CELLULAR AND SOLUBLE MEDIATORS OF DELAYED-TYPE HYPERSENSITIVITY AND THEIR USE IN CORRECTING ANERGY

by

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ABSTRACT

A chamber technique was developed allowing recovery of cytokines and T-cells from human DTH reactions. These cells were cloned, and were enriched, relative to the blood, for T-cells reactive to the antigen eliciting the DTH. Antigen-specific CD4+ clones, had two phenotypes; an IL-2/IFN-g producing group not providing help for Ig synthesis, and a group providing help but not producing IL-2 or IFN-g. Cytotoxicity and TNF production were found in both groups. TNF and IFN-g were present in the chambers over DTH reactions. Anergic patients demonstrated deficient mononuclear cell delivery to DTH sites which was restored to normal by co-injection of MLC supernatants with antigen. T-cell clones from such sites were similar to those from normal DTH sites. Additional experiments established that MLC supernatants contained 2 distinct factors, one restoring DTH in anergic patients, and another, which protected anergic rats from lethal peritonitis.

SOMMAIRE

Une technique en chambre a ete developpee pour la recuperation des cytokines et des cellules T à partir des reactions DTH humaines. Ces cellules ont ete clonees et enrichies par rapport au sang normal, car les cellules T reagissent aux antigenes associes aux reactions DTH. Les antigenes specifiques CD4+ clones ont deux phenotypes: un phenotype que produisait le IL-2 et IFN-g mais ne contribuant pas à la synthese de l'Ig, et un groupe contribuant à la synthese de l'Ig, mais ne produisant pas de IL-2 ni de IFN-g. Une cytotoxicite et une production de TNF a ete constatee pour les deux groupes. On a constate la presence de TNF et de IFN-g dans la chambre apres les reactions DTH. Dans le cas des patients anergies, on a constate une production deficiente de cellules mononuclees au site de la reaction DTH. Cette production a pu etre restauree a son niveau normal par l'injection conjointe de surnageant de culture mixte de lymphocytes (MLC) avec l'antigene. Les cellules T clonees à partir de ces endroites etaient identiques à celles produites à des sites DTH normaux. Des experiences supplementaires ont permis d'etablir que le surnageant de MLC offrait deux facteurs distincts: un premier restaurant le DTH pour les patients anergies et un autre qui protegeait les rats anergies des peritonites mortelles.

Original Contributions to Knowledge

1) A method was developed for obtaining the cells and mediators directly from human DTH reaction sites without contamination by circulating cells. This method, the "Sombrero" Skin Window Chamber technique, is demonstrated to provide a faithful, quantitative reflection of the underlying DTH reaction. The method is reliable and easy to perform, with no observable negative side effects.

2) The T-cells involved in DTH reactions have until now been poorly characterized, largely as a result of their inacessability. While many functions have been attributed to these cells, largely on the basis of *in vitro* data from circulating cells, direct evidence of their characteristics has not been available. In this study, the cells from skin window chambers over DTH sites were cloned using either PHA or the Ag eliciting the underlying DTH reaction. TDTH cells were found to consist of both CD4+ AND CD8+ cells. They were enriched, relative to the blood, for cells specific for the antigen(Ag) eliciting the DTH reaction.

3) The functional capacities of Ag-derived TDTH clones were determined. Cytotoxic activity and TNF production were found among both CD4+ and CD8+ clones, as was IFN-g production. IL-2 production was limited to CD4+ clones.

4) IL-2 & IFN-g production were linked among CD4+ clones, both those from the blood and TDTH clones specific for the eliciting Ag. There was an inverse relationship between the capacity to produce IL-2 or IL-2 & IFN-g and the ability to provide help for Ig synthesis. While a similar observation has been made among murine T-cells, this is the first evidence of such a dichotomy in human TDTH cells. Evidence is also presented to suggest that the capacity to provide B-cell help was inversely related to the production of IFN-g, and that stimulation by different reagents results in different lymphokine production profiles of individual clones.

Original Contributions to Knowledge cont'

5) CD8+ clones from DTH sites specific for the eliciting Ag are largely cytotoxic cells. A small number of these display NK-like cytotoxicity. The majority of specific CD8+ TDTH clones also produce IFN-g.

6) TNF and IFN-g were both detected in high levels in the fluid from skin window chambers when the chambers were above DTH reaction sites. This represents the first direct evidence of the production of these cytokines at DTH sites.

Taken together, the above information indicates that there is a functional dichotomy in human CD4+ TDTH cells, with one group producing IL-2 and IFN-g and not providing help for Ig synthesis, while the other fails to produce these cytokines but does provide help for Ig synthesis. Cytotoxicity and TNF production are found in both groups, and also in CD8+ cells, which also produce IFN-g. These groups of cells are also present in the circulation. The cells at human DTH reactions are enriched for cells reactive to the eliciting Ag, and produce detectable quantities of TNF and IFN-g.

7) Using the skin window chambers, anergy to recall skin test Ag in hospital patients is demonstrated to be reflected in an abnormally low mononuclear cell delivery to skin test sites. Mononuclear cell delivery is also abnormally low when an antigen to which the patients are not sensitive is injected.

8) The supernatants from mixed lymphocyte cultures (MLC) have been previously demonstrated to restore, locally, an observable DTH-like reaction in anergic patients if co-injected along with an Ag to which the patient is sensitized. This restoration was reflected in a significant increase in the number of mononuclear cells delivered to such sites. This increase results in normal levels of cell delivery. Hospitalized controls also demonstrate improved cell delivery, albeit their response to the co-injection of MLC supernatants is much lower than that seen in anergic patients. The restoration of normal levels of cell delivery in anergics, and the improvement seen in reactive controls, is dependent upon a prior sensitivity to Ag.

Original Contributions to Knowledge cont'

Evidence is also presented that suggests that the anergic patients have a defective response to non-specific inflammatory mediators.

9) The nature of the DTH-like reaction seen in sensitized anergics co-injected with Ag + MLC supernatant, and its relationship to normal DTH, was probed by examining the functional capacities of T-cell clones from these sites. As with normal DTH reactions, the cells from these sites are enriched, relative to the blood, for cells specific for the eliciting Ag. These cells were cloned and found to consist of both CD4+ and CD8+ cells, and to produce IL-2, TNF and IFN-g. A few also gave help for Ig synthesis. Overall these cells are indistinguishable from those of normal DTH reactions. This represents the first evidence that the restored reaction seen in anergics co-injected with Ag + MLC supernatants, is comparable to normal DTH.

10) The ability of MLC supernatants, when co-injected with Ag, to restore normal levels of cell delivery in anergics, and the normal functional capacities of the delivered cells, led to the investigation of their ability to afford protection against infecting organisms. A model of peritonitis was established using anergic rats. With this model it was demonstrated that MLC supernatants confer protection against a lethal bacterial peritonitis in anergic animals.

11) The factor that confers protection against lethal peritonitis in anergic animals is present in high levels in human MLC supernatants. It has a molecular weight less than 10kD, and is produced as a result of cellular activation. Evidence is presented that implies that the factor does not afford protection by attracting/activating PMN. This factor was shown to be distinct from the factor(s) in MLC supernatants that restore DTH, which has a molecular weight of >50kD.

Taken together, the results show that anergy in hospital patients is reflected in a decreased delivery of mononuclear cells to DTH sites, which can be corrected by the co-injection with Ag of a factor or factors of MW > 50kD, present in MLC

Original Contributions to Knowledge cont'

supernatants. The resulting response is a true DTH reaction. A second factor with MW < 10kD, also present in human MLC supernatants, can afford protection from lethal infection in anergic animals.

PREFACE

The experimental work for this thesis was done while the candidate was under the supervision of Dr. H. Rode in the Dept. of Surgery, McGill University. All work contained in the thesis is that of the candidate. The skin window chamber technique was derived from the method of Morris *et. al.* (134). The neccessary modifications to the chamber were designed and carried out by the candidate.

The screening of hospital patients and skin testing of all subjects was carried out by several research nurses attached to the research group of which the candidate is a part. They were: Mary Broadhead RN, Lise Laporte RN, Louise Chartrand RN and Mary DeSantis RN. Normal controls were obtained and screened by the candidate.

The mononuclear cell culture methods used were either modified or developed by the candidate. All cell culture work, with the exceptions noted below, were carried out by the candidate. Some PBM isolations from blood were performed by Mrs. Magda Jass, under the supervision of the candidate. All cytokine preparations were prepared and fractionated by the candidate. B-cell help cultures and Ig production assays were performed by Mrs. Manon Blain and Ms. Doris Hellstern, under the supervision of the candidate. Assays for TNF activity were performed by Dr. Leopoldo Sanchez-Cantu, under the supervision of the candidate. All other functional and LK assays were carried out by the candidate.

Bacteria used in the study were grown by Ms. Betty Giannias, under the supervision of the candidate. Zone of inhibition and bacterial cytotoxicity studies were carried out by the candidate. The animal model of peritonitis in anergy was developed by the candidate from separate models as described in chapter 6. All experiments involving animals were designed and carried out by the candidate. Various individuals assisted in carrying out these studies, they were: Dr. John Marshall, Dr. Juan-Carlos Puyana, Dr. Ihor Zakaluzny, Dr. Leopoldo Sanchez-Cantu, Mrs. Dale Eshelly, and Ms. Corrinne Lacey.

PREFACE cont'

Computer analysis, calculations, database use and statistical analysis were performed by the candidate using commercial software. The thesis was prepared by the candidate.

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I would like to aknowledge my gratitiude for the contributitions of the following individuals: Dr H. Rode, whose patience, encouragement, and the example he set for me were absolutely essential to my development and aquisition of the skills of a scientist; to Dr. J. Gordon, whose wisdom, encouragement and creativity have been helpful and an inspiration throughout my research, and to Dr. R. Bissonette, who as "big-brother" and confidante taught me a great deal about the design of practical experiments and survival amidst the rigors and frustrations of graduate school.

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Finally, I would like to express my admiration and gratitude to the many patients I worked with at the Royal Victoria Hospital. Faced with many important and even devastating problems, these people were willing to take the time and inconvenience to indulge a young man's curiosity, at some risk and with no benefit to themselves, in order to aid others, and the pursuit of knowledge. They may serve as an example, and an inspiration to us all. This thesis is dedicated to them.

Abbreviations used in the Thesis*

Ab:	antibody
ADCC:	antibody-dependent cellular cytotoxicity
Ag:	antigen
Ag+'ve:	antigen reactive, ie a subject positive either *in vivo* (by skin test) or *in vitro* (by LTT) - eg. PPD+'ve HA = an anergic hospital patient reactive *in vitro* to PPD
APC:	antigen presenting cell
ATCC:	American Type Culture Collection
Anergic(s):	When capitalized, this refers to anergic surgical patients
C3b:	complement component 3b
CAN:	Candida antigen
CD:	clusters of differentiation
CDX+:	denotes a clone/cell preparation expressing the CD antigen X
CFA:	complete Freunds's adjuvant
CK:	cytokine(s), refers in this thesis specificaly to the cytokine preparations from mixed lymphocyte cultures used to modify *in vivo*: 1) the skin test response of patients, or rats 2) the response of rats to infection
CMI:	cell mediated immunity. Includes but is not limited to DTH.
CMV:	cytomegalo virus
CPM:	counts per minute
Cr:	chromium
CS:	contact sensitivity, a form of DTH to soluble, reactive chemicals
dH2O:	distilled water
ddH2O:	double-distilled water
DNCB:	di-nitro-chloro-benzene, commonly used contact sensitizer
DTH:	delayed-type hypersensitivity
EBV:	Epstein-Barr virus
ELISA:	enzyme-linked immunosorbant assay
ETAF:	epidermal-derived thymocyte activating factor
FACS:	flourescence activated cell sorter

Abbreviations used in the Thesis cont'

GM-CSF:	granulocyte/monocyte-colony stimulating factor
GVH:	graft vs. host disease
3H:	tritium
HA:	hospitalized anergic patient(s)
HBSS:	Hanks balanced salt solution
HIV:	human immunodeficiency virus
HLA-XX:	human leukocyte antigen XX
HR:	hospitalized reactive patient(s)
hr(s):	hour(s)
Ia:	immune associated Ag
i.d.:	intradermal
IFN-x:	interferon-x, x may be: a (alpha), b (beta), g (gamma)
Ig:	immunoglobulin
IL-X:	interleukin-X
IP-10:	IFN-g induced protein found in monocytes and keratinocytes
K:T:	killer to target cell ratio
KLH:	keyhole limpet haemocyanin
LDCC:	lectin dependent cellular cytotoxicity
LIF:	leukocyte migration inhibition factor
LK:	lymphokine(s)
LTT:	lymphocyte transformation test
MBP:	myelin basic protein
MIF:	migration inhibition factor
MLC:	mixed lymphocyte culture(s)
MoAb:	monoclonal antibody(s)
MPCA:	monocyte procoagulant activity
MPIF:	monocyte procoagulant activity inducing factor
MTT:	(3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide)
Ni:	nickel
NK:	natural killer(cell)
O.D.:	optical density
PAP:	peroxidase-anti-peroxidase (antibody) complex
PBM:	peripheral blood mononuclear cell(s)

Abbreviations used in the Thesis cont'

PGEx:	prostaglandin-Ex
PHA:	phytohaemagglutinin
PMN:	polymorphonuclear cell(s)
PPD:	purified protein derivative of Mycobacterium tuberculosis
PWM:	pokeweed mitogen
RIA:	radioimmunoassay
RPMI:	Roswell Park Memorial Institute medium 1640
SDC:	single donor culture
SE:	standard error of the mean
STS:	skin test score ie. the sum of the diameters of the skin test responses
SW:	skin window(chamber)(s)
Txxx:	T-cell; participating in xxx or originating from an xxx site, or belonging to the functional group xxx. eg. TDTH: T-cell participating in DTH or originating from a DTH site.
TH1:	T-Helper cell, type 1.
TET:	tetanus toxoid antigen
TNF:	tumour necrosis factor(s). Refers to both TNF-a & TNF-β unless specified
TPA:	13-o-tetradecanoylphorbol -12-acetate
U:	units
V-V(+):	*Vicia villosa*(adherent)
ZAS:	zymosan activated serum

*: letters or words in parentheses may be included or implied by the context in which the abbreviation is used. Some commonly accepted abbreviation may not be listed.

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Chapter 1: Cell Mediated Immunity and delayed-type hypersensitivity: an historical perspective

"Every medical man must learn the art of drawing the best conclusions he can from data that are often incomplete and sometimes highly confusing. He will find immunity an excellent training ground."

W. W. C. Topley

Vaccination probably originated in asia. From Constantinople, vaccination against smallpox was introduced to England in 1718 by Lady Mary Montagu (1). There followed the development of cowpox vaccination against smallpox by Benjamin Jesty and the subsequent investigation of the phenomenon, and popularization of the practice, by Jenner towards the end of the century (1). Jenner's investigation was thorough and scientific, and included a description of what we now recognise as DTH (2). These events, preceeding as they did the Germ theory of disease, were not fully exploited as the stepping-stones to further knowledge until almost a century later.

Immunology as a science had its origins in the search for effective vaccines by european microbiologists, notably Pasteur and Koch, in the 1870's and 80's (3). Following the establishment of the Germ theory of disease, the scientific investigation of how the body reacts to infectious organisms, and what could be done to augment that response, began in ernest. Metchnikof's phagocytic theory of immunity was introduced in 1883 (3) and while *in vivo* phagocytosis is often the result of co-operation between the cellular and humoural arms of the immune system, it can be argued that this represented the first concept of the cellular basis of immunity.

Following his discovery of the causative microrganism of tuberculosis (1), it was Koch who first noticed the unusual aspects of the reaction of previously infected Guinea pigs to intradermal injection of tubercule bacteria. This reaction was notable for its slow rate of evolution (relative to other skin reactions), requiring 24 hrs for visible indications of a

response, and thereafter the rapid necrosis and sloughing off of dead tissue from the site. He also noted that while this reaction was slower than some other skin responses it was dramatically more rapid and energetic than seen in the skin in the first encounter with the bacteria, which took 10-14 days to become noticeable (4). Thus Koch had described the two essential characteristics of CMI, a hypersensitivity (resulting in tissue destruction), and the anamnestic (immune) recall response.

The concept of hypersensitivity was more formally developed by 1906 when Von Pirquet introduced the term "allergy" (5). This term has come to refer exclusively to the swift reactions mediated by IgE antibodies, but it was then applied generally to all visible forms of immune reactivity. Although the difference that underlay antibody and cellular mediated skin reactions was not then known, Zinsser recognised the tuberculin-type reaction as distinct from the other more rapid skin reactions (6).

The fundamental discovery by Landsteiner and Chase (3) and Chase (6) that the DTH skin reaction was due to the action of cells, later discovered to be lymphocytes, and not by serum elements lead to a rebirth of work in the field of cellular immunology (3) which had existed to some extent in the shadow of serology for several decades. The predominant role of lymphocytes in the transfer of CMI was extended to show that it is the T and not the B lymphocytes that mediate this response, and that the transferred T-cells must be specific for the antigen used in the skin test (7).

As noted by Koch, a primary sensitizing exposure to antigen is necessary to produce the secondary anamnestic response which results in the typical visible signs of DTH. Up to this time, most of the information regarding CMI was derived from histological studies and adoptive transfer experiments (5). The role of the antigen specific lymphocytes in the secondary response was uncertain.

