culture, and is too small to account for more than the low level of spontaneous Ig synthesis seen in normal cultures [417]. Also, as shown by time course studies in the present data, and in other culture systems [406], Ig producing cells are present from the moment of isolation.

Increased spontaneous Ig synthesis by PBLs *in vitro* is not unique to anergic surgical patients. It has previously been described in patients with liver disease [332,334] graft versus host disease [438], autoimmune disease [418,419,420,421] burns [242], and recently, in post-operative patients [422]. The etiology of the activation *in vivo* in these diseases is unknown.

Age is not associated with an increase in spontaneous synthesis; in fact, a decrease was observed [174].

There are two groups of hypotheses regarding the origin of these *in vivo* activated B cells in surgical patients. The first is that they represent a kind of "immune Brownian motion", with non-specific activation of the B cell system due to neurohormonal alterations, bacterial products, or secondary effects of other activated specific or non-specific aspects of host immune systems. The second is that they represent a traffic manifestation of systemic humoral immune responses to unknown antigens, possibly bacteria.

Acute illness in man is associated with increased corticosteroid levels. The influence of this on blood MNCs was tested by the addition of exogenous hydrocortisone in concentrations equivalent to, and two logs greater, than those achieved in ill patients. In otherwise unstimulated cultures, this caused a modest increase in IgG synthesis compared to untreated cultures of LC cells, so that quantities were similar to those produced by MNCs from DTH reactive patients. The already increased amounts of IgG produced by MNCs from anergics was further increased. The effect was comparable for both concentrations of hydrocortisone. In PWM stimulated cultures, either dose of hydrocortisone caused some increase in IgG synthesis, mostly in cells from LC subjects. These effects in LC cells, especially in cultures without mitogen, are similar to the pattern of total IgG synthesis by patient MNCs. A possible mechanism for the interaction between corticosteroid and the immune system has been suggested by the recent demonstration of IL-receptor induction on blood lymphocytes by the hormone [423]. Another feature of increased steroid levels is lymphopenia [308], which was also observed in the present study in the more ill patients. There are thus two abnormalities in surgical patients that parallel the changes caused by elevated corticosteroid levels.

Another possible explanation for an increased non-specific spontaneous Ig synthesis is *in vivo* activation by bacterial products. Endotoxemia has been described in acutely ill patients [94], and is an important aspect of the pathogenesis of sepsis [424]. Lipopolysaccharide is uniformly present in gram-negative bacteria, and is a well-known activator of murine B cells [425]. Human lymphocytes also proliferate in response to many bacterial products [426]. This is mainly a property of B cells [427]. The effect is dependent on monocytes, relatively independant of T cells [112], and may progress completely to Ig synthesis with minimal proliferation [428,429]. Bacterial products are thus capable of producing a polyclonal response in human B cells, and may account for some of the observed *in vivo* activation seen in surgical patients with reduced DTH responses. Since LPS activation is relatively T cell

independant, functional defects in T cells in these patients would likely not affect this process.

The neutrophil cannot be considered an immunologically inert cell, and may play a role in the *in vitro* synthesis of Ig. Increased numbers of neutrophils were present in cell cultures from the more ill surgical patients. Experiments showed that the presence of neutrophils, although in greater numbers than observed, except in occasional patients, decreased Ig synthesis *in vitro*. This could be simply a result of competition for media substrates, but there are data showing the inhibition of B cell differentiation by soluble material released by neutrophils [430]. Neutrophils may also release factors that participate in T cell activation [431], and these could also affect *in vitro* MNC responses. There may thus be important interactions between antigen-specific and non-specific aspects of the host defence system.

The other major hypothesis to explain increased spontaneous Ig synthesis in the blood of ill patients is that these circulating activated B cells are part of systemic immune responses to unknown antigens, and appear in the blood in the course of the lymphocyte traffic involved in such responses. They would thus be producing specific rather than non-specific, Ig. Antibody producing cells in the blood after vaccination have been described in animals many years ago [432,433,434,435,436]. In man, data from the present study, as well as other studies, show that early in the course of humoral immune responses to both protein [136,437] and polysaccharide antigens [144], such spontaneous antibody-secreting cells appear in the blood.

The list of possible antigens that a surgical patient could be responding to includes blood transfusions, self-antigens released through tissue injury, and bacteria, which the host could encounter either by invasive sepsis, or translocation from the gut or other body sites.

Heterologous blood components are frequently given to surgical patients. Immune responses to non-host HLA antigens are well known, and could in some instances account for the *in vitro* observations in surgical patients.

Trauma and surgery both result in tissue injury. The release of self-antigens could result in an increase in circulating Ig producing cells, similar to autoimmune disease.

A possible source of antigen in patients with infections is bacterial antigens. Previous studies have provided some evidence that anti-bacterial antibody responses occur in septic patients, and invasive bacterial infections have been shown to increase *in vitro* IgG synthesis

[438]. The *in vivo* data supporting a humoral immune response to infecting bacteria in septic man have relied on the measurement of serum antibody levels. Serum levels of IgG, M and A antibody to homologous LPS and CGL all increased within 7 days of gram negative bacteremia [439]. Anti-O antibody showed the greatest increase. Others have also found evidence of increased IgG and IgM anti-bacterial antibody in septicemic patients [440,441,442]. Although responses were detected in most patients, the magnitude was greater in patients with intact DTH responses. In skin test reactive patients, the mean IgG and IgM titers were 124 and 53, compared to 53 and 13 for anergics. In bacteremic cancer patients, 67% responded to homologous bacteria, but only 5% to CGL [443,444]. Pseudomonas is a special case; while 68% of patients with invasive sepsis have a serum response to exotoxin A, only 24% responded to the homologous LPS [445]. The response to pulmonary pseudomonal infections has primarily been studied in cystic fibrosis [446,447,448,449]. Anti-pseudomonal antibody can be demonstrated in most patients. The major portion of the antibody response is directed against the O antigens. Other studies have been done in pulmonary sepsis in non-cystic patients in respiratory ICUs [450]. With regard to anaerobic bacteria, there is evidence of natural IgM antibody against Bacteroides sp., with a serum IgG response in infected subjects [451,452].

Studies relying purely on serum antibody content, especially in surgical patients, are prone to problems of fluid and protein shifts, as well as blood product transfusion. They also have generally used older techniques for antibody determination, sometimes difficult to relate to modern methods [453]. The use of *in vitro* MNC cultures, such as those used in the present studies, avoids these problems. There are no data that demonstrate *in vitro* synthesis of anti-bacterial antibody by MNCs from septic patients. It is known, however, that whole bacterial vaccines elicit antibody responses, including the appearance of MNCs in the blood that produce antibody *in vitro* [454].

There is thus some evidence that supports the occurrence of anti-bacterial antibody responses in infected patients. This provides one possible explanation for the observed increase in Ig synthesis by blood MNCs from some patients.

However, not all patients have clinical evidence of systemic sepsis. Another source of antigen must be postulated. One possibility that is supported by some evidence is bacterial translocation across the gut mucosa. Support comes from studies of the phenomenon of translocation in man, the pattern of Ig isotypes produced in increased amounts in surgical

patients, and their response to PWM stimulation *in vitro* seen in this study, the demonstration of the presence of anti-bacterial antibody secreting cells in gut-associated lymphoid tissue, and the decrease in spontaneous Ig synthesis in germ-free animals.

The movement of bacteria across the intestinal wall into the host was initially described in laboratory animals in 1966 [455]. Translocation has since been demonstrated for both enteric bacteria [456,457] and fungi [458] in animal models, especially following burns. In recent studies, there is evidence that hemmorhagic shock alone is associated with bacteremia and endotoxemia in 50 and 87% of rodents subjected to a mean arterial pressure of 30 mm Hg [459]. Although this is an extreme degree of hypotension, the same study reported positive blood cultures taken within 3 hrs of admission in in 56% of human trauma patients without bowel perforation when the admission systolic blood pressure was less than 80 torr. The extent to which bacterial translocation plays a role in the ill surgical patient is currently a subject of much interest. The phenomenon could account for several features of the present observations on abnormal Ig synthesis in surgical patients.