How the sensitized cells directed this response was unknown until the discovery that T-cells released soluble mediators that directed the action of other cells. The first of what came to be known as lymphokines was the blastogenic factor described by

Gordon and McLean (8) and Kasakura and Lowenstein (9), which later became known as IL-2. Since then the number of soluble mediators released by lymphocytes and monocytes has risen to well over 100 (6). The study of these factors, the cells that release them and their effects upon target cells has represented much of the recent work in this field and has led to an understanding of many of the mechanisms involved in DTH.

Using immunohistological staining of CMI lesions, and the adoptive transfer of sensitized cells into naive recipients the T-cells responsible for directing CMI were generally found to be CD3+,CD4+,CD8- (10) however cells with other phenotypes have also been shown to be involved (11).

The role of CMI in the response to infection has been demonstrated in both human and animal diseases. In animals, numerous infections, usually by obligate or facultative intracellular organisms, were shown to be controlled primarily by CMI responses, among the most important from an experimental viewpoint were: tuberculosis, salmonella, listeria and more recently leishmania (12,13). In humans, immunity to many organisms was found to depend upon CMI, including tuberculosis, Mycobacterium leprae, Candida, leishmania and numerous others, including many viruses (5,13,14).

In the 1950's and 60's, the observation of patients born with congenital T-cell deficiencies further demonstrated the essential nature of CMI responses (15). More recently, aquired T-cell immunodeficiencies such as anergy in surgical patients and the immunodeficiency resulting from infection with HIV-I have also confirmed that T-cell directed CMI responses are critical in maintaining freedom from infection (2).

The advent of tissue transplantation in the 1960's, both as experimental model and theraputic treatment, resulted in the confirmation of the dominant role of CMI responses in both the graft rejection (15,16) and the graft vs host reaction (7). CMI was also shown to play a role in the immune reaction to neoplasms (5), and the importance of immunoregulation of CMI *in vivo* has been demonstrated by the discovery of the role of self-destructive CMI reactions in both infectious diseases such

as leprosy, and autoimmune diseases such as rheumatoid arthritis and multiple sclerosis (15).

The study of CMI, which commenced at the very beginnings of the science of immunology, continues to this day. It has been of great value in understanding many aspects of immunity and will no doubt continue to be so. Early studies were primarily histological and phenomenological. These gave way to more precise histological methods, and to the techniques of adoptive transfer and *in vitro* cell culture. Yet unlike the immune responses which take place in dedicated tissues (spleen, lymph node, thymus etc.) and those that release their soluble mediators into the blood, the functional capacities of the cells responsible for the secondary responses of most forms of CMI have not been studied directly owing to the difficult task of extracting these cells from their reaction sites without altering them or "contaminating" them with cells from the peripheral circulation. Initially this inability was of little consequence, however the recent breakdown of classical functional categories such as "helper" and "suppressor" cells into many smaller and more specific groupings, such as cells that secrete particular CK or "induce" or "transduce" functional activities in other cells, and the realization that the immune system may be compartmentalized into heterologous systems based in different "home" sites such as spleen, gut, and skin makes an examination of pure populations of these cells at the clonal level an important objective.

Forms of Cell Mediated Immunity

CMI is a complex array of different immune responses. They differ in both the primary and secondary effector cells that dominate the reactions (5) and to some extent, the sites in which the reactions occur, and their duration. Among the reactions considered to be mediated in whole or in part by CMI are some anti-tumor responses (7,17), allograft rejection (18,19), the graft vs host reaction (7,20), destruction of virally-infected cells (21,22,23), certain autoimmune diseases (24,25), and responses against some bacteria, primarily

intracellular pathogens (5,7). Many of these responses have features that share processes or components with DTH, and thus, evidence regarding the mechanisms of these forms of CMI has often been assumed to apply also to DTH which has been considered the prototypic reaction for most forms of CMI.

The generation of sensitized cells is required for the subsequent demonstration of DTH. The majority of this thesis focuses on the recall component of DTH and I will treat it as a separate entity, of which the primary reflection is the tuberculin type skin reaction. Analogies to other CMI responses will be presented as they were drawn historically, or when they serve to reinforce or illustrate the workings of DTH.

DTH

This reaction was originally defined by the slowness of its visible appearance, as compared to the swifter skin responses of the Ab-mediated reactions. Thus the name delayed-type hypersensitivity represents an operational definition, which cannot alone encompass all the forms of immune response now grouped under this term.

DTH is characterized by the infiltration of T-cells and monocytes at the site of Ag deposition. DTH reactions in the skin occur in two forms, both of which may occur naturally. The tuberculin type reaction may be induced by infection (4), non-infectious exposure (so called "natural immunity") or other as yet undefined events leading to the DTH-mediated autoimmune reactions described above. Contact sensitivity may arise to such environmental hapten antigens as nickle and various plant oils, rubber and various drugs (26). Contact sensitization in both animal models and in man is readily accomplished by either topical application of the sensitizer, usually suspended in a neutral oil or other irritant, or by intradermal injection of the agent (27). The induction of tuberculin-type DTH is more difficult. The majority of protein antigens, if injected in saline alone will give rise to antibodies which can interfere with the expression of DTH (5) or give rise to the more ephemeral Basophil dependent reactions described below (12,28).

Several strategies have been developed to generate long-lasting tuberculin-type DTH responses. Immunization with CFA is often effective, although in some cases this may also give rise to antibodies that produce an Arthus reaction that interferes with the expression of DTH. This may be avoided by conducting the subsequent DTH tests in the period prior to the appearance of circulating antibody (29), by the use of highly conjugated carriers of weak immunogenicity or by subsequent boosting 5-12 days after the priming exposure to antigen (5).

The DTH reaction is directed by Ag-specific T-cells (5). The major secondary effector cell is the macrophage (5), while cytotoxic T-cells may also be present (7) and may dominate the response, particularly those against viruses, allo-antigens and contact sensitizers (30).

When the reaction is provoked in the skin and directed against soluble or particulate antigens it is usually referred to as a "tuberculin-like" reaction (5). When the antigen used is a reactive hapten, painted on the skin the resulting response is termed "contact sensitivity" (5). Although this reaction is provoked by a special type of antigen (31) and may involve a different APC than the tuberculin-type reaction (32), the two are often treated as a single entity (5).

The experimental provocation of DTH typically induces a reaction in which the inflammatory component is first notable by 12-24 hrs and lasts no longer than 96 hrs (5). When induced instead by persistent Ag, such as those produced by viable organisms or cells, the longer duration of the reaction and the various other events that take place in infection can influence the immune reactions and the DTH response may have many different appearances which do not appear to meet the older operationally defined criteria for this response.

In these cases the responses are considered to be DTH reactions by virtue of their dependence upon Ag-specific T-cells and the predominance of the resulting lesions by macrophages and/or cytotoxic T-cells (5). Thus allograft rejection (7), some anti-tumor responses (33), anti-viral responses (7) and many anti-bacterial reactions, including immune granulomas (7) are

considered to be DTH reactions.

Other DTH-like responses

CMI reactions which do not involve macrophages and/or Tc as secondary effectors are also known. The early phase of CMI responses in the Guinea pig (34) and the Rabbit (5) for example are dominated by PMN which may decrease or increase in proportion depending upon the degree of ongoing tissue destruction (5).

Hypersensitivity responses which involve basophils as the secondary effector cells are known as Jones-Mote reactivity (28) or Cutaneous Basophil hypersensitivity (35). This reaction can be induced experimentally by immunization with protein antigens in incomplete Freund's adjuvant (36) and is at least partially mediated by antibody (36) and possibly suppressor cells (37). This form of CMI is believed to play an important role in the response to parasites, notably blood-sucking arthropods in the skin and helminth parasites in the gut (7). Eosinophils play an important role as secondary cells in T-cell mediated responses to many parasites notably *Schistosoma* and *Trichinella* (7).

T-cells may also interact with B-cells to produce germinal centers at sites of Ag deposition to result in Ab formation *in situ* (7) which may give rise to an ADCC-directed lesion closely mimicking a classic granuloma in gross appearance (7). Still other T-cell directed lesions may be produced as a result of the effects of LK released by T-cells at the site upregulating normal functions of bystander cells. T-cell induced hyperactivity by goblet cells resulting in lesion formation has been reported (7), and the GVH lesion found in spleen and bone marrow is partially attributed to the effects of LK from the graft-derived Tcmi cells upon host hematopoietic cells (7). NK cells may act as either primary or secondary participants in CMI (7). They are able to both produce and respond to many of the LK involved in T-cell mediated CMI (38). They appear to play an important role in anti-tumor responses, and also in some parasitic and bacterial infections (17). NK cells may be responsible for the rejection of parental bone marrow grafts in F1 recipients (19) and some anti-viral responses (22).

Thus CMI responses are a diverse group of reactions of which those directed by Ag-specific T-cells are a subset, and DTH reactions a subset of those Tcmi responses.

Quantitation and Histology: a cautionary tale

Histological studies were responsible for much knowledge regarding DTH (5). These efforts were valuable in determining or confirming the cells involved in DTH, their activation states and Ag specificity (5) and their importance is not to be minimized. However, with the evolution of a more quantitative outlook in immunology it is worthwhile to take a second look at these studies and the conclusion drawn from them.

In histological studies of DTH, often no mention is made of results from normal skin (39-43), or normal skin is represented by Ag-injected sites of unimmunized subjects (44). When "normal" skin is mentioned it is often dismissed as having "few cells" (45) or "small numbers" (46,47) of lymphocytes present, or contrasted to DTH reactions as in Platt *et al* (44) in which "small perivascular mononuclear cell clusters" were observed in control biopsies but dismissed by comparison with the "more exuberant infiltration" seen in responder biopsies.

Detailed histological studies of DTH often report qualitative measures such as a "+, +++, etc" scoring scheme (42) or at best, marginally quantitative measures such as the percentages of cells within a given feature (such as perivascular clusters) (43,44,47). Such measures have no scalar reference. These percentages vary widely from author to author, to the extent of disagreement over the presence (44) or absence (47) of cell subsets (CD8+ cells) or even lymphocytes in general (44,48) in the epidermis. This type of report must be seen as essentially qualitative and only marginally quantitative despite reporting percentages of cell types.

These shortcomings were acceptable at the time the studies were performed. Histological studies are labor-intensive. To match the scales of the samples to the magnitude of the lymphocyte presence in "normal" skin would have required a substantial increase in the number and size of samples prepared

and scanned. Since there was little evidence for a dynamic, homeostatic regulation of the skin immune system until recently, there was little incentive to scale up to the work required.

As early as 1949 Andrews and Andrews reported that lymphocytes made up as much as 1-4% of the cells in the basal layer of normal skin (44). Thus there would seem to be reason to consider a role for lymphocytes in the skin in its normal homeostatic function(s) and/or the early components of the DTH reaction.

In recent years such studies have been performed. Using large numbers of samples, and external linear scales, a better appreciation of the events in normal skin has come to light. Bos *et al* conducted such a study using 24 samples from six different sites of different subjects (49). They express their results from vertical sections in terms of cells/10mm horizontal line, with the actual cell counts and length of the horizontal line scanned given in terms of mean, median and range. While the range is still large, their results indicate that there are in the order of 105 T-cells/cm2 in normal skin. The presence of this number of cells would open the possibility of a significant participation in DTH reactions by the T-cells already present in the skin, prior to Ag-deposition.

Such a possibility would not have seemed reasonable in the light of previous reports of "few cells" etc. present in such skin. Qualitative histological studies are of great value in illuminating the DTH reaction, but they should not be over-interpreted, particularly when judging the significance of cells or events that are infrequently observed.

Cells Involved in the DTH Reaction

Histological description of DTH

Early histological studies first identified the infiltrate in recall DTH reactions as being composed of mononuclear cells (5). This influx was resolved by histochemical analysis into both lymphocytes and monocytes (39). The lymphocytes were later shown by immunohistochemistry to be T-cells, both CD4+ and CD8+ (5). The application of detailed immunohistochemical studies to the analysis of the cells infiltrating DTH reactions has permitted the description of the reaction given below.

The early events in DTH are a generalised vasodilation and edema (5). Within 4-6 hrs of the injection of Ag, perivascular infiltrates of leukocytes are evident (34). By 6 hrs, mononuclear cells come to predominate the infiltrates while some basophils may be present, even in man in response to Ag such as PPD (5). The mononuclear cells are clustered perivascularly (44) and consist largely of T-cells with a pronounced enrichment for CD4+ cells relative to the blood (44). Approximately 1% of the cells are Leu 7+. One third of the cells are monocytes with the bulk of the remainder being T-cells (39,47) of which very few express activation markers. Interestingly, these same events take place whether the subject is sensitized to the antigen or not (44,50).

After 12 hrs, mononuclear cells dominate the perivascular regions. Resident macrophages which originally made up the bulk of the macrophage population have by this point been joined by an equal number of monocytes from the circulation. The majority of these new monocytes are of the interdigitating type and express HLA-DR. Twenty percent of the remaining monocytes also appear to be activated and express both HLA-DR and C3b receptors. The CD4+:CD8+ ratio decreases to levels only slightly greater than those found in the blood (47). A doubling in the number of Langerhans cells is seen as are small numbers of T-cells, scattered throughout the dermal interstitium (47).

At 18 hrs there is a slight increase in the number of mononuclear cells in the perivascular regions expressing Leu 7. The number of T-cell expressing the activation antigens T9 and CD25 (Tac) increases. The decrease in the perivascular CD4:CD8 ratio continues but the infiltrate remains slightly enriched for CD4+ cells relative to the blood (44). Small numbers of T-cells are found in the interstitium. A weak expression of T9 is also seen on keratinocytes.

In non-responders the CD4:CD8 ratio remains fixed at 1. T-cells and infiltrating macrophages are rarely seen in the interstitium, and very few of these express activation markers (47). The reaction sites of non-responders do not progress beyond this appearance and the mononuclear influx will gradually diminish (47).

After 24 hrs the infiltrate is increased. Perivascular regions show little change in cellular composition, but the numbers of all cell types increases, the one exception being the relative proportions of monocyte types. There is an increase in the proportion of activated macrophages and a correspondingly lower contribution by both resident and interdigitating macrophages (47). The expression by T-cells of activation antigens, particularly T9, increases (44). A modest increase in the numbers of Langerhans cells in the dermis occurs, while in the epidermis they are variously reported to increase (47) or decrease (26,31,51) by small amounts. The extent of interstitial infiltration of both T-cells and macrophages increases (5). The majority of infiltrating macrophages are of the activated type, expressing both HLA-DR and C3b receptors (44,47). The CD4:CD8 ratio returns to the ratio seen in the blood (44,47). Occasional Leu 7+ mononuclear cells and T9+ keratinocytes are found (47).

T-cells are found in the epidermis for the first time, and they are found in close association with Langerhans cells (47). These T-cells have been reported to be either both CD4+ and CD8+ (44) or exclusively CD4+ (47).

After 48 hrs the infiltration of all areas of the skin by T-cells and of all areas except the epidermis by macrophages is seen (39,33,37,51). The proportion of activated macrophages in

the perivascular regions reaches its peak (approx. 80%) and macrophages of the resident and interdigitating types are infrequent (47). The numbers of Leu 7+ cells in the perivascular region increase to a peak value of approx 3% (44). Langerhans cell numbers remain constant in all areas (44,47,51). There is a dramatic increase in the proportion of T-cells expressing activation antigens, including CD25 and also HLA-DR(in a smaller proportion of the T-cells). The T-cells in the epidermis appear to contain a greater proportion of activated cells than the other regions (44). Keratinocytes begin to express HLA-DR (44,51) and to proliferate, resulting in epidermal thickening with Langerhans cells concentrated in the upper regions.

By 72 hrs the lymphocyte and macrophage infiltrates begin to wane (5,47). Keratinocyte proliferation and HLA-DR expression continue and may increase (44,51). Langerhans cell numbers double in the dermis in the period from 48-72 hrs (51) while their numbers in the epidermis drop precipitously as the upper epidermal regions are shed (51), or may remain constant (47), presumably to be shed somewhat later.

Over the next 10 days the numbers and distribution of T-cells and macrophages decreases and may return to normal or remain slightly elevated (52). Langerhans cells gradually decrease in the dermis as they appear to migrate upwards and repopulate the epidermis (51). Induration and edema decrease rapidly and the reaction grossly returns to normal (44,51). Within the tissue, CD4+ cells continue to dominate the infiltrate (52). HLA-DR but not DQ expression by keratinocytes increases from days 4-17, particularly in small focal patches (52). While a T-cell influx in all areas may remain for up to 45 days or more (52), the infiltrates eventually decrease (5,51,52) and the fixed elements of the skin return to normal.

Evidence from other techniques

Much has been learned about the functional capacities of the cells involved in DTH by the employment of the adoptive transfer technique. Indeed the original demonstration by Landsteiner and Chase that DTH is a cell mediated reaction was carried out using

this technique. By depleting and/or enriching T-cell populations from antigen sensitized animals that show DTH responsiveness, and subsequently transferring these cells into naive recipients, their involvement in the recall component of DTH has been determined. In this way it has been shown that the cells that mediate DTH reactions to soluble and allo-antigens are CD3+, CD4+, L3T4+, CD8-, Ia+ (10,33,53-58). While these cells may suffice to produce the Ag-specific inflammatory component of DTH, the full complement of reactions involved in the CMI response to many intracellular pathogens requires both CD4+ and CD8+ Ag specific cells (59-65) and both cells are sometimes required for the formation of immune granulomas (62). Cytotoxic cells of both NK (64) and T-cell (65) lineage may also be required for the full development of DTH-driven CMI effector function to some pathogens, particularly viruses (23).