The pattern of isotypes produced spontaneously in increased amounts by surgical patients, especially those that were more ill, supports the hypothesis that they are responding to polysaccharide antigens. The major increases in spontaneous Ig synthesis are in IgG and IgA, with less or no increase in IgM. In the present study of subjects vaccinated with PPS, a similar pattern was observed. Greater quantities of IgG and IgA than IgM anti-PPS were produced *in vitro* after *in vivo* activation. Lymphoblasts secreting IgM antibody may be subject to different traffic patterns than the other isotypes [460]. Another study has shown that the normal spontaneous IgC synthesis is mostly IgG_2, consistent with anti-polysaccharide antibody responses [461]. These data thus show similarities between the *in vitro* manifestations of an antibody response to polysaccharide antigens, and the observed abnormalities in Ig synthesis *in vitro* by blood cells from ill surgical patients.

Studies of gut lymphocytes have provided evidence of humoral immune responses to bacteria without invasive sepsis. Cells secreting anti-bacterial antibody have been demonstrated in the submucosa of the intestine of normal subjects [462]. The existence of traffic involving these cells could account for some spontaneous antibody-secreting cells in the blood, especially IgA. Circulating cells spontaneously secreting antibody found been found after oral antigen administration in man [463]. These cells represent lymphocyte traffic [464]. An immune response to such gut-origin antigens, especially if a breakdown in the

integrity of the gut barrier during acute illness allowed an increase in humoral immune activity, would increase the number of such cells in the circulation. This could account for the observed data on *in vitro* Ig synthesis by blood MNCs, and would support the hypothesis that surgical patients with elevated spontaneous total Ig synthesis are responding to bacterial antigens, possibly of gut origin.

There are data from germ-free animals that show the importance of intestinal bacteria in causing spontaneous Ig synthesis. In mice, the background synthesis of IgG and IgA, but not IgM, was shown to be dependent on stimulation of the immune system by external antigens [465]. Bacterial antigen in the germ-free state decreased the number of spontaneous Ig secreting cells. Even the low level of spontaneous Ig-producing cells in normals may thus be related to a background of intermittent or continuous response of the humoral immune system to intestinal bacteria.

Athough the majority of Igs are not produced by blood cells, increased spontaneous synthesis *in vitro* by circulating MNCs would predict elevated serum Ig levels, if such cells are present in the blood as a traffic phenomenon. This was observed. Although more strongly associated with septic outcome than the results of DTH skin testing, more ill surgical patients were found to have elevated IgG, and IgA levels, but normal IgM. This isotype pattern is once again parallel to the observations in the *in vitro* culture system; spontaneous synthesis of IgG and IgA are elevated, whereas IgM is normal. The fact that these *in vivo* increases were most significant in septic patients is consistent with the concept that the circulating spontaneous Ig secreting cells, detected *in vitro*, are a true biologic manifestation of *in vivo* activation of the B cell system, very possibly by contact with bacterial antigens.

The effect of mitogen stimulation of cell cultures, althought there are other possible mechanisms, is also consistent with active host anti-bacterial humoral immune processes. The most interesting feature of the effect of PWM on *in vitro* Ig synthesis by patient MNCs is the observation that PWM either failed to increase, and in some patients, even caused a significant reduction, in Ig synthesis. These abnormal patterns were more prevalent in the more ill patients, and affected IgG and IgA, but not IgM. It is unknown whether PWM is simply affecting the activity of spontaneously synthesizing cells, or completely inhibiting this population, and activating another. Regardless, the possible explanations for this *in vitro* observation include abnormal regulatory cells, monocyte inhibition, soluble mediator

abnormalities, or once again the possibility that the blood cells are a traffic-related manifestation of ongoing *in vivo* antibody responses to unknown antigens.

The effect of PWM in the culture system used in the present studies was shown to be dependant on T cells. Changes in the balance of regulatory T cells may affect *in vitro* Ig synthesis [143]. It has previously been shown that there is a population of Leu-7$^+$ large lymphocytes that regulate antibody synthesis *in vitro* [466], and that CD8$^+$ cells can inhibit both total and specific Ig synthesis *in vitro* after vaccination *in vivo* [467]. Analysis of regulatory T cell percentages in surgical patients showed an increase in Leu-7$^+$ cells, and a reduction in helper:suppressor ratios in patients with reduced DTH responses. These imbalances are possible contributors to the observed effect of PWM *in vitro* on MNCs from these patients.

Increased percentages of monocytes present in cultures from anergic patients may also inhibit PWM stimulated responses. In the present culture system, as has been demonstrated previously [468], monocytes were necessary for expression of the response to PWM stimulation. A ratio of lymphocyte:monocyte of 10:1 was found to be optimal for the conditions used in these studies. Ratios of less than 5 were inhibitory. This is a level sometimes present in patients with reduced DTH, as was demonstrated both in counting by morphology, FACS, and latex bead ingestion studies. Thus, abnormalities in the cellular composition *in vitro*, in either T cells or monocytes, may alter PWM responsiveness in the manner observed in patients.

Several soluble mediators are involved in the regulation of B cell function. These include B cell growth and differentiation factors [469], colony-stimulating factor, and IL-1 and IL-2 [470]. Increased IL-1 secretion is seen in cultures of MNCs from patients with acute inflammatory illnesses [471]. Cells from thermally injured subjects, which show increased spontaneous Ig synthesis *in vitro* [236], similar to the patients with reduced DTH responses in this study, produce decreased amounts of IL-2, and increased amounts of IL-1 [472]. It is not clear at present how these changes are related to abnormalities in *in vitro* Ig synthesis. Abnormalities in the function of these mediators in anergic surgical patients have not been described.

During acute illness, the synthesis of several proteins in the host is increased. These molecules, collectively called acute phase reactants, include fibrinogen, and C-reactive protein (CRP). Fibrinogen-derived peptides may regulate some aspects of lymphocyte protein synthesis [473]. CRP has been shown to decrease PWM-induced Ig synthesis by binding

to, and activating, monocytes [474]. Although absent from the *in vitro* cultures, increased levels of CRP in the serum of ill surgical patients, together with the observed monocytosis that is carried over into the cell cultures, could play a role in the decreased response to PWM *in vitro*. Such a mechanism, or some other systemic response to stress, could mediate the observed deleterious effect of even psychologic stress on plaque-forming cell responses [475].

The final possible explanation of the differential effect of PWM on isotype synthesis *in vitro*, lies in the hypothesis that MNCs were harvested from surgical patients during the course of an immune response to unknown antigens, possibly bacteria.

One characteristic of the phenomenon of appearance of spontaneous antibody-secreting cells in the circulation early after antigenic encounter is that the synthesis of IgG and IgA antibody, but not IgM, can be down-regulated by PWM stimulation *in vitro*. This is the same isotype pattern observed in studies on the effect of PWM on total Ig synthesis *in vitro*. It is therefore possible that some or most of the spontaneous total Ig measured in the supernatants of cultures of MNCs from ill surgical patients is specific antibody to unknown antigens, being produced by lymphoblastoid cells that can be inhibited by PWM. MNCs activated *in vivo* to produce Ig, present in either the bone marrow [410] or gut-associated lymphoid tissue [411], can be down-regulated by PWM *in vitro*. The temporary presence of these cells in the blood due to traffic could produce the observed increase in spontaneous Ig synthesis, and the common non-response to PWM, with the occasional significant negative response. The fact that a significant reduction in total Ig synthesis with PWM was observed only occasionally is consistent with the data from the present studies, which show a short window of only a few days in the course of an antibody response when this effect is present.

However, the hypothesis that the increased Ig that is produced spontaneously *in vitro* is all antibody to unknown antigens is not consistent with the disparity between the quantities of culture supernatant total Ig that can be accounted for by the amounts of specific antibody produced *in vitro* after *in vivo* vaccination. This suggests either multiple synchronous responses, or concomitant polyclonal activation of the B cell system. The presence of large lymphocytes in the circulation, which are actively dividing [476] and resemble those produced by mitogen stimulation *in vitro* [477,478], may be a histologic corollary of a traffic-based manifestation of either a specific immune response [479], or such a polyclonal activation of B cells. An increase in serum Ig levels, only a fraction of which was specific antibody, has also been observed after immunization [480]. This polyclonal activation could cause even more

spontaneous Ig synthesis *in vitro* than would occur in the course of an immune response to a specific antigen.