The Role of Ag-specific cells in DTH

The involvement *in situ* of Ag-specific T-cells was first demonstrated by Najarian and Feldman (66). They and other groups subsequently used 3H-Thymidine labelling in donor animals during the sensitizing process (67,68) to generate labelled Ag-specific cells which were transferred to naive recipients. The recipients were then skin tested and autoradiography used to identify the labelled cells in DTH lesions. These studies established that only a very small proportion (<1%) of the cells present at DTH sites are specific for the Ag used to elicit the reaction (5).

This experimental design results in very few positive (labelled cells) being detected in the lesions. To eliminate this and other design limitations, McCluskey *et. al.* carried out an elegant series of experiments (69). In the first, the recipient animal was labelled sytemically with 3H-Thymidine prior to the adoptive transfer. In subsequent skin tests, approximately 90% of the cells in the lesion were labelled. In the second experiment, unlabelled animals were passively immunized with cells from two different donors, one of which had been labelled. The donors were sensitized to two different antigens. Regardless of which set of cells were labelled, or

which Ag was used to skin test the animal, between 0.8-8% of the cells at the site were labelled. The percentage of labelled cells was consistently higher in the site tested with the Ag used to generate the labelled cells, but the increase was slight. These results implied that there was a small Ag-specific infiltrate, but that the transferred cells also accumulated non-specifically at the skin test sites. This was confirmed in the third series of experiments in which animals were actively immunized. After 7-9 days, at which time the Ag-specific cells were presumed to have ceased proliferating, the animals were labelled with 3H-Thymidine for several days and then skin tested. The resulting lesions consisted almost entirely of labelled cells.

The interpretation of these experiments was that the majority (greater than 90%) of mononuclear cells present at DTH lesions are not Ag-specific, but rather are proliferating cells of irrelevant specificity from the circulation that accumulate at such sites non-specifically (69,70).

These early studies reported little evidence for the accumulation or enrichment of Ag-reactive cells at DTH sites, relative to the blood. Very low numbers of Ag-specific cells were enumerated, making this observation suspect. Interestingly, in some of the early studies described above, it was noted that using the contact sensitizer DNCB, the proportion of Ag-specific cells seen in the epidermis was 5 to 20-fold greater than in the lesion as a whole (67,68). A recent study (71) has reported a marked Ag-specific accumulation of hapten-specific lymph node cells at sites of CS reactions when sufficiently high concentrations of hapten were used. The authors attributed the requirement for higher doses of sensitizer to the impaired ability of their cultured Ag-specific cell lines to traffic into skin and lymph node sites, a phenomenon also reported by others (72,73).

One of the first attempts to directly quantitate the numbers of Ag-specific cells present at human DTH sites, has recently been reported by Kapsenberg *et. al.* They prepared T-cell clones from the cells isolated from punch biopsies of

Ni+2 CS sites, and found that approximately 10-15% of the cells from DTH biopsies were Ni+2 reactive (74). Although these cells may have been contaminated by cells from the peripheral circulation this would seem to be a higher proportion of reactive cells than one would expect to find in the blood. Thus it appears that while the proportion of Ag-reactive T-cells within DTH sites may be small, there is some evidence to support the idea that DTH sites are somewhat enriched for cells reactive to the antigens driving the reaction.

That a small number of Ag-specific cells may be sufficient to drive a DTH reaction was confirmed by Marchal and his colleagues in an elegant series of experiments involving the injection into murine footpads, along with Ag, of Ag-specific T-cell lines (75) and clones (76). They demonstrated that a single Ag-specific cell, placed in the footpad was capable of provoking a DTH reaction. Although one cell alone was capable of generating a DTH reaction, the evidence indicated that not all T-cells can evoke DTH reactions. Marchal's results pointed to the the possibility of a numerically rare Ag-specific TDTH phenotype, responsible for initiating the subsequent recruitment of effector phenotype cells.

To date, the studies regarding Ag-specific cells in DTH have determined that very few Ag-specific cells are required to generate a DTH reaction, but the actual numbers and proportions of such cells that are present at DTH sites is uncertain.

Regulatory cells in DTH

Numerous types of cells are important in regulating DTH reactions. Much of the evidence regarding regulation of DTH has been generated in adoptive transfer experiments. While both CD4+ and CD8+ cells are required for DTH expression, cells of both phenotypes may serve to suppress the response (5,53,55,77). The suppression of DTH is itself part of a complex cascade, most clearly demonstrated in CS in which a CD3+, CD4+, CD8-, I-J+ suppressor-inducer cell produced an Ag-specific suppressor-inducer factor which is assembled with an I-J region product from CD3+, CD4-, L3T4-, I-J+ cell (53,78). This factor then acts via a CD3+, CD4-, CD8+, I-J+ transducer cell to induce

suppressor effector cells which may themselves act via soluble factors (78,79).

Non-specific contrasuppressor cells with the phenotype of CD3+, CD4+, L3T4+, I-J+, V-V adherent, have been shown to be required in order to passively transfer DTH immunity to contact sensitizers in naive mice (78). These cells apparently function to overcome the innate generation of suppression by resident cells in the spleen (78).

In interpreting the results of adoptive transfer experiments it is prudent to note that it has long been recognised that lymphoblasts preferentially accumulate at DTH sites regardless of their Ag-specificity (80-82) and that *in vitro* activated T-cells retain this characteristic (72,73). In contrast, *in vivo* activated lymphocytes will, after a short period in which they may be non-specifically recruited to any site of inflammation, tend to recirculate into the tissue in which they encountered Ag (83). Thus a regulatory cell, activated *in vitro* and transferred *in vivo* may exhibit abnormally enhanced or non-specific trafficking to inflammatory sites, where it may exert a greater than normal influence. Thus some of the regulatory pathways demonstrated utilizing this technique may be artifactual.

Antigen presenting cells in DTH

Cells of the macrophage/monocyte lineage have been shown to function both as antigen presenting cells (APC) and effector cells in DTH. Their role as APC had been inferred from *in vitro* experiments that have shown that both the CD4+ TDTH effector cells and CD8+ cytotoxic cells must see processed antigen in the context of Class II and Class I Ia antigens respectively (5,6).

There are numerous cells in the skin capable of functioning as APC. In the dermis these are primarily "resident" macrophages (47) which may be involved in the continuous activation of T-cells in the perivascular region of "normal" skin (47,49). As a DTH reaction evolves, large numbers of monocytes are recruited into the dermis, however it is unclear whether some of these cells subsequently act as APC or if they function solely as effector cells (5).

In the epidermis the dominant APC is believed to be the Langerhans cell (5,6,44,47). They are extremely effective as APC *in vitro* (32,84) and are seen to be closely associated with T-cells in the epidermis in DTH reactions (44,47,51), and may be the only APC capable of stimulating some hapten-specific T-cells from contact sensitivity reactions (74). Langerhans cells are sensitive to environmental insult and this and similar sensitivity by other APC may play a role in the loss of DTH responses seen following UV irradiation *in vivo* (79) and possibly other circumstances (78).

Macrophages as effector cells in DTH

Macrophages present at DTH sites also act as effector cells in several ways. By the production of CK that attract and/or activate cells involved in DTH, or by altering the vascular endothelium they may serve to increase the non-specific influx of cells to DTH sites. They may also be able to downregulate reactions by releasing compounds like PGE2 which inhibit T-cell activation and LK production (6).

Dvorak and others have shown that fibrin deposition is responsible for the induration felt at DTH sites (206-209). The

fibrin is assembled on the surface of the macrophages and eventually forms an intracellular meshwork (85,86). The deposition of fibrin by macrophages is a result of their production of macrophage procoagulant activity (MPCA). This occurs in response to signals from (Ag) activated T-cells (6), either the soluble factor macrophage procoagulant inducing factor (MPIF)(6,87-89), or directly via a rapid unidirectional Ia-restricted cell-cell interaction (6).

Macrophages also function as cytotoxic effector cells in DTH. Their ability to phagocytose bacteria makes them natural hosts for obligatory intracellular bacteria. The expression by macrophages of enhanced intracellular killing of such organisms is a result of the effects of the T-cell LK IFN-g and perhaps other mediators with macrophage activating factor (MAF) activity (5,6). Macrophages, and their progeny giant cells, can be observed phagocytosing and killing intracellular bacteria at CMI sites (5,6,90-92) and usually *in vitro* (92,93).

The role of mast cells in DTH

A role for mast cell-T-cell interactions in the generation of secondary DTH reactions has been put forth by Askenase and colleagues (94). Their work, carried out in the mouse, argues that the an Ag-specific T-cell factor produced by cells in the spleen and lymph nodes, binds to mast cells. These "armed" cells respond to crosslinking of the membrane-bound T-cell factor by Ag in a fashion similar to their response to IgE cross-linking, with the release of vasoactive amines (95-98) which cause the formation of gaps between the vascular endothelial cells leading to the non-specific extravasation of T-cells which direct the remainder of the DTH response. This group has amassed a considerable body of experimental results in support of this theory (for a review see 94), however their claims that this represents the sole initiation mechanism for DTH and that its participation is obligatory cannot be fully supported by the evidence at hand. The claim that the mast cell deficient W/Wv and Sl/Sld mice lack detectable DTH (99) and that reserpine blocked DTH, through its ability to inhibit serotonin, in normal mice, was refuted by Galli and Hammel (100) who used more

sensitive histological and autoradiological techniques to show that these mice did demonstrate normal DTH responses. They also showed that this response was reserpine sensitive despite the innate serotonin deficient state in these mice. Given the demonstration that a single Ag-reactive TDTH cell encountering Ag in the periphery may elicit DTH and the ability of soluble products of activated macrophages to alter the vascular structure (see below), an absolute dependence for mast cell degranulation in DTH would seem unlikely, however the demonstration of isotype-like regulation of the Ag-specific factors responsible for mast cell involvement (53) by suppressor factors (101) suggests that this may be a mechanism whereby specific T-cells in one immune compartment may influence T-cell responses at distant sites. Alternatively, the influx of serum proteins into the tissues as a result of endothelial gap formation may lead to enhanced fibrin deposition at DTH sites. On balance it would appear that mast cells do play a role in some DTH responses in mice, but that this role is more of an amplifying (in either magnitude or tempo) rather than an obligatory one.

Soluble mediators of DTH

Since the description of the first lymphokine, BF or IL-2 (8), over 100 CK, have been described (5,6). Since the description of MIF in 1966 by by Bennet and Bloom (6), many of these CK have been believed to be involved in DTH. The majority of CK fall into 6 general categories: Ag-specific factors, regulatory factors, growth and/or differentiation factors, factors effecting vascular permeability, migration inhibition factors and chemotactic factors. Within these categories there are often different factors having the same or similar effects but with different target cell populations (5,6).

The majority of these factors have been produced and their functions determined *in vitro*. To comprehensively review the entire pantheon of factors postulated to participate in DTH is impractical. I will instead concentrate upon those factors whose production and/or function(s) have been demonstrated *in vivo*.

The methods used to demonstrate the involvement of these factors have utilized Ab for immunohistochemistry or blocking of the responses, extraction from the site, or the introduction of CK into tissues to evoke DTH.

Histological evidence of CK participation in DTH

The detection by immunohistological techniques of cytokine production at DTH sites has been accomplished for a few CK. MIF has been detected in both human and murine DTH reactions. Using a monoclonal Ab, Sorg *et. al.* found that the production of MIF *in vivo* was carried out not by T-cells but rather first by endothelial cells and later in the reaction with macrophages (50). They detected large numbers of endothelial cells and small numbers of dermal macrophages producing MIF in the early non-specific inflammatory phase of DTH reactions. Interestingly, MIF was also produced in the early responses of non-sensitized and tolerized controls, as well as in the response to non-specific inflammatory agents. If a DTH reaction subsequently developed approximately 1/2 of the infiltrating macrophages produced MIF. Since it has been recently shown *in vitro* that activated macrophages synthesise MIF but only release it in the presence of Ag-activated T-cells the authors claim their histologic data demonstrates that the important sources of MIF *in vivo* are endothelial cells and macrophages (82). The ability of a Ab to MIF to block the expression of DTH was demonstrated by Geczy *et. al.* (102) supports the view that MIF is important in the DTH response.

IL-2 has long been postulated to be an important LK in DTH reactions. This hypothesis was strengthened by the demonstration by many authors of the progressive expression with time of IL-2 receptors by T-cells at DTH sites (5,6,41,44,50). Fullmer *et. al.* showed that T-cells at DTH sites stained positive for IL-2 (41). This cytoplasmic staining was in a pattern indicative of IL-2 production as opposed to IL-2 uptake.

Keratinocytes at DTH sites have been shown to express HLA-DR antigens late in DTH reactions (44,51,52). This response can be induced *in vitro* by IFN-g, and the *in vivo* expression of Class II Ag has been considered to provide indirect evidence for

IFN-g production at these sites. The same argument has been used regarding the expression by macrophages from DTH sites of IP-10. This protein is induced in monocytes, endothelial cells and keratinocytes *in vitro* only in response to IFN-g. *In vivo*, the same pattern of induction was seen at DTH sites, with macrophages and endothelial cells positive for cytoplasmic IP-10 by 24 hrs and basal endothelial keratinocytes positive by 41 hrs.

Issekutz *et. al.* demonstrated that the injection of Ab to IFN-g at sites of Ag deposition blocks DTH (103,104) and others have also shown that such Ab block the DTH mediated rejection of allogeneic tumor cells in mice (105) and the injection i.p. of monoclonal anti IFN-g blocks the expression of Class II Ag on keratinocytes at DTH sites (106). Ab to IFN-a have also been used to demonstrate the involvement of this CK in the DTH response (85).

Abilities of CK to produce DTH-like responses *in situ*

Demonstration of the potential involvement of certain CK in DTH has been shown by their capacities to produce DTH-like reactions when injected *in situ*. The somewhat poorly defined skin reactive factor of Bennet and Bloom was shown to induce a DTH-like induration when injected i.d. (108). The injection of ETAF into murine footpads results in a DTH-like induration and mononuclear cell influx (205). IL-1 injection *in situ* induces very little mononuclear cell recruitment within the skin (104). TNF-a induces a moderate cellular recruitment, and it is much more potent in this regard than TNF-β (110). Issekutz *et. al.* showed that the injection of Interferon a/β (104) and IFN-g resulted in DTH-like responses (110). The inhalation of IFN-g or TNF-a induces changes in the mononuclear cell population in the lungs of rats similar to those seen in DTH-mediated reactions (112). The most potent induction of DTH-like reactions occurs when an IFN is co-injected i.d. along with either IL-1 or TNF with TNF-a/IFN-g showing the greatest synergy and potency (104,110). Similar synergy was also observed in the inflammatory response in lungs of rats receiving aerosolized TNF-a and IFN-g (112).

Direct extraction of CK from DTH sites

Another approach to the demonstration of the presence of a CK at DTH sites has been to obtain the CK directly from the site. To date several chemotactic factors have been extracted from the DTH sites of guinea pigs (113-115). These factors were prepared from punch biopsies that were minced, frozen, sliced with a microtome, dehydrated and extracted by solvents. Under these conditions, the release of intracellular contents and contamination with blood-borne mediators and cells is inevitable and so the factors cannot be certain to arise solely from soluble factors released by activated cells in the DTH site. Nevertheless, factors chemotactic *in vitro* for monocytes, lymphocytes (113,114), B-cells (115), T-cells and CD4+ cells (116) have been obtained in this fashion and some of them have been shown to be produced *in vitro* in serum-free cultures of activated mononuclear cells (117). The problems incumbent in the extraction technique for obtaining putative factors from DTH sites casts doubt over the relevance of some of the detected factors to DTH reactions. The inclusion of a B-cell chemotactic factor amongst those extracted from DTH sites (116) underlines the potential problems of such a tissue-destructive technique.

The most convincing evidence for the involvement of a CK in DTH is the demonstration of the presence of specific CK at DTH sites. As mentioned previously, to date *in situ* immunohistological analysis has identified several CK at DTH sites. The difficulties of generating suitable antibodies and detection techniques with sufficient specificity and sensitivity have limited the success of this approach. Also, the demonstration of biological activity of the identified CK still lies in question, although admittedly, one can only find the activity one looks for, and given the pleiotropic activities of most CK, it may be difficult to know what activity(s) to those should be.

A non invasive/destructive method for obtaining the secreted products from DTH sites would be of great service in providing unequivocal proof for the involvement of individual factors in DTH.

Functional Subdivisions of T-cells with regards to LK Production

T-cells have long been divided into helper and suppressor/cytotoxic subsets. Initially it was believed that these functional groups corresponded to those defined by Ab to what are now known as the CD4 and CD8 loci. This categorization is breaking down under the weight of accumulating evidence that "helper" activity is carried out by only a subset of CD4+ cells and that both suppression and cytotoxicity can be mediated by both CD4+ and CD8+ cells. The ability of CD4+ cells to mediate DTH and provide help for B-cell Ab synthesis and proliferation (118) implied that perhaps these various activities were mediated by different cell populations (118).

The ability to clone T-cells has led to a dissection of many of these functions at the cellular level. Most important, in regards to the subject of the thesis, was the observation that local DTH reactions were transferable only by cytotoxic CD4+ clones that did not provide B-cell help (24). The understanding that these functions, and perhaps also inflammation, may involve different groups of CK, has led to the idea that functional subsets may exist within the CD4+ population, and that this may be a reflection of differing patterns of CK production.

Substantial evidence has been produced in murine systems to support this contention. Mossman *et. al.* have suggested that there are two kinds of CD4+ cells, distinguishable on the basis of their actions and LK production (119). The first, the so called TH1 or "inflammatory" T-cells produce IL-2, IL-3, IFN-g, TNF-β and GM-CSF upon stimulation, mediates local DTH responses, is cytotoxic and suppresses Ag-driven Ig secretion. The second or TH2 "helper" or "regulatory" T-cell produces IL-3, IL-4, IL-5 and GM-CSF upon stimulation and provides help for Ag-driven Ig secretion (118,120,121). Both types have receptors for IL-2 and IL4, but TH1 cells lack IL-1 receptors. Both types respond to IL-2 and IL-2+IL-4, however, only TH2 clones respond to IL-4 (in the presence of IL-1 (122). Similar results with regards to LK production and function *in vitro* have recently been reported for clones of human PBM derived

T-cell clones (118,123-126), although in contradiction, Patel *et. al.*, examining a panel of 83 CD4+ PBM derived clones, found that 81 produced IL-2 and all produced IFN-g, GM-CSF and TNF, and all were cytotoxic and gave help for Ig-synthesis (204).

Mossman *et. al.* have hypothesised that their TH1 clones correspond to the T-cells that drive DTH reactions. It remains to be determined however whether such cells are present at DTH sites, and if so, whether they predominate in such reactions.

To determine if this is so, will require the ability to obtain Ag-specific T-cells from human DTH sites, free of cells from the circulation, clone them, and examine their functional capacities *in vitro* order to see if they conform to the profiles of TH1 and/or TH2 cells. Prior to the work described in this thesis, no techniques enabling such studies to be performed have been reported, and thus this remains an open question.

Chapter 2: Anergy.

As I was going up the stair,
I saw a man, who wasn't there.
He wasn't there again today.
I wish, I wish he'd stay away!

Hughes Mearns, The Psychosed

Anergy was originally described by Von Pirket in 1907 as being the opposite of allergy (2). At the time, the distinction between allergy and DTH was not formally developed. Today Anergy is defined in clinical terms as the lack of DTH to recall skin test antigens. As a result of this negative definition, attempts to study anergy itself are difficult. Like the man upon the stair, anergy just isn't there. It is more useful to approach the problem of studying anergy by conceiving of it as a syndrome of unknown etiology, characterized by a loss of cell mediated immunity, with an increased risk of infection. In this context, the study of DTH, and the mechanisms responsible for its regulation, can be seen to be part of any attempt to understand anergy. Many disease processes, notably the congenital defects in immune function, have served to illuminate the fundamental processes of immunity. Studying anergy through studying DTH, may also reveal some of the basic workings of CMI.
There is as yet no evidence to directly link the increase in infections seen in anergy with the loss of DTH. It has been proposed that the loss of DTH is a reflection of a more fundamental defect at or close to the root cause of anergy (128). Some support for this view can be implied by the observation that most of the infections that occur in anergic patients are by organisms not thought to be controlled by CMI (129). Admittedly though, many of the infections are due to "traditional" targets of CMI such as *Candida* (129,130), and the dogma regarding the participation of T-cell mediated immunity in host defence against different pathogens may be oversimplified (63).

Anergy shares another characteristic with the man upon the stair, for those suffering from anergy, nothing could be more desirable than for it to stay away. It is a serious condition

carrying a substantially increased risk of infection and mortality for which there is as yet no effective therapy, and while the study of anergy may be useful in providing basic knowledge regarding CMI, the application of that knowledge to produce potential therapies for the underlying defects associated with anergy is a goal of paramount importance.

Anergy has the dubious distinction of being both negatively and operationally defined. For the purpose of this thesis, anergy will be defined as follows:

Human: DTH response less than 5mm to 5 putative recall Ag (131) (Normal response = DTH>5mm to two or more Ag)

Rat: DTH response < 5mm to KLH in previously sensitized animals (153) (Normal response 5mm, typically 6-12mm).

Minor differences may be encountered in discussing the work of other authors, and these will be noted as they arise.

The fundamental cause(s) of anergy in humans is unknown. Infection by HIV and possibly other agents can produce anergy, as may some forms of radiation, anti-neoplastic and immunosuppressive compounds.

In surgical patients free of such agents it typically occurs in concert with or following: cancer, trauma, malnutrition, burns, sepsis and advanced age (2,127,131). It is only this form of anergy that will be discussed in this thesis, and the term "anergy" will be presumed to relate solely to this/these form(s) of the anergic state.

To what degree any of these conditions are merely associated with, as opposed to causative factors of, anergy is unknown. Anergic patients are at increased risk for post-operative sepsis and septic mortality (129,131,133) regardless of whether the anergy existed pre-operatively or developed in the post-op period (129,131). While this association has long been recognised (2), it was not until 1975 that McLean *et. al.* recognised the potential predictive value of skin tests with regards to sepsis and septic mortality in surgical patients (133).

In a study of 2202 surgical patients, Christou *et. al.* examined multiple possible correlations between physiological functions/illness and sepsis and septic mortality. The resulting

analysis revieled a numerical relationship between skin test score and sepsis/mortality (129). Alterations in other factors such as serum albumin were also found to be predictive, however DTH score remained the best predictor of sepsis and septic mortality in surgical patients. When combined, DTH score and serum albumin levels could be used to predict outcome with even greater reliability.

Defects in Host Response associated with Anergy

Non-specific host defences

Alterations in the adherence and chemotaxis of neutrophils obtained from peripheral blood have been noted in surgical patients (129) but their relevance to anergy remains unclear. Morris *et. al.* found that surgery depressed *in vitro* chemotaxis, and *in vivo* delivery of PMN to Boyden chambers filled with 50% autologous serum placed over tape-stripped sites (134). While these patients had significantly lower DTH scores pre-operatively than younger normal controls, the post-operative decrease in PMN chemotaxis did not correlate with the observed decrease in *in vivo* PMN delivery (134). Interestingly there was a 10-fold higher *in vivo* delivery seen in pre-op patients as compared to the normal controls (135). Anergic patients however did not show this increase, resulting in PMN delivery 1/10 of the hospital reactives but essentially identical to normal controls (134). These results may indicate that anergic patients are failing to respond to inflammatory processes to the same degree as reactive hospitalized controls (135).

Pre-op studies have also shown that PMN adherence is elevated in all patients, but more strikingly so in anergic as opposed to reactive patients. A similar situation exists for PMN chemotaxis however it is depressed in patients rather than increased. So too are total serum proteins, albumin and hemoglobin. A pronounced neutrophilia has been found in all surgical patients with anergic patients exhibiting the most pronounced effect (129).

Overall there are differences between the non-specific host defence and physiological parameters of pre-op patients and

normal controls. The degree to which the differences in age and disease state between these two groups may account for these observations is unknown. However, if the pre-op patients are stratified according to their skin test response, the deviations from the normal values are generally more profound in the anergic population (134).

Alterations in immune functions

Several deviations in B-cell activity are associated with anergy. These defects appear to be confined largely to responses to protein Ag (136). Both *in vivo* and *in vitro* production of specific antibody to tetanus toxoid is deficient in anergic subjects (a similar but smaller defect is also seen in the reactive patient population) while the response to pneumococcal polysaccharide is normal (136,137). Serum levels of IgG and IgA are elevated (136) and *in vitro* the numbers of B-cells spontaneously secreting these isotypes are also abnormaly high (138). The elisa-spot technique used to detect this spontaneous secretion requires only a few hours of culture, and thus this is interpreted to reflect the presence of these cells in the circulation *in vivo* and not an abnormal response aquired *in vitro*. Interestingly, the production of specific Ab by PBM in a primary *in vitro* response to to sheep erythrocytes has also been reported to be somewhat elevated in anergic patients (138).

By definition, *in vivo* T-cell function is profoundly altered in anergy. DTH skin test responses are obviously depressed or absent, and there has been a report of the acceptance by one anergic patient of a full thickness HLA-incompatable skin graft for 44 days without micro or macroscopic evidence of rejection (133). Following primary exposure to DNCB and KLH, reactions to residual Ag remaining at the site do not occur in anergics and sensitization by this exposure does not occur as evidenced by a subsequent lack of sensitized cells in the peripheral circulation (139).

Paradoxically, T-cell responses *in vitro* are not affected by anergy. Proliferative responses to alloantigens (140) and soluble Ag (PPD, 140) in previously sensitized anergic patients

are all normal. Cell mediated lympholysis and the response to crude preparations of T-cell growth factor have also been reported to be normal (141). These results led to the hypothesis that the cells of anergic patients were capable of mounting CMI responses, but that the "anergic environment" somehow interfered with the expression of CMI.

This hypothesis was tested by Rode *et. al.* by injecting autologous PBM, pre-cultured with PPD *in vitro* i.d. into anergic patients. Sixty-three percent of anergic patients reactive to PPD *in vitro* gave a positive skin test reaction to these cells, conditional to the cells being viable (140). Thus at least one of the defects in anergy would seem to result from an *in vivo* block of lymphocyte activation.

The use of CK to investigate anergy

The role of CK in the re-establishment of DTH was also addressed by Rode *et. al.* (139,140,142,143). In a series of experiments they established that the secondary (recall) DTH reaction could be demonstrated in the majority of previously sensitized anergic patients when the skin test Ag was co-injected with CK alone. Effective Ck preparations were made from the supernatants of either soluble Ag (139,140,142,143) or allo-Ag (143) stimulated PBM, from either reactive or anergic subjects. This effect was shown with both PPD (140,142,143) and KLH (140). The active component(s) were Ag non-specific (139) and non MHC-restricted (139,143). Histological examination showed that only the injection of CK plus Ag to which the anergic subject was reactive *in vitro* resulted in a mononuclear cell infiltrate typical of a DTH reaction (143). In these studies, miniscule amounts of culture supernatants, as little as 0.1ml per injection, sufficed to induce the effects described.

Not only can CK restore the ability to mount a secondary response to Ag in anergic patients, but it also restores the capacity to mount a primary sensitization to Ag. When a sensitizing dose of KLH was co-injected with CK, sensitized lymphocytes were found in the peripheral blood 14 days later, as opposed to anergics injected with KLH alone, who had no such

cells. These cells proliferated in response to KLH *in vitro*. A DTH reaction was obtained when these newly sensitized anergics were skin tested with KLH+CF (139).

In summary, anergy is associated with many physiological derangements, which are often present in surgical patients, and also with alterations in some non-specific host defence parameters. The severity of these abnormalities is greater in anergic than reactive patients. *In vitro* responses of both T and B cells are normal or slightly enhanced, indicating that APC, T & B-cells of anergic patients are functionally competent. *In vivo* B-cell responses to protein Ag are defective, and there are an abnormal number of Ab secreting cells in the circulation, but curiously *in vivo* responses to polysaccharide Ag are normal. T-cell responses are absent *in vivo*, but they can be restored by the co-injection of Ag with CF. The underlying cause(s) and the defective mechanisms responsible for anergy are themselves unknown. As a result of the *in vivo-in vitro* dichotomy of response, *in vivo* methods are required to study anergy.

Animal Models of Anergy

Anergy is characterized by a loss of DTH reactivity and an accompanying increase in susceptability to infection. However, the lack of knowledge regarding the underlying cause(s) of anergy in humans has made it difficult to design appropriate animal models of the condition. Two techniques are currently available for inducing anergy to recall Ag in animals. Long-term protein deprivation results in a wasting syndrome which includes anergy in mice and rats (111,127). A third degree thermal trauma model, originally developed by Walker and Mason (144), also results in decreased CMI responses. The mechanisms responsible for anergy to recall Ag in these two models are uncertain, however the same can also be said for anergy in surgical patients.

In addition to depressed DTH responses, other effects of the thermal trauma model include fluid loss (which may be replaced by i.p. and s.c. injection), lowered serum Ig and fibronectin

levels, increased susceptability to infection (145), depressed B-cell responses, lowered *in vitro* responses to Ag and mitogens (146) and increased activity of CD4+ suppressor-inducer cells (147).

Animal models of infection

A rapidly disseminating peritonitis is frequently encountered in anergic patients following abdominal surgery (148). There are numerous animal models of infection and peritonitis but I will confine myself to a discussion of the models developed to imitate the clinical characteristics of peritonitis, and to the particular model utilized in the thesis and its direct antecedents.

Rat models of peritonitis designed to simulate clinical peritonitis, using fecal materials, were created in the late 1960's (149,150). These models however led to little or no lethality (151). The inclusion of the irritant Ba2SO4 with rat feces in the peritoneal cavity of rats by Onderdonk *et. al.* (152) and Lowenstein *et. al.* resulted in a model which mimics human peritonitis in having a rapid generalizing peritonitis, lethal within three days (151). It was subsequently shown that the organism responsible for lethality in this model was primarily *E. coli.*, with *B. fragilis* required for abscess formation in survivors (151). A subsequent refinement in which these and other bacteria obtained from cultures were used *in lieu* of fecal material, resulted in a useful and reproducible model of peritonitis (151). This model was later used to demonstrate that anergy, occuring secondary to peritonitis in rats, was predictive of lethal outcome (153), and was adapted for use in the studies to be described in this thesis.

Use of cytokines to treat infections in animals.

Non-specific immunomodulators were first described by Gaston Ramon in 1925 (154). Jules Freund expanded upon his work and developed affective immunizing adjuvents. Further study by numerous investigators has led to a proliferation of non-specific immunopotentiating compounds useful in immunization

in animals (155) and humans (154) and in infectious processes in animals (155).

The potential of numerous CK to participate in combating infections was well recognised from *in vitro* experiments carried out in the 1970's. In 1975 Kampschmidt and Pulliam showed that the administration of crude preparations with IL-1 like activity inhanced survival of infected rats (156). Until highly purified mediators were available the pleiotropic effects of crude CK preparations, the known potential for synergy between CK and the belief that impractically large quantities of culture supernatants would be required to conduct *in vivo* tests, discouraged investigation.

The recent availability of large quantities of recombinant CK has led to a proliferation of studies of the *in vivo* effects of CK in combating infections, including those caused by extracellular pathogens. In 1987 Ozaki *et. al.* demonstrated that recombinant human IL-1a improved the survival of rats infected with *Klebsiella pneumoniae* or *Pseudomonas aeruginosa* (157). Similar results were reported by others involving infections with *Listeria monocytogenes* and *K. pneumoniae/Staphylococcus aureus* (158,159). These studies utilized up to 200,000 Units/mouse with 50% effective doses in the range of 1,000 to 10,000 Units/animal. They also reported that the administration of the IL-1a by the same route as the bacteria (ie. iv-iv or ip-ip) was most effective in treating these infections (158). Additionally they found that when the injections were given ip the CK was most effective if given 48 hrs prior to infection, while if the route of injection was iv the CK was most effective if given concominantly with the bacteria (158).

IL-2 was shown to be protective against *E. coli.* infection in mice if administered by the same route as the bacteria in doses of approximately 4x104 Units/mouse (160). IFN-g has also been shown to improve survival in *E. coli./K. pneumoniae* infected mice after 15 days of treatment with 7,500 Units daily (161).

All these studies share in common the use of large quantities of recombinant cytokines to treat infection in immunonormal animals. The optimal strategy typically required the injection of both CK and bacteria by the same route, and often pre-treatment with CK was most effective. None of the investigations paralleled the work of Rode and colleagues which utilized minute amounts of a mixture of presumably both known and unknown CK, to restore normal immune function in anergic subjects in a site-dependent fashion. Thus while these two lines of investigation share features in common, they derive from a different approach to the application of CK to treat infections.

Anergy is clearly a dangerous and intractable condition that threatens surgical patients. The current lack of knowledge regarding some of the basic processes of CMI and how these are defective in the anergic state has hampered attempts to understand and treat anergy. While it is known that some CK can reverse the effects of anergy locally, how they do this and the implications of this capacity for both the study of DTH and the treatment of anergic patients remain unresolved.

Scope Of The Thesis

The cells involved in DTH remain poorly characterized, primarily as a result of the difficulties involved in recovering them from DTH sites. The development of a technique for recovering the cells from human DTH sites is the subject of Chapter 3.

The functional capacities of the T-cells involved in DTH have largely been inferred from those of cells present in the blood and organs of the immune system. Which functions are actually present in the population carrying out DTH responses remains unknown.

Having gained access to the T-cells from human DTH sites, the cloning of these cells and the determination of their antigenic specificity are described in Chapter 4. The T-cell clones from DTH sites that were specific for the antigen eliciting the reaction were submitted to analysis of their functional capacities. The production of cytokines *in situ* at DTH reaction sites was also evaluated. These studies will also be related in Chapter 4.

In Chapter 5, the nature of the defective DTH response in anergic patients was examined using the same techniques as were applied to normal DTH reactions. Additionally, the nature of the response that results when anergic patients are skin tested with antigen coinjected with the supernatants of mixed lymphocyte cultures (MLC) was also investigated. The antigenic specificity and functional capacities of the T-cells from such skin test sites were determined and compared to those from normal DTH reactions, with a view towards assessing their potential for involvement in anti-pathogen responses.

As a result of the investigations related in Chapter 5, the potential theraputic value of the cytokines in MLC supernatants in treating infections in anergic subjects was examined. These experiments were carried out in a rat model of anergy and are related in Chapter 6.