In summary, MNCs from surgical patients spontaneously produce more IgG and IgA *in vitro* than cells from healthy subjects. This spontaneous activity is more pronounced in patients with reduced DTH responses. IgM synthesis is not increased. The presence of these cells in the blood implies *in vivo* activation of the B cell system in surgical patients, especially those more severely ill, with reduced DTH. Stimulation with PWM resulted in either no difference, or decreased total Ig synthesis. This effect was seen mostly in ill patients with reduced DTH, and affected only IgG and IgA, not IgM. This pattern is identical to the observations made in the anti-PPS *in vitro* studies, and is consistent with the hypothesis that ill surgical patients are responding to bacterial antigens.

4. CELL PHENOTYPES

There are alterations in the phenotypes of circulating MNCs in surgical patients. In patients with decreased DTH, surface Ig^+ cells are reduced, while monocytes, HLA-DR^+ and Leu-7^+ cells are increased, and the helper:suppressor ratio is reduced. Increased numbers of large cells are present in the circulation, and there are more cells that are positive for T cell subclass markers, than for a global T cell marker.

Selective recovery of some phenotypes during cell isolation may account for some differences, since it is known that cell separation procedures can give rise to a deviation in T:B ratios [358,481]. However, cells from all subjects were isolated in the same manner.

Some differences in the phenotypes of blood cells from patients suggest a process of *in vivo* activation of the immune system. Large cells are present, and there is an increase in HLA-DR^+ cells. Although it is not known which of monocytes, T cells or B cells contribute most to the increased in HLA-DR^+ cells, its expression is generally viewed as a marker of activation [482,483]. After vaccination, an increase in HLA-DR^+ cells in the peripheral blood has been described [484], beginning after one day, and continuing for 2 weeks [485]. For T cells, this increase occurs in the $CD4^+$ subset [486]. A similar increase of HLA-DR^+ MNCs has been described in autoimmune diseases [487], which also show increased spontaneous Ig synthesis by blood MNCs. However, similar antigens may be present on the surface of neutrophils at some stages of differentiation as well [488,489], and could have contributed to the

analysis. The present data do not allow a determination of the source of the increased percentage of cells bearing HLA-DR$^+$ markers. Nonetheless, this may be an indication of *in vivo* activation, possibly secondary to host immune responses to unknown antigens.

Changes in the prevalence of B cell markers are also suggestive of *in vivo* activation. The observed decrease in sIg$^+$ cells, with a concomitant increase in synthetically active lymphoblastoid B cells, known to be sIg$^-$, was more marked in patients with reduced DTH, and is also consistent with *in vivo* activation of the humoral immune system.

Some of the observed differences in *in vitro* Ig synthesis by PWM stimulated cells from anergic patients may be accounted for by changes in the cellular composition of the cultures. Data are presented that show the inhibitory effect of monocytes on *in vitro* Ig synthesis, at a level consistent with the increased numbers found in some of the patients with reduced DTH. Whether there are differences in subpopulations of monocytes in the patients is not known [490]. Cells that are positive for Leu-7, a marker for natural killer activity, were also elevated in patients, and can play a down-regulatory role in *in vitro* cultures [395]. Finally, there is a change in the balance of CD4 and CD8 cells, favoring suppression, that may alter the *in vitro* Ig response to PWM stimulation [143,491,492].

The finding of an excess of T cell subclass marker positive cells compared to cells that are positive for a pan-T cell marker, has been reported previously in the blood of aged subjects [493]. In surgical patients, this was clearly associated with reduced DTH responses. Because thymocytes acquire CD4 and CD8 before expressing a pan-T cell marker in the process of differentiation [494,495], this may represent a diminished capacity of the anergic patient to terminally differentiate T cells, which results in the appearance in the blood of increased numbers of less mature T cells.

In summary, there are changes in the phenotypic composition of the blood in surgical patients. Since few immune processes occur in the bloodstream, the exact significance of the findings cannot be stated. However, the overall pattern of phenotypes in surgical patients suggests activation of the immune system, worse in the more ill subjects with reduced DTH responses.

5. HUMORAL IMMUNITY AFTER TRAUMA

The study of host defence mechanisms in trauma is extremely useful because it is a cause of acute acquired immunodeficiency, quite different from other more chronic disease processes. *In vivo* antibody responses were studied in relation to altered physiologic status, DTH, and outcome. *In vitro* Ig synthesis was also measured.

A relatively well group of trauma patients was studied. This resulted from the exclusion of subjects that did not survive at least 14 days after injury, and accounts for the zero mortality rate and infrequent episodes of sepsis. DTH responses in this group were initially depressed in some subjects, but recovered rapidly. This depression was more marked in patients with blunt compared to penetrating injuries. Similarly, APACHE II scores, initially elevated, improved rapidly and in parallel with changes in DTH responses. In blunt and penetrating injuries, the degree of injury correlated with the degree of physiologic impairment early after injury, with a trend towards worse DTH responses with greater injury severity. In thermal injuries, there was a direct relationship between degree of injury, clinical course, and changes in DTH responses and physiologic status. These data show that trauma has a dose-dependant acute effect on DTH responses, that parallels physiologic derangements. Only when there is improvement in global physiologic status does the DTH skin test response recover.

The antibody response to TT administered to trauma patients in the emergency room was normal in comparison to lab control subjects. Within the trauma group, however, there was variability in the degree of antibody responsiveness. When the anti-TT responses were examined in relation to degree of physiologic abnormalities and DTH responses, the best correlations were found with STS and APACHE II scores immediately after injury, with a progressive decline in the strength of the relationships thereafter. In the context of the dynamic changes in the host after injury, these data show that there is a critical period for the determination of the magnitude of the antibody response. This decisive period occurs within the first few days after antigen encounter, and determines the magnitude of the antibody response measured weeks later.

The responsiveness of trauma patients was compared to that of non-trauma patients. In comparable skin test categories, the anti-TT responses of trauma patients were better than non-trauma subjects. Apart from age, there were other major clinical differences between these groups of patients that may be related to humoral immunity. Depressed DTH

responses were a transient phenomenon in the trauma patients, with nearly all subjects showing normal responses two weeks after injury. The DTH responses of the non-trauma patients remained suppressed over this period of time. There was also no mortality and minimal sepsis, even in the anergic trauma patients, compared to the non-trauma patients. These data again illustrate the acute, reversible nature of depressed DTH in trauma patients, and the lesser association of anergy in this setting compared to non-trauma illness, with reduced humoral immunity, and increased sepsis and mortality.

There were some differences in *in vitro* Ig responses in trauma patients compared to controls and other patients. Stimulation with PWM caused less suppression of anti-TT synthesis early after vaccination than in controls. This finding could be explained by a left shift in the kinetic response curve, which would indicate accelerated humoral immune mechanisms in trauma patients.

Stimulation with PWM was also unable to elicit the synthesis of large amounts of specific antibody late after vaccination. This phase of the response to TT vaccination is dependant on the generation of a second, long-lived population of B cells, which probably represent a memory function. These data suggest, that although the population of B cells that produce the early lymphoblastoid response is normal, the second population of anti-TT specific memory B cells is not produced by TT vaccination of trauma patients.

The changes in total Ig synthesis after traumatic injury were similar to the changes seen in non-trauma patients, especially those with reduced DTH responses. There was an increase in spontaneous Ig synthesis, with no response to PWM stimulation. The kinetic pattern is similar to what would be expected if the trauma patients were responding to antigens encountered early after injury. Such antigens could be self antigens, released by injury, or bacterial antigens, recently demonstrated in the circulation of trauma patients early after injury [459].

In summary, the antibody response to a protein antigen in trauma patients is preserved, even in those with reduced DTH. The difference between these, and other surgical patients, is the rapidly acquired, transient nature of anergy. Study of the *in vitro* synthesis of specific Ig suggests that, although the serum response is normal, trauma patients may not generate antigen-specific memory cells after vaccination. The pattern of spontaneous and PWM stimulated total Ig synthesis in vitro was closer to normal than other patients, even in those with reduced DTH responses.