Chapter 3: Skin Window Chambers, a method for recovering the cells and cytokines involved in delayed-type hypersensitivity

Introductory comments

Studies of cells involved in delayed-type hypersensitivity have been primarily carried out by histological and, more recently, immunohistochemical techniques (44). While these approaches have yielded substantial information regarding the types and surface antigens of responding cells, they can only provide a static picture of a dynamic process (5).

Direct studies of the functional capacities of cells involved in DTH have not been practical due to the inaccessibility of these cells. To this point, only skin biopsies have been available as a source of these cells for study (74). Biopsies have the disadvantage of being an invasive technique yielding only small numbers of cells which are partially contaminated with blood cells.

In order to be able to readily pursue further studies on the cells in a DTH lesion, a non-invasive technique which allowed recovery of a sufficient number of cells without contamination with blood was required. Methods derived from the classic Rebuck skin window but employing chambers instead of the coverslips utilized by Rebuck *et al* had been used to recover PMN from non-specific sites of inflammation (134, 163-165).

The purpose of the work described herein was to modify these techniques in order to provide access to the cells and CF from DTH sites of humans.

METHODS

Subjects

The subjects were 62 healthy volunteers, 24 female and 38 male, ranging in age from 18-72 years. Informed consent was obtained from all participants. The subjects were skin tested for DTH reactivity by the injection i.d. of 0.1ml of 5 standard test antigens: Candida (Dermatophyton O, 1:100(diluted with Albay buffered saline(0.5% NaCl + 0.275% Nabicarbonate + .4% phenol) Hollister Stier), Mumps (MSTA, Connaught Laboratories), PPD (Tubersol, Tuberculin PPD(Mantoux), 50 TU/ml), Connaught Laboratories), Trycophyton (1000 pnu/ml, Hollister Steir) and Varidase (Streptokinase 1000 U/ml-Streptodornase 250U/ml, Hollister Steir). Reactions greater than 5mm in diameter were considered positive. All subjects responded to two or more of the antigens.

Serum

Blood was obtained by venipuncture and was defibrinated by agitation with sterile glass beads for 20 min. Serum was obtained by centrifugation of the defibrinated blood @ 200g for 20 min. Serum used to fill the chambers was pooled from two healthy type-AB donors, which had been screened for: anti-Hepatitis antibody, Hepatitis virus, and anti-HIV-I antibody. In certain experiments autologous serum diluted to 10% v/v with normal saline (Travenol) was used with or without heat inactivation @ 65t for 10 min.

Skin window chambers

The chambers placed over the DTH sites were small plastic "Sombrero"-shaped bubbles1 (Fig. 3.1b). A hole had been melted through the top of the chamber and a rubber injection port from a 10cc bottle of sterile water (Travenol) glued in place with Epoxy. The resulting chamber had an approximate volume of 0.8ml. To prevent loss of cells by adherence, the chambers were siliconized using a water based siliconizing compound (Siliclad, Johns Scientific). The chambers were then gas-sterilized with ethylene oxide prior to use. It was found that each chamber

could be re-used an indefinite number of times following vigorous cleaning with Sparkleen (Fisher) in an ultrasonic cleaning bath. The rubber injection port required replacement after 2-4 uses, due to deterioration of the Epoxy during sterilization and cleaning.

Preparation of the DTH site and chamber application

The preparation of the site followed the method of Morris (134) which uses a modification of the tape-stripping method of Mass *et. al.* (165). Briefly, the volar surface of the forearm was shaved (if required) and sterilized with 2% Iodine (Royal Victoria Hospital) and 70% alcohol. A gas sterilized adherent plastic tape (3M) with two 1.6 cm diameter holes approximately 6 cm apart was applied as a template. The stratum corneum was removed from both template holes by multiple (100-150) applications of sterilized clear (Transpore, 3M) tape (Fig. 3.1a). Stripping was completed when the skin attained a glistening appearance. All tape stripping was performed by the same person (M.J.M.). A standard skin test dose of antigen (or saline) was injected intradermally in 0.1ml into each site. The template was then removed and each site covered with a sterile "sombrero" skin window chamber (Fig. 3.1b) which was secured in place with 2 pieces of microfoam (3M) tape with holes large enough to slip over the body of the chamber. Two additional pieces with small holes permitting access to the injection port were then applied. The chambers were filled with serum through the injection port, using a 3cc syringe (Becton-Dickinson (B-D)) fitted with a 24-gauge needle (B-D) and employing an additional 24-gauge needle as an air vent. Sufficient volume was flushed through (approx 2.5ml) to assure that no bubbles remained within the chamber (Fig. 3.1c). A final piece of Transpore tape was placed over the chambers to secure them and the forearm then wrapped with a sterile elastic bandage to protect the preparation (Fig 3.1d). At the desired time, usually 24 and/or 48 hrs later, the fluid was removed and the chambers rinsed with approximately 5ml of sterile saline (Travenol). The chamber fluid and the saline wash were pooled and used for analysis. The chambers were then removed and a swab taken of the sites for

bacteriological analysis. The sites were covered loosely with a sterile dressing and allowed to dry.

Cell enumeration

Cells from the skin window chambers were collected by centrifugation (10 min @ 100g) and the chamber fluid frozen at -20°C for storage. The cells were resuspended in RPMI 1640 + 40% Normal Human Serum, and then enumerated with a hemocytometer following staining with Turks solution. Confirmation of cell numbers was obtained by differential counting of a cytocentrifuged slide preparation stained with Wright's stain.

Cytocentrifugation and staining

Cells for cytocentrifuge preparations were suspended at 2x105 cells/ml in PBS + 0.5% BSA (Fraktion V, Boehringer-Mannheim) Microscope slides (Fisher) were prepared in a Shandon cytocentrifuge using Shandon filter cards by coating them with 200 µl of 1% BSA in PBS (GIBCO) and centrifuging for 10min at top speed. Five hundred µl of cell suspension was added to each holder and the slides spun for 5 min at 200 rpm and air dryed. The slides were then stained with Wright's stain in an automatic staining apparatus.

Preparation of Peripheral Blood Lymphocytes

Blood samples were mixed with heparin (Grganon) to a final concentration of 10U/ml and diluted 1:1 with HBSS (GIBCO). They were then layered over Ficol-Hypaque (Pharmacia) and centrifuged for 20 min at 400g. The cells at the Ficol-Hypaque-Plasma interface were removed and washed twice in HBSS, counted and resuspe.·ded in culture medium.

Lymphocyte responsiveness

To assess lymphocyte responsiveness, skin window chamber cells or PBM containing 40,000 mononuclear cells were cultured in 0.2ml RPMI + 20% Pooled human serum with and without one µg/ml PHA (Wellcome Laboratories) in 96 well V-bottom plates (Linbro) in a 5% C02 atmosphere at 37°C.

After 66 hrs, one µCi of 3H-Thymidine(specific activity 2µCi/mg, NEN) was then added to each well and the

cells incubated for a further 18hrs, at which time the plates were frozen and thawed and the wells harvested with a MASH apparatus (M.A. Bioproducts). The contents of the wells were deposited onto glass fiber filter discs which were washed with dH20 and transferred to scintillation vials, dryed for 2hrs at 65°c. One and a half ml of scintillation cocktail (Omniflour (Dupont) 1.6% in toluene (Fisher)) was added to each vial and the radiolabelled DNA counted in a scintillation counter (Packard Instruments).

Statistics

In the experiments shown in Figs. 3.2 and 3.5, paired chambers were placed on a single individual. Comparisons of cell deliveries in these experiments were analyzed by the Wilcoxan Paired Signed Ranks test. Comparisons between total cell delivery in 0-24 + 24-48 hrs vs 0-48 hrs were made using data from different individuals (Table 3.2). These experiments, were tested by the one-tailed unpaired Wilcoxan Rank Sum Test. All statistical procedures were carried out using a statistical analysis program (STATS-PLUS, Human Systems Dynamics). When necessary, tabular p-values were obtained from Choi (166).

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11 The chambers from which the "Sombrero" skin window chambers were constructed were: Capsule en Makrolon

purchased from:

Trametal,
21 Quai Debonneuil,
9410 St. Maur,
France

RESULTS

Modifications to the Boyden chamber technique.

The original technique which was used to collect the cells from DTH sites was the Boyden chamber method of Morris *et. al.* (134). This technique had been shown to work well to collect PMN from non-specific inflammatory sites but when it was applied to the DTH reaction it was found that the combination of the non-specific inflammation with the induration of the DTH reaction produced excessive swelling under the chambers. This resulted in considerable pain and discomfort, along with minor infections around the rim of the chamber. Several modifications were tested in order to produce a less irritating technique which would be more acceptable to experimental subjects and would produce less non-specific inflammation. These changes, shown in Table 3.1, resulted in the technique described in the materials and methods section which was used throughout to obtain the cells from skin reaction sites.

Delivery of mononuclear cells to skin window chambers depends upon the presence of antigen

The hallmark of a DTH reaction is the delivery of mononuclear cells to the skin in response to Ag. The initial experiments looked at the numbers and types of cells delivered into the chambers upon the initiation of a DTH reaction.

The 24hr delivery of mononuclear cells and PMN to paired chambers above sites injected with saline or antigen to which the subject was reactive was determined in 11 experiments (Fig 3.2) In all but one instance the delivery of mononuclear cells was augmented in the presence of antigen yielding a mean increase of 15-fold. With two exceptions, the number of neutrophils recovered from chambers with antigen was similarly increased. In this and subsequent experiments, the results from the initial enumeration with Turk's stain and the differential count on Wright's stained cytocentrifuged preparations were comparable. The mononuclear cells were a mixture of lymphoid and macrophage like cells (Fig. 3.3) approaching 25% of the cells in the chambers.

Kinetics of Mononuclear cell delivery

To determine the kinetics of mononuclear cell delivery to the chambers, two techniques were employed. In the first experiment, two chambers were placed on two PPD reaction sites of one individual. The contents of alternate chambers were removed at various times and the cells enumerated. The chambers were then refilled with serum and harvested again at later time periods. The results are shown in Figure 3.4. Mononuclear cells were found in the chamber in significant numbers in 12 hrs, and increased rapidly in the following 12 hrs.

In the previous experiment, the cells and fluid were removed from the chambers and not replaced. If the cells delivered to the chambers and any factors present in the fluid were indeed involved in the DTH reaction it was possible that they would continue to carry out their effector functions while in the chamber, with an influence upon the underlying DTH. To investigate this possibility, the total delivery of mononuclear cells to chambers which were harvested at 24 hrs (and the chambers refilled with serum) and again at 48 hrs was compared to the delivery obtained when the chambers were left undisturbed for 48 hrs. The results are shown in Table 3.2. The delivery of cells to chambers left undisturbed for 48hrs was significantly greater than the sum of the deliveries from 0-24 & 24-48 hrs. Similar results were obtained from a small number of experiments utilizing paired chambers on individual subjects (data not shown). Accordingly, subsequent experiments were carried out by harvesting the cells and fluid from the chambers after 48hrs.

Cell delivery is dependent upon previous sensitivity to antigen

The previous experiments implied that increased cell delivery may be an antigen-specific function. To test this hypothesis a series of seven paired experiments comparing cell delivery to one skin test antigen to which the subject was sensitive as ascertained by skin testing, with the delivery to another antigen to which the subject was not reactive were performed. In this series the chambers were left in place for

48hrs. As seen in Figure 3.5, again with one exception, antigen to which the subjects were reactive induced an increase in the number of both mononuclear and PMN harvested compared to those recovered from chambers placed over non-reactive antigen sites.

Responsiveness of skin window chamber cells to PHA

The functional capacity of lymphocytes harvested from the chambers was tested in terms of their proliferative responses to PHA, and were compared to those given by PBM from the same subjects. The reactions obtained (not shown) were positive but very low, which we attributed to the high numbers of residual neutrophils in these cultures which we were unable to remove.

Bacteriological results

Swabs were taken from both chambers of 50 individuals. All were cultured for the presence of bacteria. Four of 100 were positive for *Staph. species* or *Staph epidermidis*.

Table 3.1: Modifications to the Boyden Chamber Technique

Modification	Effect
Siliconization of chamber	Increased number of cells recovered
Enlargement of abrasion site	Increased number of cells recovered
Use of plastic "Sombrero" chambers	Reduction in non-specific & induced inflammation Reduced PMN delivery Improved comfort for subjects Reduced incidence of infection at chamber site
Use of foam tape	Reduced incidence of chamber leakage Improved comfort for subjects

Table 3.2: Comparison of Total Mononuclear Cell Delivery in 48 Hrs With & Without the Removal of Cells at 24 hrs.

Time Period(s)	N	Median Mononuclear Cell Delivery	p
0-24 + 24-48	12	0.30 x 106	
0-48	17	1.86 x 106	<.05

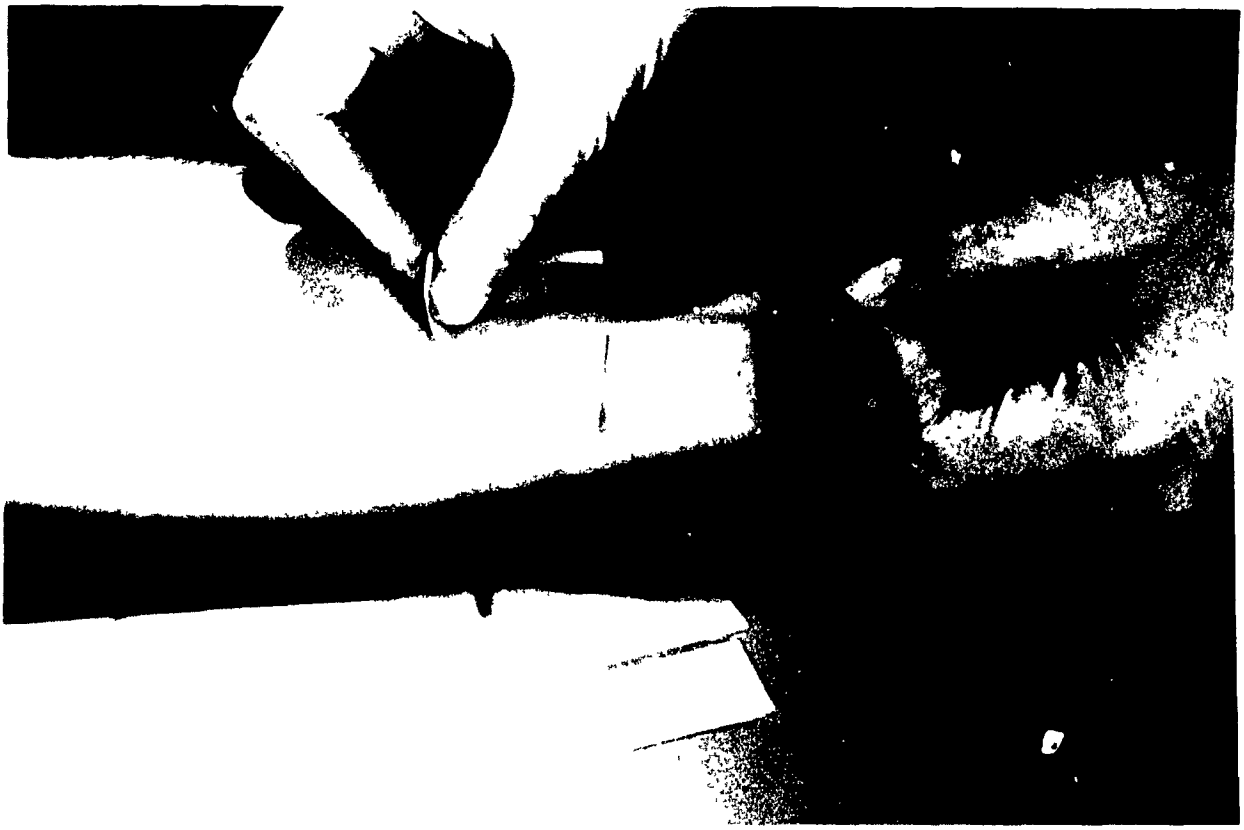

fig 3.1a

Fig. 3.1: Application of skin window chambers. The lower arm was sterilized with iodine and alcohol. A template was then applied and the stratum corneum removed by tape stripping (A). Antigen or saline was then injected into the sites and the template removed. The "sombrero" skin window chambers were then placed over the sites (B). The chambers were securely taped in place and filled with serum (C). The preparations were then covered with an elastic bandage (D).

fig 3.1b

fig 3.1c

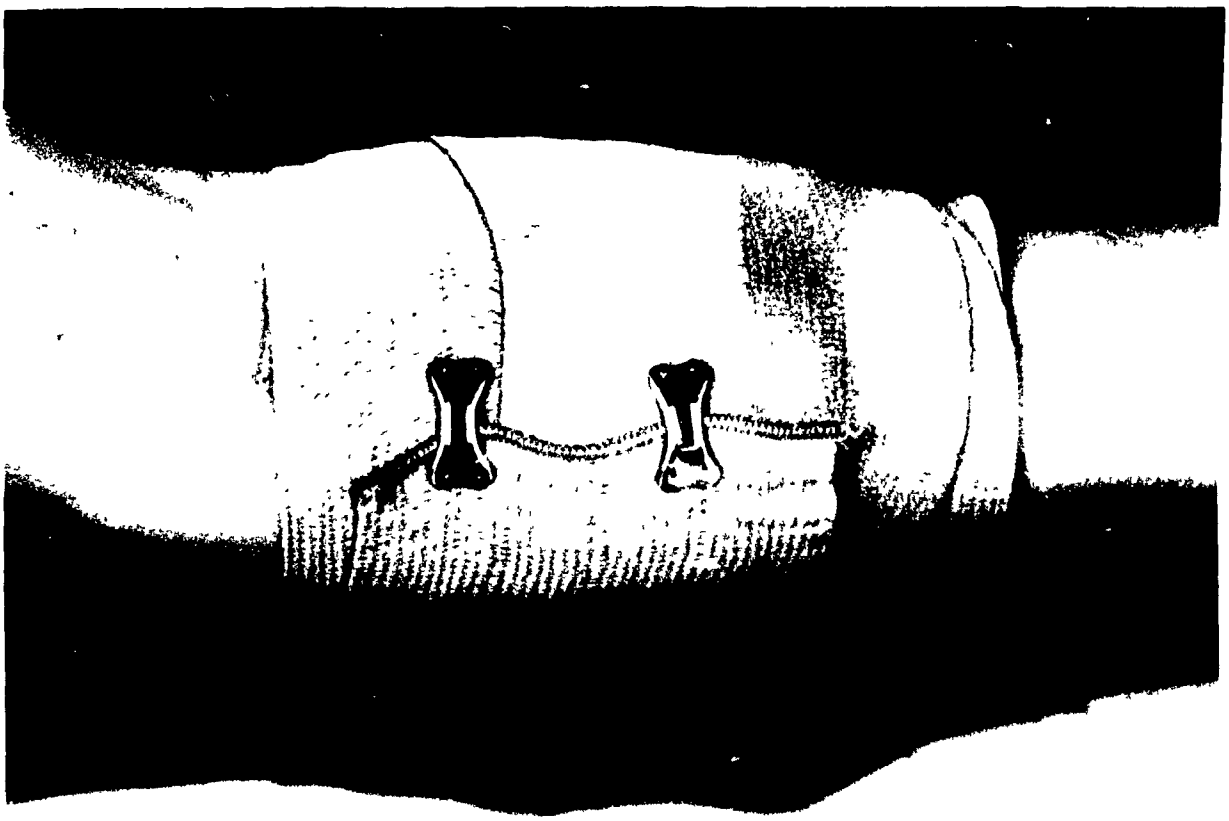

fig 3.1d

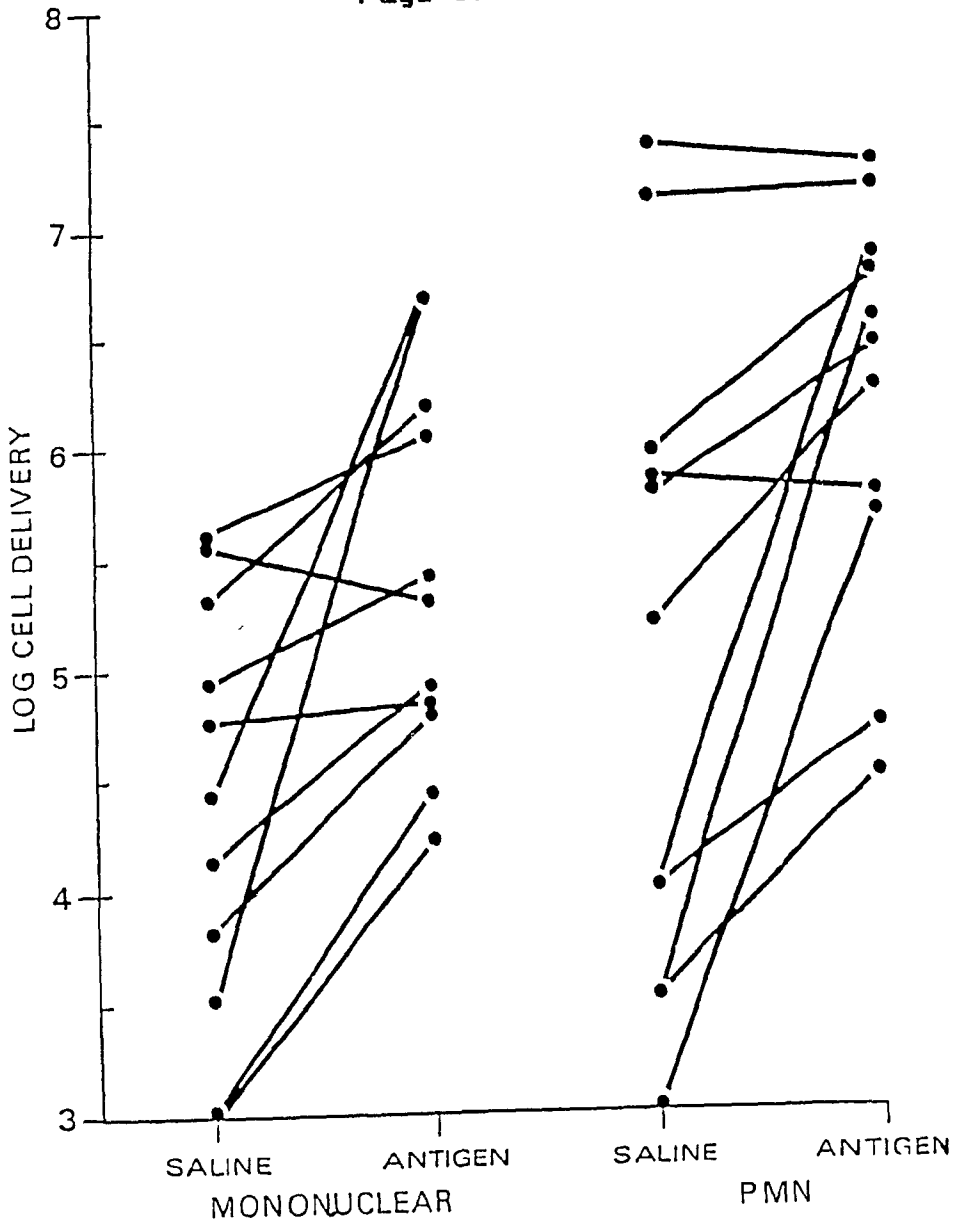

FIG. 3.2: Antigen dependence of cell delivery to skin window chambers. Two chambers were placed on each subject, one over a site injected with antigen eliciting a DTH reaction (antigen) and the other injected with saline (saline). After 24hr the chambers were harvested and the cells delivered were enumerated. The data shown represents the cell deliveries to chambers from individual subjects. the increased delivery of both mononuclear and polymorphonuclear cells when antigen was injected was significant ($p<0.003$).

fig 3.3a

fig 3.3b

Fig. 3.3: Characteristic mononuclear (A) and polymorphonuclear (B) cells delivered to a skin window chamber after 24hrs over an antigen reactive site.

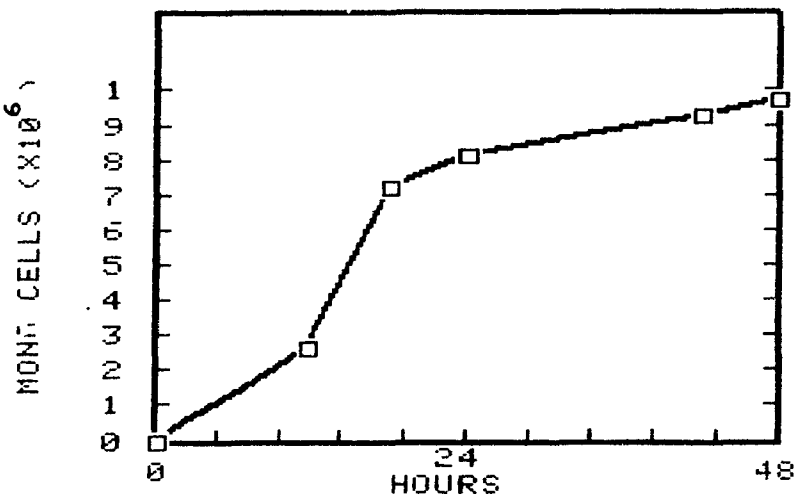

Fig. 3.4: Kinetics of mononuclear cell delivery to skin window chambers. Two chambers were placed over DTH sites of a single individual. The chambers were harvested alternately at various times and re-filled with serum. The cumulative cell delivery was plotted for each time point.

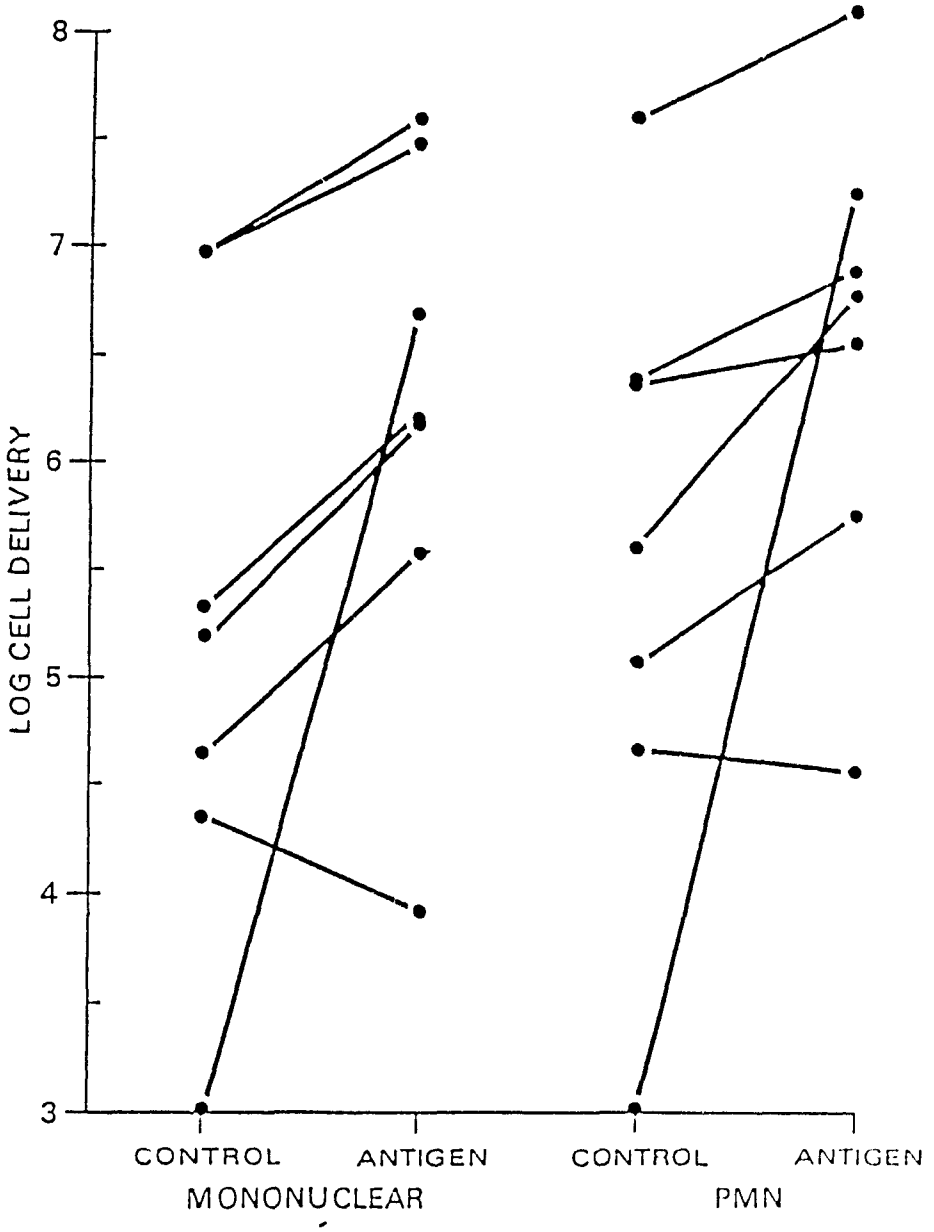

Fig. 3.5: Dependence of cell delivery upon previous sensitivity to antigen. Two chambers were placed on each subject, one over a site injected with antigen eliciting a DTH reaction (antigen) and the other injected with an irrelevant antigen to which the subject gave no response (control). After 48hrs the chambers were harvested and the cells delivered were enumerated. The data shown represents the cell deliveries to chambers from individual subjects. The increased delivery of both mononuclear and polymorphonuclear cells when antigen to which the subject was reactive had been injected was significant ($P<0.02$).

DISCUSSION

The recovery of mononuclear cells from human DTH reactions is a requirement for the determination of the precise phenotypes of these cells. The "sombrero" skin window chamber technique permits the recovery of substantial numbers of such cells.

The method was modified from skin window techniques used to study the migration of PMN to sites of local injury (134). Very few, if any mononuclear cells were recovered using these methods. By adopting such a technique for a study of the DTH response, whose hallmark is the accumulation of mononuclear cells, it is not unexpected to find PMN contamination of the mononuclear cell influx. By preparing the skin sites with gentle tape stripping, substituting a light-weight plastic chamber with a flat broad and smooth lip, and through the use of foam tapes, we reduced the attendant non-specific inflammation and thus the influx of PMN. By injection of antigen we achieved the attraction of mononuclear cells typical of DTH reactions although accompanied by a PMN accumulation. We attribute the latter to an increased inflammation seen in skin in contact with chambers covering a DTH reaction. The majority of PMN in the latter preparations appeared to be activated (Fig 3.6).

The presence of serum in the chambers may have contributed to the PMN influx. We chose 100% serum for the chambers because we felt that it might provide the optimal milieu to promote survival and recovery of functional mononuclear cells. Subsequently, it was found that the reduction of the serum content in the chambers to 10% reduced PMN delivery selectively, whereas heat inactivation further decreased all leukocyte influx (data not shown).

When the antigen was deposited in the chamber in lieu of injecting it into the skin, mononuclear cell delivery was increased as compared to chambers without antigen, however the numbers of cells delivered was substantially less than when the antigen was injected (data not shown). Accordingly the antigen was injected throughout the work reported herein.

Although there was variation in the absolute numbers of cells delivered from individual to individual, the results from paired chambers are consistent enough to allow significant results to be obtained from modest numbers of subjects. Unpaired analysis required only slightly larger group sizes to obtain significant results.

The recovery of cells from a DTH site without contamination by cells from the circulation is one of the goals of this approach. Since AB serum was used to fill the chambers, erythrocytes would be expected to be found in the chambers if the i.d. injections were performed improperly (resulting in vascular damage). Such failed injections occured only occasionally, and were obvious at the time because of the visible presence of blood at the injection site. When chambers were placed over these sites, erythrocytes were detected in the chambers. Such incidents were rare, and the data presented here comes only from sites injected correctly.

The hallmark of DTH is the development of a mononuclear cell infiltrate in the dermis and epidermis within 6-48 hrs of exposure to an antigen to which the subject was previously sensitized (5). The delivery of mononuclear cells to the skin window chambers followed similar kinetics. Benign, non-immunogenic irritants provoke small cellular infiltrates in comparison to DTH reactions (167). Thus the increased numbers of mononuclear cells delivered to chambers over Ag+'ve sites compared to those over saline sites indicated that the mononuclear cell delivery to the chambers is primarily dependent upon the DTH reaction in the underlying skin.

DTH responses are controled by Ag-specific T-cells. When an Ag to which a subject is non-responsive is injected, there is an initial cell delivery which wanes within 6-12 hrs and is much smaller than the eventual infiltrate seen at Ag+'ve sites (44,50). Similar results were found when paired chambers were placed over Ag+'ve and Ag-'ve sites of individuals. In all but one instance there was a markedly higher delivery of mononuclear cells to the Ag+'ve sites, demonstrating that the delivery of these cells was a reflection of an immune response to specific

antigen in the underlying skin. Thus the technique provided a faithful rendition of the DTH reaction.

In the current model of the DTH reaction, the initial T-cell influx is small in size until a cell responsive to the antigen(s) present encounters processed antigen in the context of self-MHC on an antigen-presenting cell. These antigen-reactive T-cells produce lymphokines which result in the attraction of much larger quantities of mononuclear cells (5).

When the cells delivered within the first 24 hours were removed from the chambers, the influx in the subsequent 24 hours was smaller than that seen in the first day, and the total cell delivery over the 48 hour period was approximately 1/6 of that seen in chambers left undisturbed for 48 hours. This would suggest that the cells delivered to the chamber in the first 24 hours contain significant numbers of lymphokine-producing cells which are responsible for recruiting large numbers of cells in the later phase of the reaction.

The mononuclear cells recovered from these chambers responded to PHA *in vitro*, indicating that there is a functional T-cell component to the influx. It should be possible to clone these cells, enabling us to examine directly the functional capacities, such as the antigen sensitivity and production of lymphokines, of the T-cells responsible for DTH.

DTH is directed by cells that are believed to carry out many of their functions via soluble mediators (CF). Previous attempts to study these CF have been hampered by the lack of an appropriate source of material (5). The serum recovered from the chambers is likely to have been conditioned by the responding cells. Thus it should be a good source of the cytokines produced by cells in the DTH reaction.

Any method applied to research involving human subjects must be both useful and practical. This technique was well tolerated by all subjects, and had a leakage rate of less than 7%. Contamination, chiefly *Staph. epidermidis* occurred in less than 4% of the chambers, with no ill effects to the subjects.

In total, placing pairs of chambers on 100 subjects for periods of up to 72 hours, none chose to withdraw from the study. Thus the technique can be applied with confidence that it is safe, and will be accepted by test subjects.