6. ANTI-BACTERIAL IMMUNITY AND BIOLOGICAL RESPONSE MODIFIERS

The ultimate objective of the study of host defence mechanisms is to decrease the prevalence and effects of sepsis in patients, by defining correctable abnormalities in immunity [496]. In the context of the humoral immune system, there are several relevant considerations. These are the general concepts of biological response modification as related to humoral immune systems, whether or not an anti-bacterial antibody response occurs in patients, and the relationship of anti-bacterial antibody to outcome in sepsis.

There are several general concepts of biological response modification related to the findings in this study of humoral immunity. Host anatomy and physiology determine the microenvironmental milieu in which wound healing and anti-microbial resistance mechanisms operate. The association observed in the present study between physiologic derangement and altered immunity is consistent with this concept, and suggests that restoration of normal physiology may restore humoral immunity [497]. The corrective value of restoring nutritional status has been shown for several abnormalities of host defense [216,498,499], including serum Ig levels [500] and specific antibody responses to protein antigens [213,214,217].

Surgery itself, although generally immunosuppressive, may aid in the restoration of immune function by treating disease. Although there are no data available related to humoral immune function, it is known that other aspects of immunity can be improved by tumor resection [501], burn wound excision [502], and correction of biliary tract disease, bowel obstruction, hypovolemia and visceral abscesses [19].

Antigen-specific approaches to immunomodulation utilize the concepts of active or passive vaccination to increase specific antibody levels. The potential value of this approach rests on the knowledge that the level of anti-bacterial antibody at the time of sepsis, is positively related to outcome. Zinner showed that high titers of IgG antibody against O antigens or CGL of infecting bacteria in bloodstream infections was associated with a significant reduction in mortality [503]. IgG antibody levels were generally higher than IgM, which were not significant. Very similar data were obtained by others [445,504].

The normal response to bacterial polysaccharide antigens seen in this study implies that surgical patients would produce anti-bacterial antibody. This would justify attempts at active vaccination against gram-negative sepsis. There have been studies of this in some patient

groups, especially with Pseudomonas [234,505] but consistent good results are not available [497]. Most anti-bacterial antibody is formed against the surface O antigens. Unfortunately, there is substantial diversity in the structure of these surface polysaccharide antigens among different gram-negative organisms. These difficulties with antigenic diversity, together with the problems of vaccine preparation and timing of immunization, are unresolved issues in the field of active immunization.

The presence of antibody responses to the polysaccharide antigens of bacteria, however, does not necessarily insure the timely delivery of effector antibody molecules to sites of sepsis [506,507,508]. Interest has therefore also turned to strategies of passive vaccination. Observations on the role of LPS in the pathogenesis of sepsis have suggested that antibody to the core structure of LPS, which is similar among many gram-negative organisms, would be therapeutically useful. Passive immunization with human antisera against Pseudomonas [509], and Escherichia coli [96] have proven effective in human trials. However, the ideal source and composition of antibody is unknown [497]. It is also unknown which isotype is most protective, and what the respective roles are of anti-O and anti-CGL antibody [96].

Some aspects of the present study will provide useful information in developing strategies of specific host immunomodulation. The close correlation between *in vivo* antibody responses, and the spontaneous synthesis of antibody early after vaccination by blood MNCs, provides a window for testing non-specific immunomodulators that may be useful in upgrading appropriate aspects of a native antibody response. Also, the peripheral blood is evidently a source of lymphoblastoid cells secreting antibody early after vaccination that could be used in fusions to provide human monoclonal antibodies.

In summary, antibody responses to protein antigens are reduced in surgical patients. Responses to the more common bacterial antigens, polysaccharides, are normal. This, together with the observed patterns of in vitro B cell function, suggest that surgical patients are capable of anti-bacterial antibody responses. Until more data are available on the relationship between infection and endogenous antibody production, and the mechanism of action of antibodies of various classes in different body locations, the impact of the host's own antibody responses to infecting bacteria will remain problematic. It may be that the role of humoral immunity in surgical patients has already been summarized in antiquity by Ovid, *Too late I grasp my shield after my wounds.*

APPENDIX 1: CLASSIFICATION OF DEGREE OF SURGERY

Surgical procedures were classified according to general assessment of magnitude, from class 1 (lowest) to class 4 (highest). The procedures are listed below.

Class 1

Appendectomy
A-V-Fistula
Breast Biopsy
Colostomy Closure
Hemmorhoidectomy
Herniorraphy
Panniculectomy
Rectal Fulguration
Sclerosis of Esophageal Varices
Ureterolithotomy

Class 2

Amputation
Cholecystectomy
Drainage Abscess
Femoro-Popliteal Bypass
Hepatic Artery Cannulation
Hiatus Hernia Repair
Hysterectomy
Lung Biopsy
Mallory Weiss Repair
Mastectomy
Neck Dissection
Open Reduction and Fixation
Parathyroidectomy
Repair Gastroduodenal Perforation
Salpingoophorectomy
Skin Grafting of Burns
Splenectomy
Thoracotomy
Thyroidectomy

Class 3

Adrenalectomy
Anterior Resection
Cholecystoduodenostomy
Choledochojejunostomy
Closure of Hartmann's Procedure
Common Bile Duct Exploration
Esophageal Transection
Hartmann's Procedure
Hemicolectomy
Iliac Aneurysm Repair
Internal Pseudocyst Drainage
Lobectomy
Partial Gastrectomy
Pneumonectomy
Repeat Neck Exploration

Class 4

Abdominal Aneurysm Resection
Abdominoperineal Resection
Aortic Reconstruction
Aortocoronary Bypass
Esophagogastrectomy
Hepatic Resection
Pancreaticoduodenectomy
Park's Pouch
Proctocolectomy
Portal-Systemic Shunts
Radical Cystectomy
Radical Nephrectomy
Total Gastrectomy
Total Colectomy

APPENDIX 2: APACHE II CALCULATION

The APACHE II score is the sum of points for abnormal physiology, age, and chronic health evaluation. Points are given for abnormal physiologic and laboratory values as shown below. The most abnormal value in a given 24 hour period is used to calculate the score for that day. Age points are assigned as indicated. A history of chronic liver, cardiovascular, respiratory or renal disease is scored with 2 or 5 points, if the patient had elective surgery, or if the patient had emergency or no surgery, respectively.

AGE POINTS

Age	Points
< 44	0
45-54	2
55-64	3
65-74	5
>75	6

PHYSIOLOGY AND LABORATORY POINTS

POINTS	+4	+3	+2	+1	0	+1	+2	+3	+4
Temperature	>41	39-40.9		38.5-38.9	36-38.4	34-35.9	32-33.9	30-31.9	<29.9
Mean BP	>160	130-159	110-129		70-109		55-69	40-54	<39
Heart Rate	>180	140-179	110-139		70-109		55-69	40-54	<39
Resp Rate	>50	35-49		25-34	12-24	10-11	6-9		<5
PaO_2					>70	61-70		55-60	<55
Serum CO_2	>52	41-51.9		32-40.9	22-31.9		18-21.9	15-17.9	<15
Sodium	>180	160-179	155-159	150-154	130-149		120-129	111-119	<110
Potassium	>7	6-6.9		5.5-5.9	3.5-5.4	3-3.4	2.5-2.9		<2.5
Creatinine	>3.5	2-3.4	1.5-1.9		0.6-1.4		<0.6		
Hematocrit	>60		50-59 9	46-49 9	30-45.9		20-29.9		<20
White Count	>40		20-39.9	15-19 9	3-14.9		1-2.9		<1

Glasgow Coma Score Points = (15-actual Glasgow Coma Score, see appendix 3)

APPENDIX 3: MODIFIED INJURY SEVERITY SCORE DETERMINATION

The following tables show the points given for various injuries in each organ system. Injuries in each region were assigned a severity score from 0 to 5 based on clinical estimate of degree of injury and associated mortality, as indicated below.

DESCRIPTION OF SEVERITY SCORES

severity score	%mortality	description
0	0	no injury
1	0	minor injury
2	0	moderate injury
3	< 5%	severe injury, not life threatening
4	< 25%	severe, life threatening injury, survival probable
5	> 25%	severe injury, life threatening injury, survival uncertain

BODY AREA: NEUROLOGIC

For neurologic injury, points are assigned according to the result of the Glasgow coma scale, as shown.