In conclusion, the skin window chamber technique described here provide an opportunity for the first time to recover, clone and analyze exclusively both the cells involved in DTH, and the cytokines and other factors that they may produce.

Chapter 4: The Human T-DTH Cell, a clonal analysis

Introductory comments

The skin window chamber technique detailed in the previous chapter was developed to allow the recovery of mononuclear cells and their products from DTH sites, without their contamination with cells from the circulation, and gives the opportunity to analyse the cells and to determine their functional capacities directly.

The cells from DTH sites were cloned using the same Ag as was used to elicit the DTH reaction. The resulting clonal population was then defined according to surface marker expression.

The fraction of T-cells present at DTH sites that were specific for the Ag inducing the DTH has previously only been estimated in animals using methods that are impractical in humans (66,68,69).

Cloning skin window chamber cells using Ag as the stimulant, and using limiting dilution frequency analysis, the accurate determination of the frequency of Ag-specific T-cells at DTH sites in humans to be made for the first time.

In terms of function, Mossman *et. al.* have recently proposed that murine CD4+ TH can be divided into two populations, one directing inflammatory and cytotoxic processes and the other providing help for Ig synthesis (119). The existance of a similar functional dichotomy in human TH populations has not been fully established (118). One reason for this has been that in neither the human nor the mouse have the appropreate TH been shown to be present at Ag-specific inflammatory sites, the prototype of which is DTH.

The clones derived in the course of the frequency analysis were submitted to functional analysis, permitting a detailed picture of the capacities of the cells present at DTH sites to be determined.

Activated cells expressing the high affinity receptor for IL-2 (5,4,6,82) & IL-2 producing T-cells have been found at DTH sites (41), and among clones capable of mediating DTH (118,120). These same clones were also shown to be cytotoxic. Thus it was logical to expect IL-2 production and cytotoxicity by TDTH cells. TNF is a potent cytotoxic CF (6). Both a and β TNF can produce a DTH-like reaction (104,110). IFN-g has been shown to produce a DTH-like lesion when injected i.d. (103,104). It has also been shown to synergise with both IL-1 and TNF to produce a DTH-like reaction at lower doses (104,110).

The production of both IL-2 & IFN-g has been used to help define the TH1 inflammatory T-cell in mice (119). IL-2 production and the capacity to give B-cell help have been associated with two independent T-cell populations in the mouse (121,119). B-cells are not normally found at DTH sites (5). Consequently it has been assumed that helper cells for Ab synthesis would not be found at DTH sites.

Accordingly, the capacity of T-cell clones from DTH sites to carry out the functions described above were determined in order to define the T-cell population participating in DTH.

Additionally, the fluid from skin window chambers also allowed for direct confirmation that specific cytokines were produced *in vivo* at DTH sites in humans.

The results of these descriptive and functional tests will be discussed in this chapter.

Materials and Methods

Subjects

The subjects utilized in these experiments were volunteers who gave informed consent for the proceedure. The subjects were 2 females and 4 males, aged 21 to 68 yrs. One subject whose cells were used to generate T-cell lines (Po) was a hospital patient. All other subjects were healthy laboratory volunteers without underlying illness. All subjects were reactive as defined in the previous chapter. Only antigens to which the subjects were reactive (ie DTH/5mm) were injected. Subjects were skin tested at least 48hrs prior to the placement of skin window chambers over newly injected sites.

Preparation of serum

Serum was prepared from 10cc of peripheral blood obtained by venipuncture 24 hrs earlier. The blood had been collected into Vacutainer tubes (B-D) without anticoagulant and allowed to clot at room temperature for 2 hrs. The clot was then detatched from the walls of the tube using a sterile pasteur pipette, and allowed to shrink for 4 hrs @ 4℃. The tube was then centrifuged @ 400g for 10min and the serum collected. The serum was recentrifuged as before, and then drawn off. Just prior to the preparation of the chamber sites, 1cc of serum was diluted with 9cc of normal saline (Travenol).

Peripheral blood mononuclear cell preparations

Following the recovery of cells from the skin window chambers, blood was drawn by venipuncture and defibrinated by swirling with sterile glass beads for 20min. The defibrinated blood was centrifuged @ 400g for 20min and the serum removed and filtered through 0.22 μM sterile filter. A volume of HBSS equal to the volume of serum recovered was then added to the cells and the mononuclear cells recovered by centrifugation over Ficol-Hypaque as described in chapter 3. Samples of serum were frozen @ -20℃ for later use.

Epstein-Barr virus preparation

EBV was obtained from the supernatants of B95-8 marmoset cells obtained from Dr. Jose Menezes (St. Justine Hospital, Montreal). The cells were grown in RPMI + 10% FCS in 250ml flasks (Falcon) for 7 days. The supernatants were collected by centrifugation @ 400g for 20min and filtered through a sterile 0.22uM filter (Millipore). Aliquots were frozen @ -70c for storage and thawed by vigorous agitation in a 37c water bath immediatly prior to use.

EBV-transformation and establishment of B-cell lines

PBM were suspended in RPMI + 20% FCS + 2.5ug/ml of cyclosporin-A (hereafter referred to as EBVt medium) (Sandoz) in 13x100mm glass test tubes (Canlab) at a concentration of 106 cells/ml. One ml of cell suspension was centrifuged @ 400g for 10 min and the supernatant discarded. 0.2ml of EBV suspension was added and the cells incubated for 1hr @ 37c, with the cells being agitated every 15 min. Following the incubation, 0.8ml of EBVt medium was added and the cells incubated @ 37c in a 5% CO2 atmosphere. The medium was replaced weekly until colonies were visible. These colonies were transferred to 50 ml culture flasks (Falcon) in 7ml of EBVt medium. When the cells were growing rapidly the medium was replaced thrice weekly with RPMI + 10% FCS.

Antigen reactive PBM cultures (Lymphocyte transformation tests)

One hundred thousand PBM, suspended in 100ul of RPMI 1640 + 20% NHS, were placed in flat-bottomed microwells of 96-well plates (Falcon). One hundred ul of RPMI + 10% NHS containing antigen preparations at various concentrations was then added to each well. The cultures were grown for 7 days in a 5% CO2 atmosphere. 1uCi of 3H-Thymidine (S.A. 20 Ci/mmol, NEN) was added in 20ul of RPMI 1640 to each well for the last 6 hrs. The plates were then frozen at -20c thawed and the wells harvested with a MASH apparatus, and 3H-Thymidine incorporation into DNA determined as described in chapter 3). Positive response was defined as a stimulation index

greater than or equal to 5 and greater than or equal to 4,000 new CPM.

All cultures were performed in either duplicate or triplicate. The antigen preparations used in the cultures were:

PPD (Connnaught, lot# A28): 20µg/ml
PPD (SSI, lot# RT40): 20µg/ml

TET (Merieux, lot# GP3.2): 0.64 U/ml

CAN (Merieux, lot #02A3M7): 20 U/ml

The antigen preparations from Merieux were the kind gift of Rhone-Poulenc Ltd.

T-cell cultures

1: T-cell growth medium

T-cell cultures were maintined in medium which contained IL-2 derived from two sources; supernatant from the cell line MLA 144, cultured in RPMI 1640 + 5% FCS + glutamine (.02mM, GIBCO) and/or the supernatants from PBM cultured for 24 hrs in RPMI 1640 + glutamine (.02mM, GIBCO) + 10% FCS + 1 µg/ml of Protein-A (Pharmacia). The supernatants were harvested by centrifugation @ 200g for 20 min. The MLA 144 supernatants were heat inactivated @ 60% for 30 min and ultracentrifuged @ 100,000 rpm for 30 min to remove virus particles. Individual preparations of MLA 144 supernatant were tested in the CTL-L IL-2 assay as described below and sufficient supernatant added to the TCGM to induce maximal proliferation.

The complete T-cell growth medium was:

RPMI 1640	
glutamine	.02mM
MLA 144 supernatant	2-20%
PBM Protein-A supernatant	10%
NHS	15%
gentamycin(GIBCO)*	0.1mg/ml
fungizone(GIBCO)*	2.5µgm/ml

* when neccessary

2: Recovery of Cells from DTH sites

The preparation of skin window chamber sites and the recovery of cells from the chambers was carried out as detailed in the previous chapter with only minor variations. All chambers were harvested at a single time point of 48 hrs.

In some experiments, to minimize the chance of leakage, a thin layer of the human thrombin-fibrinogen sealant TISSEAL was applied to the rim of the chamber immediately before placing it over the site. The TISSEAL was allowed to set prior to the filling of the chambers with 10% autologous serum. TISSEAL was the kind gift of Immuno Canada.

3: Bulk T-cell lines

Various numbers (5,000-100,000) of PBM or Skin Window cell suspensions (containing 5,000-100,000 Mononuclear cells) were cultured in TCGM with either PHA (1µg/ml Wellcome) or antigen (PPD @ 20µg/ml Connaught; Candida @ 20 U/ml, Merieux; Tetanus 0.64 U/ml, Merieux) in 24-well plates (Linbro). PBM (10⁶/well) irradiated with 5000 rads from a ^{137}Cs source (Gammacell 40, AECL, 135 rads/min) were included as feeder cells. PHA stimulated cultures utilized either autologous or allogeneic feeder cells. Antigen stimulated cultures utilized autologous feeder cells. Fresh medium was added thrice weekly and the cultures were re-stimulated every 2 to 3 weeks.

4: Establishment of T-cell clones by Limiting Dilution

T-cell clones were established in 96-well flat bottom plates (Falcon). One hundred thousand PMB, irradiated as above, were used as feeder cells, autologous PBM were utilized for Ag stimulated cultures and either allogeneic or autologous irradiated PBM for PHA stimulated cultures. Varying numbers of mononuclear cells were added to the wells using concentrations that did not give growth in all wells. The final culture volume was 200µl of TCGM with/without either PHA(1µg/ml, Wellcome) or antigen as described above. The cultures were grown @ 37° in a 5% CO_2 atmosphere. Approximately 100µl of medium was removed by suction every 7 days, and the wells refilled with fresh TCGM.

The plates were examined under 100X magnification at 14 and 21 days. Wells showing growth had their contents transferred to 24-well plates. Clones were expanded in the same manner as the bulk T-cell lines (see above). Prior to stimulation or the use of the clones in surface phenotyping or functional assays, the

well contents were layered over Ficol-Hypaque and the viable lymphocytes recovered as described in Chapter 3.

5: Frequency Analysis

The determination of the frequency of responding cells in the limiting dilution analysis (LDA) was carried out using a modification of the proceedure of Taswell (177). The number of wells showing growth at each dilution was recorded and used to calcul ce the log10(% non-responding wells)(heretofore referred to as the LogNR). The number of CD3+ cells in the PBM preparation used as responders was determined by immunocytochemistry as described below. The ratio of CD3+ cells: total mononuclear cells was calculated and this ratio was used to determine the actual number of CD3+ cells/well used in the LDA as follows:

$$\%CD3+ \text{ cells/well} = \#\text{ mononuclear cells/well} \times \frac{\#CD3+ \text{ cells}}{\#\text{ mononuclear cells}}$$

The -logNR was plotted vs the number of CD3+ cells/well using a least-squares linear regression program (HSD, Curvefit) on a microcomputer. The equation of the best-fit straight line for the data, and the r2 coefficient were obtained from the program. The unit frequency of responding cells was determined according to poisson statistics as the point on this line corresponding to 37% negative responding wells. The number of cells/well at this point was considered to be the frequency of responding cells. Since all T-cells in the PBM preparation should be capable of responding to PHA, the reciprocal of the unit frequency for this population was considered to be the overall cloning efficiency for the LDA of that subject.

In the case of the skin window cell preparations, direct evaluation of the number of CD3+ cells via immunohistochemistry was not possible. Accordingly the observed frequency of response from the cultures was calculated in terms of mononuclear cells/well. The frequency of respose was obtained by multiplying this value by the unit frequency of CD3+ cells (ie the reciprocal of the cloning efficiency) observed for the PBM preparations.

In one experiment, as a control, 100µl of RPMI 1640 +10% NHS was added *in lieu* of responders, to feeder cells.

6: Determination of the frequency of Ag-specific T-cells

To determine the frequency of T-cells responding to Ag, limiting dilution cultures were set up as above except that in place of PHA, antigens (Candida, PPD and Tetanus) were used as stimulants as described above. To correct for the actual number of T-cells in the responding cell preparations the observed frequency of responding cells was divided by the frequency of PHA-reactive cells as shown below:

$$\text{Adjusted Frequency of Ag-reactive cells} = \frac{\text{Observed Frequency of Ag-reactive cells}}{\text{Frequency of PHA-reactive cells}}$$

(Immuno)histology

1: Cytocentrifuge preparations

Cytocentrifuge preparations were prepared as described in chapter 3.

2: Cell Typing slides

Slides for use in the typing of cells by immunoperoxidase were prepared following the method of Bross (178). Briefly, Gum arabic was prepared by mixing 10gm of Sucrose (Sigma) with 10 gm of Gummi Arabicum (BDL) with 40ml of ddH2, and the reagents dissolved by gentle swirling in a 37° waterbath. The mixture was cooled to RT, 0.5ml of NaN3 (0.5%, Fisher) added, and the mixture aliquoted and frozen at -20°. Polysiloxan was prepared by mixing 8ml Polysiloxan (DMPS 2X Sigma) with 42ml of Propanol (Fisher) and 0.8ml H2SO4 (18M, Fisher). Poly-L-Lysine solution was prepared by dissolving 100mg Poly-L-Lysine (Sigma) in 200ml ddH2O, and adding 20ml of Glycerin (Fisher), 0.5ml HEPES (Fisher, pH 7.5, 1M in PBS) and 2ml NaN3 (0.5%, Fisher) and aliquots frozen @ -20°.

Microscope slides (Fisher) were cleaned with 100% Ethanol and dryed. Small (approx 20µl) drops (8-12/slide) of Gum Arabic solution were applied to the slides using a 1ml syringe (B-D) tipped with a 27-Gauge needle (B-D). The slides were dryed

overnight at RT, and then coated with Polysiloxane solution, allowed to dry overnight, and soaked for 24hrs in Extran 300 (BDH, , 1:20 in ddH2O). The slides were washed vigorously with ddH2O and dried. Twenty Ul of poly-L-lysine solution was added to each of the hydrophilic spots on the slides which were stored upright at RT until use.

3: Immunohistochemical detection of surface antigens

Immunohistochemistry slides were placed on ice and poly-L-lysine removed by suction. The spots were washed four times with cold PBS (pH 7.4). Cells to be typed were washed in cold PBS and suspended @ 105-106/ml. 20Ul of the cell suspension was added to each spot, and the cells allowed to attach for 1 hr. The PBS removed by suction and replaced with MAG (blocking solution .1% Gelatin (GIBCO) .01% BSA, .05% NaN3 in DMEM) for 20 min. The cells were then washed thrice with PBS. Fifteen Ul of 1° antibody was then added to the spots and incubated for 1hr. The spots were then washed thrice with PBS and 15Ul of fixer (0.05% gluteraldehyde (Fisher), 1% glucose (Fisher) pH 7.8) added. The fixing solution was replaced after 15 min. After the second 15 min period the fixer was replenished and the spots allowed to sit an additional 15 min. They were then washed three times with PBS and replaced with MAG for 20 min. The 2° antibody (Rabbit anti-mouse Ig, Cedarlane 6002, diluted 1:2500 with MAG + NHS 1:625 + Normal Swine serum, Cedarlane 1100 1:625) was then added in 15Ul and incubated for 1 hr. The spots were washed and the 3° antibody solution (Swine anti-rabbit Ig, Cedarlane 6200, diluted 1:20 with MAG + NHS 1:10) was added, and incubated for 1hr.

The cells were then washed and the pre-formed peroxidase-(rabbit)anti-peroxidase complex (Cedarlane Z116, diluted 1:20 in PBS + MAG 1:20) added to each spot and alowed to incubate for 1hr. The spots were then washed and 15Ul of the substrate (DAB, Sigma, .05mg/ml in TRIS buffer (.025M, pH 7.6) plus .015% H2O2 (Fisher) added to the spots. The slides were allowed to develop at RT for 20 min. The substrate solution was washed away with three changes of PBS and the slides air dried, and counterstained with 1% methyl green in ethanol (Gurr). The

slides were examined by light microscopy at a magnification of 40X. Positive cells displayed a complete ring of dark brown substrate around their edges.

Functional Assays

1: Cell Mediated Cellular Cytotoxicity

The cytotoxic function of T-cell clones was assessed as follows. NK-like cytotoxicity was determined using K562 cells as targets in the absence of lectin. Lectin dependent cellular cytotoxicity (LDCC) was assessed using K562/U937/EBV-transformed B-cells as targets in the presence of 1% PHA (GIBCO). Target cells, either K562, U937 or autologous EBV-transformed B-cells were labelled with 51Cr by suspending 10x106 cells in 0.3-0.5ml of RPMI 1640 + 10% FCS and adding 100µl of 51Cr (NEN, 312 Ci/mmol). The cells were then incubated for 1 hr @ 37c, after which they were washed 3 times with HBSS. The cells were then allowed to incubate @ RT for 30 min in med & washed again. The cells were resuspended @ 200,000 cells/ml, in RPMI 1640 + 10% FCS with and without 1.0µg/ml PHA (GIBCO). One hundred µl of the targets were placed in the wells of 96-well flat bottomed plates. Clones to be analysed were suspended in RPMI 1640 + 10% FCS and added to the wells in various numbers. Killer:Target ratios varied from 2-10. After 6 hrs, 100µl of supernatant was removed, placed in 12x75mm glass tubes and radioactivity counted in a γ-counter (LKB). Wells which contained only target cells + 100µl of medium *in lieu* of Killer cells were used as controls. Spontanious release was determined by counting 100µl of supernatant from these wells. Total label was determined by suspending the target cells in these wells, and counting a 100µl sample from the wells.