SEVERITY	INJURY DESCRIPTION
0	GCS = 15 (no injury)
1	GCS = 13 to 14
2	GCS = 10 to 12
3	GCS = 7 to 9
4	GCS = 4 to 6
5	GCS = 3

GLASCOW COMA SCALE (GCS): points are assigned as follows.

Eye opening
4 Spontaneous
3 To speech
2 To pain
1 None

Best verbal response
5 Oriented
4 Confused
3 Inappropriate
2 Incomprehensible
1 None

Best motor response

6	Obeys command
5	Localizes pain
4	Withdraws
3	Flexion to pain
2	Extension to pain
1	None

BODY AREA: FACE & NECK

SEVERITY	INJURY DESCRIPTION
0	no injury
1	abrasion/contusion of ocular apparatus or lid
	whiplash without X-ray abnormality
	vitreous or conjunctival hemorrhage
	fracture teeth
	minor facial and scalp lacerations
2	undisplaced facial bone fracture
	whiplash with X-ray abnormality
	laceration of eye
	retinal detachment
	major uncomplicated facial or scalp lacerations
3	loss of eye, avulsion optic nerve
	C spine fracture without cord damage
	displaced facial fractures requiring surgery (all Lefort fractures)
	blow-out fractures of orbit
	facial lacerations or avulsions involving deep structures
4	compound facial or skull fractures (cerebrospinal fluid leak)
5	injuries with major airway obstruction
	spine fracture with quadriplegic
	avulsion of vessels

BODY AREA: CHEST

SEVERITY	INJURY DESCRIPTION
0	no injury
1	muscle ache or chest wall stiffness
	superficial chest wall wounds
2	simple rib fractures
	simple sternal fracture
	major chest wall contusion
	transverse spine fracture
3	multiple rib fractures
	hemothorax
	diaphragmatic rupture
	pulmonary contusion
	pneumothorax
4	open chest wounds
	pericardial injury without tamponade
	pneumomediastinum

	myocardial contusion
	flail chest
5	laceration of trachea, hemomediastinum
	aortic laceration
	myocardial laceration or rupture
	pericardial injury with tamponade

BODY AREA: ABDOMEN

SEVERITY	INJURY DESCRIPTION
0	no injury
1	muscle ache, seat belt abrasion
2	major abdominal wall contusion
	rectus hematoma
	transverse spine fractures
3	contusion of abdominal organ
	retroperitoneal hematoma
	extraperitoneal bladder rupture
	thoracic/lumbar spine fracture
	renal or ureteral laceration
	single small bowel or mesenteric laceration, perforation, or serosol tears
4	minor laceration of abdominal organs
	intraperitoneal bladder rupture
	spine fractures with paraplegia
	renal or ureteral rupture
	multiple small bowel laceration or perforations
	duodenal hematoma
	single large bowel perforation or laceration
5	rupture, or severe laceration of abdominal vessels, or organs (except kidney)
	multiple large bowel laceration or perforations

BODY AREA: PELVIS AND EXTREMITIES

SEVERITY	INJURY DESCRIPTION
0	no injury
1	minor sprains
	dislocations
	fracture clavicle
	closed fracture of hand, foot
	soft tissue hematomas
	minor lacerations
2	compound fracture of digits
	undisplaced long-bone or pelvic fractures
	fracture scapula
	soft tissue avulsion
3	displaced long-bone or multiple hand/foot fractures
	single open long-bone fractures
	pelvic fracture with displacement
	laceration of major nerves/vessels
	de-gloving injuries
	closed hip fracture

4	multiple closed long-bone fracture amputation of limbs
5	multiple open long-bone fractures pelvic crush injury

APPENDIX 4. COMPUTER PROGRAMS

The computer programs written in BASIC for evaluation of data from the SPRIA and SPCIRA respectively are listed on the following two pages. Data input is by prompt. Data for the standard curve is requested initially, and calculations performed. The values are plotted as a scatter plot, and the range of log dilutions to be used for regression is entered. The line and formula are printed. Unknown values are entered for calculation. The programs run on a Sharp Model 1500 Pocket Computer.

```
10:" ":GOSUB "START"
20:GOSUB "SP CPM"
30:GOSUB 'SP PLOT"
40:GOSUB "SP LINREG"
50:GOSUB 'AP CPM"
60:GOSUB "AP CALC"
70:END
1000 'START"
1010:CLEAR :USING :TEXT :COLOR 0:
     BEEP ON :RESTORE
1020.WAIT 00.PRINT "SPRIA 2.0  CWN
     11 11 83'
1030:BEEP 1:INPUT "enter SP#: ';K0
1032: J0="M"
1034:INPUT 'Is type(enter G, M, or
     A)',M0
1040:BEEP 1:INPUT "enter Ag (TT KLM
     PPS):",L0.CLS
1042:BEEP 1:INPUT "Total CPM added:
     ";ZZ:CLS
1050:LPRINT "SPRIA ";J0;M0;'-A-";L0
1060:GRAPH
1070:LINE (129,10)-(150,35),0,3,0
1072:LINE (60,10)-(95,35),0,3,0
1073:TEXT :COLOR 0:LF 1
1074:LPRINT "SP";K0:CSIZE 1
1080:LF 1:LPRINT "SPRIA 2.0 Carl No
     hr 11.11.83"
1090:LPRINT "TIME: ';TIME
1092'LPRINT "Total CPM added= ";ZZ
1100:RETURN
2000."SP CPM"
2010:DIM AA(5,5)
2020:FOR R=0TO 5
2030'READ AA(0,R)
2040.NEXT R
2050:DATA 1,2,3,4,5,6
2060:WAIT 99:BEEP 1 PRINT "enter CP
     M for log D ="
2070.FOR R=0TO 5
2080:CLS  BEEP 1.PRINT "log D ";AA(
     0,R)
2090.FOR C=1TO 3
2100:CURSOR (2+C*6)
2110:INPUT AA(C,R)
2112:REM READ AA(C,R)
2114:REM DATA 242436,240193,243948,
     153736,176652,171720,59616,609
     39,60506
2116:REM DATA 14207,14470,13070,442
     0,0,5270,4014,3675,3197
2120.NEXT C
2130 NEXT R
2140:TEXT :CLS :CSIZE 1:LF 2
2150:LPRINT TAB 1;' log D';TAB 14,'C
     PM',TAB 23,'CPM',TAB 32,' CPM"
2160.COLOR 1
2170:FOR X=0TO 5
2180:LPRINT USING "#### #';AA(0,X),
     TAB 10;USING "########";AA(1,X)
     ,TAB 19,AA(2,X);TAB 28;AA(3,X)
2190:NEXT X
2200:TAB 0:LF 2.COLOR 0
2210'LPRINT TAB 1, 'log D";TAB 13,"A
     MEAN';TAB 24,'SEM ;TAB 31,'%SE
     M'
2220 DIM BB(6):COLOR 1
2230:FOR R=0TO 5
2240:FOR C=1TO 3
2250:BB(C)=AA(C,R)
2260'NEXT C
2270'GOSUB 'MEAN SEM %"
2280.AA(4,R)=BB(4)
2290 LPRINT USING "#### #";AA(0,R),
     TAB 10;USING '########";AA(4,R)
     ;TAB 28;BB(5),TAB 38;USING '##
     #';BB(6)
2300:NEXT R
2310:USING :RETURN
3000:"SP PLOT"
3010:LF 32:GRAPH :CSIZE 1:COLOR 0:
     GLCURSOR (0,0)'SORGN
3020:LINE (16,8)-(216,388),,,0
3030:COLOR 1
3040:FOR R=0TO 5
3042:AA(5,R)=LOG AA(4,R)
3045:X=28+R*38
3060:GLCURSOR (X-6,-16):LPRINT AA(0
     ,R):GLCURSOR (X+5,-4):ROTATE 3
     .LPRINT "-"'ROTATE 0
3070:FOR C=1TO 3
3072:IF AA(C,R)=0LET Y=-1:GOTO 3090
3080:Y=290-(((LOG AA(4,0))-(LOG AA(
     C,R)))*(LOG (AA(4,5)))*40))
3090:GLCURSOR (X,Y):LPRINT "o"
3100'NEXT C
3110:NEXT R
3120:GLCURSOR (9,120):ROTATE 3:
     LPRINT "LOG CPM BOUND":ROTATE
     0
3122:GLCURSOR (185,-25):LPRINT "log
     D'
3124:GLCURSOR (0,-80)
3130:RETURN

4000:"SP LINREG"
4010:BEEP 1:INPUT "do linreg for lo
     g D= ";J:J=J-1
4020.INPUT "to log D= ";K:K=K-1
4050.CLS :A=0:B=0:C=0:D=0:E=0:F=0:N=K-J
     +1
4060:FOR R=JTO K
4090:A=A+AA(0,R)
4100.B=B+AA(5,R)
4110:C=C+AA(0,R)*AA(5,R)
4120:E=E+AA(0,R)^2
4130:F=F+AA(5,R)^2
4160:NEXT R
4170:G=(C-(A*B)/N)/(F-B^2/N)
4180:H=A/N-G*(B/N)
4190:RR=(ABS (C-(A*B)/N))^2/((F-B^2
     /N)*(E-A^2/N))
4200:X=INT (H*10E3+.5)/10E3
4210:Y=INT (10^(H/ABS G)+.5)
4220:M=INT (G*10E7+ 5)/10E7
4230:DIM Z(2)
4240:Z(0)=290-((AA(5,0)-((J+1-H)/G)
     )*AA(5,5)*40)
4250:Z(1)=290-((AA(5,0)-((K+1-H)/G)
     )*AA(5,5)*40)
4252:Z(2)=(Z(0)-Z(1))/2+Z(1)
4260'COLOR 3
4270:LINE (28+J*30,Z(0))-(28+K*30,Z
     (1))
4280:LINE (16,Z(0))-(216,Z(0)),2,2
4290:GLCURSOR (28,Z(0)+6):LPRINT "C
     PM=";AA(4,J)
4300:LINE (16,Z(1))-(216,Z(1)),2,2
4310:GLCURSOR (28,Z(1)+6):LPRINT "C
     PM=";AA(4,K)
4311.LINE (16,Z(2))-(216,Z(2)),2,2
4312:GLCURSOR (28,Z(2)+6)'LPRINT "C
     PM=";INT (10^(LOG AA(4,K)+(LOG
     AA(4, J)-LOG AA(4,K))/2))
4320'GLCURSOR (0,-50):COLOR 0
4330'LPRINT "LINEAR REGRESSION FORM
     ULA IS.'
4340:TEXT :CSIZE 1.LF 1
4360:LPRINT "U/ML= STD U/ML *10^(lo
     g D-';:LPRINT TAB 5,M,' +";"(lo
     g CPM # ";G; "))"
4370.LF 1
4380:LPRINT "R^2= ";RR
4390:LPRINT "M=   ",M
4400:LPRINT "X int=  10^";X
4410:LPRINT 'Y int= ';Y
4420:RETURN
5000:'AP CPM"
5010'TEXT :LF 2:COLOR 0:USING
5020:BEEP 1:INPUT "enter AP #: ';O0
     .CLS
5092:BEEP 1:INPUT "number unknowns(
     <=12)';U:CLS
5100:DIM CC#(2,U-1)*12
5105:DIM DD(23,U-1)
5110.FOR R=0TO U-1
5122:BEEP 1:WAIT 0'INPUT "name :  ";
     CC#(0,R)
5124:BEEP 1:WAIT 0:INPUT "unit no .
     ';CC#(1,R)
5130:BEEP 1:WAIT 0:INPUT "date .  ";
     CC#(2,R):CLS
5154'FOR C=0TO 18STEP 6
5160.DD(C,R)=J+C/6
5165:FOR Z=C+1TO C+2
5170:BEEP 1:WAIT 0:PRINT LEFT#(CC#
     (0,R),4);" log D',DD(C,R);' CP
     M",Z-C
5180:CURSOR 19:INPUT DD(Z,R)'CLS
5182:NEXT Z
5190:NEXT C
5200:NEXT R
6000:'AP CALC":DIM Y(4):DIM X(4)
6001:FOR R=0TO U:COLOR 0
6002:GRAPH :LINE (0,0)-(250,0),5,0:
     TEXT .LF 2.USING
6004:LPRINT "NAME: ";CC#(0,R)
6005:REM LPRINT "UNIT: ";CC#(1,R)
6006:LPRINT "DATE: ";CC#(2,R)
6007'LPRINT "SPRIA:";J0;M0;"-A-";L0
6008:CSIZE 1.LPRINT "AP";O0
6009:LPRINT "Using SP",K0
6010:LPRINT "SPRIA 2 0 Carl Nohr 11
     11.83"
6011:LPRINT "TIME: ";TIME
6016:LF 1
6020:LPRINT TAB 1;"log D";TAB 15;"C
     PM";TAB 23;"CPM";TAB 31;"CPM"
6022:COLOR 1
6026:FOR C=0TO 18STEP 6
6030:LPRINT USING "####.#";DD(C,R);
     TAB 10;USING "########";DD(C+1
     ,R);DD(C+2,R);DD(C+3,R)
6034:NEXT C
6036:LF 1:COLOR 0
6040:LPRINT TAB 1;"log D";TAB 13;"A
     MEAN";TAB 23;"SEM";TAB 29;"% S
     EM"
6046:COLOR 1
6050:FOR C=0TO 18STEP 6
6054:FOR Z=1TO 3
6060:BB(Z)=DD(C+Z,R)
6064:IF DD(C+Z,R)>0LET DD(C+Z,R)=
     LOG DD(C+Z,R)
6068:NEXT Z
6070:GOSUB "MEAN SEM %"
6076:DD(C+4,R)=BB(4)
6078:DD(C+5,R)=BB(5)

6080:LPRINT USING "####.#";DD(C,R);
     TAB 10;USING "########";DD(C+4
     ,R);DD(C+5,R);BB(6)
6084:NEXT C
6100:GOSUB "AP PLOT"
6102:TEXT :CSIZE 1:LF 2:COLOR 0
6110:LPRINT TAB 1;"log D",TAB 14,"U
     /ml";TAB 22;'U/ml";TAB 30; U/m
     l"
6120:FOR C=0TO 18STEP 6
6130'FOR Z=1TO 3
6140:DD(C+2,R)=49(10^(DD(C,R)-(M+6*
     DD(C+2,R))))
6141:DD(C+2,R)=INT (DD(C+2,R)*10E4+
     5)/10E4
6142:NEXT 2
6146:COLOR 1
6150:LPRINT USING "#P##.#";DD(C,R);
     TAB 9;USING "####.####";DD(C+1
     ,R);DD(C+2,R);DD(C+3,R)
6160:NEXT C
6170:LF 2:COLOR 0
6180:LPRINT TAB 1;"log D";TAB 13;"A
     MEAN";TAB 23;"SEM";TAB 28,"%SE
     M"
6190:COLOR 1
6200:FOR C=0TO 18STEP 6
6210:FOR Z=1TO 3
6220:BB(Z)=DD(C+2,R)*10E4
6230:NEXT 2
6240:GOSUB "MEAN SEM %"
6250:DD(C+4,R)=BB(4)/10E4
6260:DD(C+5,R)=BB(5)/10E4
6270:LPRINT USING "####.#";DD(C,R);
     TAB 9;USING "####.####";DD(C+4
     ,R);DD(C+5,R);USING "######";B
     B(6)
6280:NEXT C
6282:LF 1
6290:NEXT R
6300:END
10000:"MEAN SEM %"
10010:N=3:A=0:B=0
10020:FOR X=1TO 3
10030:IF BB(X)=0LET N=N-1:GOTO 10060
10040:A=A+BB(0+X)
10050:B=B+BB(0+X)^2
10060:NEXT X
10070:IF N=1LET BB(4)=A:GOTO 10130
10080:IF N=0LET BB(4)=0:GOTO 10130
10090:BB(4)=INT (A/N+.5)
10100:BB(5)=INT ((((B-A^2/N)/(N
     -1)))/N)+.5)
10110:BB(6)=INT ((100*(BB(5)/BB(4)))+.
     5)
10120:RETURN
10130:BB(5)=0:BB(6)=0
10140:RETURN
20000:"AP PLOT"
20010:TEXT :LF 16:GRAPH :CSIZE 1:
     SORGN :COLOR 0
20020:LINE (16,8)-(216,388),,,0
20030:COLOR 1
20034:FOR Z=0TO 5
20036:X=28+Z*38
20038:GLCURSOR (X-42,-16):LPRINT (Z+
     1):GLCURSOR (X+5,-4):ROTATE 3:
     LPRINT "-":ROTATE 0
20039:NEXT Z
20040:FOR C=0TO 18STEP 6
20045:X=28+(DD(C,R)-1)*38
20050:X(C/6+1)=X
20056:Y(C/6+1)=290-(((LOG AA(4,0))-(
     LOG DD(C+4,R)))*(LOG (AA(4,5))
     *40))
20070:FOR Z=1TO 3
20072:IF DD(C+Z,R)=0LET Y=-1:GOTO 20
     090
20080:Y=290-(((LOG AA(4,0))-(DD(C+Z,
     R)))*(LOG (AA(4,5))*40))
20090:GLCURSOR (X,Y):LPRINT "o"
20096:NEXT Z
20098:Y=290-(((LOG AA(4,0))-(LOG DD(
     C+4,R)))*(LOG (AA(4,5))*40))
20100'GLCURSOR (16,Y):LINE (16,Y)-(2
     16,Y),1
20110:NEXT C
20120:GLCURSOR (9,120):ROTATE 3:
     LPRINT "LOG CPM BOUND":ROTATE
     0
20122:GLCURSOR (185,-25):LPRINT "log
     D"
20124:COLOR 1
20126:LINE (X(4),Y(4))-(X(3),Y(3))
20128'"X":LINE (X(3),Y(3))-(X(2),Y(2
     ))
20130:LINE (X(2),Y(2))-(X(1),Y(1))
20132:USING
20136:COLOR 3
20138:LINE (28+J*30,Z(0))-(28+K*30,Z
     (1))
20140:LINE (16,Z(0))-(216,Z(0)),2,2
20150:GLCURSOR (28,Z(0)+6):LPRINT "C
     PM=";AA(4,J)
20160:LINE (16,Z(1))-(216,Z(1)),2,2
20170:GLCURSOR (28,Z(1)+6):LPRINT "C
     PM=";AA(4,K)
20180:LINE (16,Z(2))-(216,Z(2)),2,2
20190:GLCURSOR (28,Z(2)+6):LPRINT "C
     PM=";INT (10^(LOG AA(4,K)+(LOG
     AA(4, J)-LOG AA(4,K))/2))
20200:GLCURSOR (8,28)
20210:RETURN
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2!" ":WAIT 00:PRINT "SPCIRA 1.0  C
  WN    24.10.83"
4!INPUT "Want to do only AP ? ";M0
  :CLS
6!IF M0="Y"THEN "INITIALIZE"
8!IF M0<>"N"THEN 2
10!GOSUB 'START'
20!GOSUB 'SP CPM'
30!GOSUB 'SP PLOT'
40!GOSUB 'SP LINREG'
50!GOSUB 'AP CPM'
60!GOSUB 'AP CALC'
70!GOSUB 'CYCLE'
80!END
1000!'START'
1010!CLEAR :USING :TEXT :COLOR 0:
     BEEP ON
1020!WAIT 00:PRINT "SPCIRA 1.0  CWN
     24.10.83"
1030!BEEP 1:INPUT "Enter SP#: ";K0
1040!BEEP 1:INPUT "For Is? (enter G
    ,M or A) ";L0:CLS
1042!BEEP 1:INPUT "total cpm added:
    ";L:CLS
1050!LPRINT "SPCIRA Is';L0;"  SP";
    K0
1060!GRAPH :LINE (152,10)-(210,35),
    0,3,B
1070!LINE (115,10)-(135,35),0,3,B:
    TEXT :CSIZE 1:COLOR 0
1080.LF 3:LPRINT "SPCIRA 1.0 Carl N
    ohr 24 10.83"
1090:LPRINT "TIME: ",TIME
1092:LPRINT "total cpm added= ";L
1100:DIM CC(2,47)
1110:DIM Q0(6)
1120:RETURN
2000!'SP CPM'
2010!DIM AA(4,11)
2020:FOR R=0TO 11
2030'READ AA(0,R)
2040:NEXT R
2050:DATA 200,150,100,75,50,37.5,25
    ,10.0,12.5,6.3,3.1,1.6
2060:WAIT 99:BEEP 1:PRINT "Enter CP
    M for xx ng: 1 2 3"
2070:FOR R=0TO 11
2080:CLS :PRINT AA(0,R),' ng:":BEEP
    1
2090:FOR C=1TO 3
2100:CURSOR (2+C*6)
2110:INPUT AA(C,R)
2112.REM READ AA(C,R)
2114:REM DATA 4533, 4003, 4052, 4305, 4
    764, 4696, 5253, 5254, 5232, 5745, 5
    880, 5616
2116 REM DATA 6588, 6469, 0, 6915, 6929
    , 6764, 7010, 7211, 7560, 8393, 8100
    , 8511
2118.REM DATA 8311, 8953, 9869, 10000,
    10600, 10091, 11002, 11615, 12097,
    13472, 13253, 13494
2120:NEXT C
2130:NEXT R
2140:CLS :CSIZE 1:LF 2
2150:LPRINT TAB 1;"NG Ig/25 ul",TAB
    13; LPM";TAB 23; CPM";TAB 33;"
    CPM"
2160.COLOR 1
2170:FOR X=0TO 11
2180:LPRINT USING "####.#";AA(0,X);
    TAB 10,USING "######";AA(1,X);
    TAB 20;AA(2,X);TAB 30;AA(3,X)
2190.NEXT X
2200:TAB 0:LF 2:COLOR 0
2210:LPRINT TAB 1;"NG Ig';TAB 12;"M
    EAN';TAB 23;'SEM";TAB 31;"% SE
    M"
2220:DIM BB(6):COLOR 1
2230:FOR R=0TO 11
2240:FOR C=1TO 3
2250:BB(C)=AA(C,R)
2260:NEXT C
2270'GOSUB 'MEAN SEM %"
2280:AA(4,R)=BB(4)
2290:LPRINT USING "####.#";AA(0,R);
    TAB 10,USING "######";AA(4,R);
    TAB 20;BB(5),TAB 30;BB(6)
2300:NEXT R
2310:USING :RETURN
3000: SP PLOT"
3010:LF 34:GRAPH :CSIZE 1:COLOR 0:
    GLCURSOR (10,0):SORGN
3020:LINE (-10,0)-(171,0)-(171, 300)
3030:COLOR 1:K=150-24*(AA(4,11)/AA(
    4,0)):REM K sets slope
3040:FOR C=0TO 11
3050:Y=LOG AA(0,C)*125
3060:GLCURSOR (170,Y):LPRINT "-";AA
    (0,C)
3070:FOR R=1TO 3
3080:X=(AA(4,11)-AA(R,C))/(AA(4,0)/
    K)
3090:GLCURSOR (X,Y):LPRINT "o"
3100:NEXT R
3110:NEXT C
3120:GLCURSOR (50,-10):LPRINT "CPM
    BOUND":GLCURSOR (0,-75)
3130:RETURN
4000!"SP LINREG"
4010!BEEP 1:INPUT "? Exclude a pt (
    Y or N) ";M0
4020:IF M0="N"THEN 4050
4030:IF M0<>"Y"THEN 4010
4040!BEEP 1.INPUT 'Drop data for NG
    = ";P
4050'CLS :A=0:B=0:D=0:E=0:F=0.N=12
4060!FOR C=0TO 11
4070:IF AA(0,C)=PTHEN LET N=N-1:
    GOTO 4150
4080:AA(0,C)=LOG AA(0,C)
4090:A=A+AA(0,C)
4100:B=B+AA(4,C)
4110:D=D+AA(0,C)*AA(4,C)
4120:E=E+AA(0,C)^2
4130:F=F+AA(4,C)^2
4140:GOTO 4160
4150:Y=LOG AA(0,C)*125:COLOR 3:
    GLCURSOR (172,Y):LPRINT "x"
4160!NEXT C
4170:G=(D-(A*B)/N)/(F-B^2/N)
4180:H=A/N-G*(B/N)
4190.R=(ABS (D-(A*B)/N))^2/((F-B^2/
    N)*(E-A^2/N))
4200.X=INT (100*H+.5)
4210!Y=INT (H/ABS G+.5)
4220.M=INT (G*10E7+.5)/10E7
4230!DIM Z(1)
4240!Z(0)=(AA(4,11)-(((LOG 1.6)-H)/
    G))/(AA(4,0)/K)
4250!Z(1)=(AA(4,11)-(((LOG 200)-H)/
    G))/(AA(4,0)/K)
4260!COLOR 3
4270'LINE (Z(0),25)-(Z(1),287)
4280.LINE (Z(0),0)-(Z(0),300),2,2
4290!GLCURSOR (Z(0)+10,120):ROTATE
    3:LPRINT 'CPM=',AA(4,11)
4300.LINE (Z(1),0)-(Z(1),300),2,2
4310!GLCURSOR (Z(1)-5,120):ROTATE 3
    :LPRINT 'CPM-";AA(4,0):ROTATE
    0
4320!GLCURSOR (-10,-30):COLOR 0
4330.LPRINT "LINEAR REGRESSION FORM
    ULA IS.'
4340!TEXT :CSIZE 1
4350!LF 1
4360 LPRINT "NG Ig/25ul= 10^(";M;
    "*LOG CPM + ";G;")"
4370.LF 1
4380 LPRINT 'R^2= ';R
4390'LPRINT 'M=  ';M
4400'LPRINT "X int= ';X
4410 LPRINT 'Y int= ';Y
4420 LPRINT
4430.LPRINT 'FOR INITIALIZATION USE
    '
4440'USING  ###.##############".
    LPRINT "M= ";G
4450.LPRINT "M= ";M
4460!USING
4470.RETURN
5000.'AP CPM'
5010'TEXT :LF 2'COLOR 0.GRAPH :LINE
    (0,0)-(250,0),5,0:TEXT :LF 2:
    USING
5020.BEEP 1:INPUT 'enter AP # ',O0
    :CLS
5030:BEEP 1.INPUT "enter culture vo
    l(ul)";V.CLS
5040!LPRINT "SPCIRA Is";L0;"  AP",
    O0
5050:GRAPH 'LINE (152,10)-(210,35),
    0,3,B
5060!LINE (115,10)-(135,35),0,3,0:
    TEXT .CSIZE 1'COLOR 0
5070!LF 2:LPRINT "Using SP";K0," w
    ith culture vol";V; "ul"
5080!LPRINT "SPCIRA 1.0 Carl Nohr 2
    4.10.83"
5090!LPRINT 'TIME:  ,TIME
5100.FOR R=0TO 47
5110!FOR C=0TO 1
5120!BEEP 1:WAIT 0:PRINT "enter CPM
    #";2*R+C+1
5130!CURSOR 16:INPUT CC(C,R):CLS
5131'REM READ CC(C,R)
5140!NEXT C
5150!NEXT R
5152!REM DATA 12640, 12043, 12559, 113
    42, 12100, 12120, 10000, 9511, 8584
    , 8156, 9073, 9099, 947
5153.REM DATA 9517, 9207, 8710, 7389, 7
    580, 6175, 5870, 5934, 6114, 6162, 6
    217, 4227, 4214, 4292
5154!REM DATA 4137, 4024, 4394, 4002, 4
    733, 4507, 4662, 4893, 4661, 7584, 8
    498, 7342
5155.REM DATA 7161, 5364, 5138, 7924, 7
    396, 6824, 6761, 6701, 6017, 8443, 9
    221, 7795, 7680, 7174
5156!REM DATA 7348, 8333, 8817, 9063, 9
    905, 9574, 8853, 10775, 11647, 9590
    , 9402, 9704, 10139, 10
5157!REM DATA 9883, 9922, 9817, 9584, 9
    299, 4121, 4110, 4102, 4251, 4072, 4
    233, 4683, 4488, 4341
5158!REM DATA 4328, 4265, 4838, 5248, 5
    907, 6525, 7447, 7225, 8410, 9297, 5
    647, 6321, 6249, 7538
5159!REM DATA 7028
5160!GOTO 60
6000!'AP CALC"
6010!BB(3)=B:LF 1:RESTORE 6200
6020:LPRINT "P WELL";TAB 10,'CPM';
    TAB 16;'CPM';TAB 23;'NG';TAB 2
    8;"SEM";TAB 32;"%SEM"
6030:GRAPH :SORGN :LINE (114,15)-(1
    14,-470):GLCURSOR (0,0):TEXT :
    CSIZE 1
6040:BB(1)="A":BB(2)="B":BB(3)="C":
    BB(4)="D":BB(5)="E":BB(6)="F":
    BB(7)="G"
6050:BB(8)="M"
6060:BB(1)=" 1, 2":BB(2)=" 3, 4"
6070:BB(3)=" 5 ,6":BB(4)=" 7, 8"
6080:BB(5)=" 9,10":BB(6)="11,12
6090.FOR R=0TO 47
6100!FOR C=0TO 1
6110!IF CC(C,R)=0LET BB(C+1)=0:GOTO
    6130
6120:BB(C+1)=(V/25)*10^(M*CC(C,R)+G
    )
6130!NEXT C
6140!GOSUB 'MEAN SEM %"
6150:CC(2,R)=BB(4)
6160:P=BB(1+INT R/8):LOCOR 1:Q=R-8
    *INT (R/8)+1
6170:LPRINT P#;BB(Q);TAB 7;USING "#
    #####";CC(0,R);CC(1,R);CC(2,N)
    ;BB(5);USING "#####";BB(6)
6180!NEXT R
6190!LF 2:COLOR 0
6200:LPRINT "WELLS";TAB 11;"MEAN";
    TAB 19; SEM";TAB 24; %SEM'
6210!GRAPH :RLINE (0,0)-(170,0):
    TEXT :LF 1:CSIZE 1
6220:FOR R=0TO 47STEP 3
6230:FOR C=0TO 2
6240:BB(C+1)=CC(2,R+L)
6250.NEXT C
6260!GOSUB 'MEAN SEM %'
6270!READ A$
6280!DATA 'A 1-6','A 7-12','B 1-6',
    'B 7-12','C 1-6', C 7-12', D 1
    -6','D 7-12'
6290!DATA "E 1-6","E 7-12", F 1-6',
    'F 7-12','G 1-6','G 7-12', M 1
    -6', M 7-12"
6300:LPRINT A$;TAB 10;USING '######
    ';BB(4);BB(5);BB(6)
6310!NEXT R
6320:IF R<47THEN 6170
6330:RETURN
7000.'CYCLE'
7010!LF 1
7020:BEEP 5:INPUT "do another AP (Y
    or N) ";M0!CLS
7030:IF M0="Y"THEN 50
7040:IF M0<>"N"THEN 7010
7050!RETURN
10000:'MEAN SEM %'
10010.N=3:A=0.A=0
10020:FOR X=1TO 3
10030:IF BB(X)=0LET N=N-1:GOTO 10060
10040!A=A+BB(0+X)
10050.A=A+BB(0+X)^2
10060.NEXT X
10070:IF N=1LET BB(4)=A GOTO 10110
10080:IF N=0LET BB(4)=0.GOTO 10110
10090:BB(4)=INT (A/N* 5)
10100:BB(5)=INT ((((ABS ((B-A^2/N)^2/(N
    -1)))/N)* 5)
10110:BB(6)=INT (1000*(BB(5)/BB(4)))*
    5)
10120:RETURN
10130'BB(5)=0:BB(6)=0
10140'RETURN
20000 'INITIALIZE'
20010!CLEAR :USING :TEXT :COLOR 0:
     BEEP ON
20030:INPUT "From SP# ",K0
20040:BEEP 1.INPUT 'For Is? (enter G
    ,M or A) ";L0.CLS
20050:INPUT "M= ";G
20060:INPUT "M= ";M:CLS
20070:DIM CC(2,47)
20080:DIM Q0(6)
20090:DIM BB(6)
20100:GOTO 50
```

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