Specific Lysis was defined as:

$$SL = 100 \times \frac{\text{Expt. Release} - \text{Spontaneous Release}}{\text{Total label} - \text{Spontaneous Release}}$$

Cytotoxic activity was defined as a specific lysis greater than or equal to 10.

2: B-cell Help

Assays of the capacities of T-cell clones to provide help for Ig synthesis was kindly performed by M. Blain in the laboratory of Dr. J. Antel according to their method (191). Briefly, 5x104 clone cells were cultured in triplicate with 5x104 allogeneic non-E-rosetting PBM (B-cells) in 200ul of RPMI + 10% FCS ± PWM (1:100, GIBCO) for 7-days. At that time, samples of the supernatants were taken for determination of the levels of human Ig.

Human Ig levels in the culture supernatants were measured in an ELISA assay utilising a goat anti-human IgG (Cappel) capture Ab. Various dilution of the supernatants were added, followed by a biotinylated anti-human Ig antibody and an avidin-peroxidase complex (Vector) and developed with OPD. Net Ig levels were determined by subtracting the levels of Ig produced by the non-E-rosetting PBM alone.

LK Production Cultures

T-cell clones at least 10 days post-stimulation were purified over Hypaque-Ficoll as described previously and then suspended in RPMI + 12% NHS ± 1ugm/ml PHA (Wellcome) or 1:100 PWM (Gibco). When possible, separate cultures were set up for each LI to be tested. Samples of supernatant were taken after 12hrs for IL-2 determination, and 48hrs for the determination of TNF and IFN-g levels in the supernatant. In some experiments, cultures employed adherent autologous APC + 20ugm/ml PPD, and supernatants were removed after 12 hrs and their IL-2 levels determined.

3: IL-2 Assay

IL-2 levels in the culture supernatants were determined by the CTL-L proliferative bioassay method of Gillis (193). Briefly, 20,000 CTL-L cells were placed in flat-bottomed 96-well microtitre plate wells in 100ul of RPMI + 10% FCS. Supernatants from the cultures were added in various concentrations. IL-2 enriched supernatants from MLA 144 cells were used in serial dilutions to construct a standard curve. All wells were incubated for 24 hrs at 37C in a 5% CO2

atmosphere. One microcurie of 3H-Thymidine was added to the cultures for the last 6 hrs. The cultures were frozen, thawed, harvested and counted as described previously.

4: IFN-g Assay

IFN-g levels in the T-cell clone supernatants, serum or the fluid from SW chambers were measured with a commercial forward sandwich RIA (Centocor). This sensitivity of this assay was 0.1 NIH U/ml.

5: TNF Assay

TNF-like activity levels were kindly detemined by Dr. L. Sanchez-Cantu, using the method of Granger *et. al.* Briefly, 25,000 L929 cells were grown overnight at 37 C in a 5% CO2 atmosphere in 96-well microtitre plate wells in RPMI + 10% FCS to establish a nearly confluent monolayer. Actinomycin D (0.1ugm/well, Sigma) was added followed by serial dilutions of the T-cell culture supernatants, serum or the fluid from SW chambers. Serial dilutions of recombinant murine TNF-a (Cetus, specific activity 2x107 U/mg) were included to generate a standard curve. After 18 hrs incubation, 20ul/well of freshly prepared MTT were added and the cells incubated for a further 4 hrs. The supernatant was removed and replaced with acid isopropyl alcohol. The plates were then read in an ELISA reader with a 550 nm filter. Results were expressed as Units/ml, with 1 unit defined as the amount of TNF required to produce a 50% decrease in absorbance relative to control cells exposed to actinomycin alone. One unit equalled 35±17 pg/ml and the final results are reported as pg/ml. Wells containing less than 0.5 units of TNF-like activity were considered negative. This assay measures the cytotoxic activity in the supernatants and may not be limited to the detection of TNF-a/β.

6: Ag Specific Proliferation

The proliferative response of T-cell clones to Ag was determined by culturing various numbers (7,000-20,000) of cells /well plus 100,000 irradiated (4,000 rads) autologous PBM in 96-well round bottomed plates (Linbro) in TCGM ± Ag (in concentrations as described previously). The cell were cultured at 37° for 72 hrs with 3H-Thymidine added for the last 6 hrs and the plates frozen, thawed, harvested and counted as described previously.

Results

Establishment of Bulk T-cell Lines from Skin Window Chambers

A large number of mononuclear cells were present in the skin window chambers, although it was difficult to determine the proportion of these that were lymphocytes. A weak proliferative response was obtained in response to PHA indicating the presence of viable T-cells (data not shown). The small response to PHA led us to question whether the culture conditions might be suboptimal. The number of PMN found in the SW chamber cells, as compared to normal PBM preparations, was thought to be responsible. Attempts to remove them by different techniques were not sucessful, because of the propensity of the cells to form aggregates, and their failure to phagocytose normally. To characterize the T-cells recovered from the chambers, it was decided to expand the cells in TCGM under conditions likely to support the growth of all T-cells. SW cell suspensions containing two-hundred and fifty thousand mononuclear cells were cultured, and the growing cells were phenotyped.

In all cases, the cells from SW chambers grew in bulk cultures. The results of cultures from chambers of different individuals, shown in Table 4.1, demonstrated that both CD4+ & CD8+ cells were present, in varying proportions.

Cloning of T-cells directly from Skin Window Chambers

Having establishing that T-cells from the chambers could be cultured in bulk it was decided to clone them directly from the chambers for functional & phenotypic analysis. Various numbers of mononuclear cells/well (from chambers) were cultured in 96-well plates, with irradiated autologous PBM as feeders & PHA as the stimulant. Cultures were also set up with different Ag, particularly the Ag used to generate the DTH reaction in each subject. Similar cultures were set up, with PBM as responder cells.

The fraction of non-responding wells for each different number of cells/well was used to determine the frequency of responding cells by a least-squares linear regression according to single-hit poisson kinetics. The results for one experiment

(Subject B) are given in Tables 4.2 & Fig. 4.1.

As shown in Table 4.2, the cells from the blood and SW chamber were cultured at various numbers of cells/well. The percentage of wells responding is shown in the rightmost column. The numbers of cells/well used were chosen so that several cultures in each series would show less than 100% response. These results were used to determine the frequency of responding cells as described previously. Clones for analysis were picked from the cultures showing the lowest percentages of responding wells.

Fig. 4.1 shows the frequency plots of the cultures from the same subject. The percentages of responding cultures resulted in straight lines with large r^2 values, indicating that the cultures were properly conducted. This, and the near-zero values of the Y-intercepts indicates that the cultures were limited by only one cell type. The slope of the lines is related to the frequency of that responding cell type within the population of cells used in the cultures. The calculation of the frequency of responding cells was performed as previously described. The results from three other experiments are shown in Table 4.3 and 4.4.

When PBM were cloned using PHA the frequency of responding cells was 1/1.08, 1/1.05, 1/1.06 & 1/1.07, corresponding to cloning efficiencies of 92.6, 95.2, 94.3 and 93.5% respectively. The frequency of responding cells from the chambers were 1/160, 1/1257, 1/48.1 and 1/16.1 mononuclear cells. Attempts to determine the ratio of CD3+ cells to tot l mononuclear cells in these preparations by immmunohistochemical techniques were unsuccesful due to high background staining. Thus these frequencies could not be normalized. Since the same culture conditions resulted in 90% cloning efficiencies for the PBM, it was concluded that the the PHA response frequencies for the skin window chamber cells could be used to normalize the Ag response frequencies of the SW cells.

Ag-Specificity of T-DTH Cells

Part 1: T-cells from Skin Window Chambers are enriched for cells reactive for the Ag inducing the underlying DTH

Using Ag as the stimulant, cloning the skin window chamber cells with Ag allowed the accurate determination of the frequency of Ag-specific T-cells at DTH sites.

The results of these experiments, in Table 4.3, show that the frequency of Ag-reactive cells was greater in the skin window chamber cells of all the subjects than in their blood. The frequency of Ag-reactive cells in the chambers ranged from 1/20.7 to 1/2.3. The degree of enrichment for Ag-reactivity relative to the blood varied from 2.6-fold to 314-fold.

Part 2: Only T-cells Reactive to the Eliciting Ag are Enriched in the Chambers

The results shown in Table 4.3 could be interpreted as reflecting a preferential recruitment &/or accumulation at the DTH site of T-cells specific for the Ag used to elicit the DTH.

It was also possible that the "reactive" cells in the Ag-stimulated cultures had been previously activated by other Ag *in vivo*, were preferentially attracted to DTH sites, as has been reported in animals (80-82), and were growing *in vitro* as a result of the presence of IL-2 in the medium. Finally, it could also be hypothesised that a small fraction of the feeder cells had escaped the lethal effects of irradiation and had proliferated in the Ag cultures.

To test these hypotheses, an additional cloning experiment was carried out using one of the subjects shown in Table 4.3 who was reactive to PPD & TET. Skin window chambers were placed over PPD-injected sites. After 48 hrs the cells harvested from the chambers, along with PBM, were cloned using PHA, PPD & TET as stimulants.

The results (Table 4.4) indicated that there was a 5-fold enrichment for cells reactive to the Ag eliciting the DTH reaction. There was no enrichment for cells reactive to TET, an Ag to which the subject gave a positive skin test. Additionally,

the cells recovered from the chamber did not grow in the cloning medium itself; an additional *in vitro* stimulus was required. The feeder cells themselves were not capable of growth.

It is valuable to compare the PPD-reactive frequencies of the PBM in the two different experiments with subject S shown in Tables 4.3 & 4.4. The two frequencies (1/150 and 1/180) were equivalent, with complete overlapping of their respective 95% Confidence Intervals. The close concordance from these different experiments indicated that the cloning technique produces reliable estimates of Ag-reactivity.

Ag-Stimulus Derived T-cell Clones Proliferate in Response to Ag

To insure that the Ag-derived clones did indeed respond to the Ag, Ag-derived T-cell clones from the chambers were tested for their proliferative response to Ag. The tests were carried out following Ag-specific expansion in 24-well plates, with a maximum of two non-specific expansions in order to minimise the potential for loss of specificity. The results, shown in Table 4.5a show that 3 of 4 PBM and 6 of 7 SW Ag-derived clones reacted *in vitro* to the Ag to which they were raised.

Clones from subject S were tested for their Ag-reactivity immediately following microscopic examination on day 21. The results, given in Table 4.5b showed that 6 of 8 clones from SW chambers and 6 of 7 PBM-derived clones responded in an Ag-specific fashion. One of the clones that did not give positive responses met one but not both of the response criteria. None of the clones showed reactivity for any Ag other than the Ag used to generate them originaly. Overall, 80% of the wells showing growth in response to Ag, were Ag-specific.

Surface Phenotypes of T-cell Clones

The clones derived from three subjects were typed by immunohistochemistry for their surface markers. The results (Table 4.6) showed that both CD4+ & CD8+ clone were generated. When cultured with PHA, the percentage of CD8+ clones obtained both from PBM and from the chambers tended to be lower than the

% of CD8+ cells in the blood. Thus results using PHA must be interpreted with some caution as there was some selection against CD8+ cells.

When cultured with Ag, the % of CD8+ clones from the window was similar to the percentage of CD8+ lymphocytes found in the blood but greater then the percentage of the antigen-specific CD8+ clones generated from the PBM. However the potential for some selection against CD8+ cells must be kept in mind in interpreting these results.

Functional Characterization of the TDTH Clones

Having performed the limiting dilution analysis, it was decided to concentrate on the Ag-specific cells from the skin window chambers in order to define the characteristics of the T-cells participating in DTH reactions. For comparison, antigen-specific clones from the blood as well as few clones grown with PHA were also analyzed. Owing to the technical probems of obtaining sufficient autologous feeder cells, the clones were expanded using allogeneic cells and PHA as the stimulant. The Ag-specific T-cell clones obtained directly from DTH sites were studied as sufficient cells became available (due to different growth rates of the clones).

The functional capacities of TDTH cells have been implied from studies employing differing but indirect methodologies for obtaining T cells involved in DTH. Cytotoxicity, both NK-like and LDCC, B-cell helper function, IL-2, TNF and IFN-g production in response to PHA (and in a few cases, to Ag) were all analyzed.

The overall results of functional tests of the Ag-derived T-cell clones from subjects B & P are shown in Tables 4.7a & 4.7b. Individual categories and other analyses are discussed in separate sections below.

Cellular cytotoxicity of T-cell Clones

The ability to lyse affected target cells is considered an important effector mechanism in CMI responses to viral, intracellular pathogens and to some tumour cells. Previously thought to be restricted to CD8+ cells, this cytotoxic capacity

has recently been reported in some CD4+ cells (168,169). The cytotoxic capacity of the T-cell clones was evaluated in a 51Cr release assay. NK-like cytotoxicity was assessed against K562 target cells. LDCC was determined using PHA and K562, U937 or autologous EBVt B-cells.

Many clones were difficult to grow in large numbers, and thus it was necessary to determine the minimum Killer:Target ratio for detecting cytotoxicity. Effector:Target ratio studies shown in Fig. 4.2 indicated that in those clones showing cytotoxicity, an E:T ratio > or = 2 was sufficient to detect cytotoxicity using a specific lysis (SL) > or = 10 as the criteria. Accordingly, only clones that were tested at K:T > or = 2, or were positive for cytotoxicity at a lower K:T were reported.

The results are shown in Table 4.8. Of the 5 CD8+ clones tested, four were capable of cytotoxic activity in LDCC, 1 clone also showed NK-like(non-lectin mediated) cytotoxicity against K562 targets. One half of CD4+ TDTH clones were cytotoxic, with seventeen percent of CD4+ clones showing NK-like cytotoxicity to K562. Clones that were positive for LDCC against one target were generally positive against both allogeneic and autologous transformed cells (p=0.039, see Tables 4.7a & b).

B-cell helper function of T-cell clones

It has been proposed that a dichotomy exists among CD4+ cells, with TH1 cells directing inflammatory responses and TH2 cells providing help for Ig synthesis (118). B-cells are not normally found at DTH sites (5). If the proposed dichotomy of TH exists, it is unclear whether TH2 cells would be present at DTH sites.

To determine if a functional exclusion existed in human DTH, the capacity of the TDTH clones to provide help for Ig synthesis was assessed in an allogeneic PWM-driven system.

A frequency analysis of the levels of new Ig production in the cultures is shown in Fig. 4.3 (Ig levels > 7000 ng/ml were grouped together). There were a large number of clones which stimulated less than 1,000 ng/ml of new Ig production. This point was chosen as the cut-off for descriminating between

helper and non-helper clones and was comparable to those used by other authors (124,125,170).

As shown in Table 4.9, approximately 1/2 of the CD4+ clones tested provided help for Ig synthesis. Interestingly, one of the three CD8+ clones tested gave help (Table 4.7a & b).

IL-2 production by CD4+ T-cell clones

IL-2 is considered to be the primary, although not the sole, proliferation-permitting signal for T-cells, particularly those believed to mediate DTH (122). Activated cells expressing the high affinity receptor for IL-2 (3,6,7,82) & IL-2 producing T-cells have been found at DTH sites (41), and among clones capable of mediating DTH (118,120). Thus it was logical to expect IL-2 production by TDTH cells.

To determine if TDTH clones produce IL-2, IL-2 levels in the supernatants from T-cell clones were determined using the CTLL bioassay. As shown in Table 4.10, approximatly 1/2 of the clones from all categories produced IL-2 upon stimulation with PHA. Stimulation of TDTH clones by the Ag used to elicit the DTH reaction and to clone them, resulted in IL-2 production by 67% of the clones. Those clones that failed to produce IL-2 following Ag-stimulation did produce IL-2 upon stimulation by PHA.

PWM failed to induce IL-2 production in two clones, one that did produce IL-2 upon PHA stimulation & one that did not (Table 4.7a, Fig. 4.5). One other clone tested did produce IL-2 following PWM stimulation, but the level of production was comparable to that seen without mitogenic stimulation (Table 4.10). None of the 7 Ag-derived CD8+ clones tested, 6 from DTH sites & 1 from blood, produced IL-2 upon PHA stimulation (Tables 4.7a & b).

TNF production by T-cell clones

TNF is a potent cytotoxic CK (6). It has been shown that both the a and β form, alone or in synergy with IFN-g can produce a DTH-like reaction (104,110). To determine if TDTH clones produce this CK, TNF activity in the supernatants of the clones was measured by the L929 bioassay.

As shown in Table 4.11, one third of the CD4+ clones produced detectable TNF activity without stimulation, & two-thirds did so following PHA stimulation. Only one of 5 CD8+ clones tested produced TNF activity following stimulation, but one of the negative clones did produce TNF activity when stimulated in the presence of irradiated EBV-transformed B-cell feeder cells (Tables 4.7a & b). No link could be made between between TNF production and: IL-2, IFN-g, IL-2+IFN-g production or B-cell help.

IFN-g production by T-cell clones

IFN-g has been shown to produce a DTH-like lesion when injected i.d. (103,104). It has also been shown to synergise with both IL-1 and TNF to produce a DTH-like reaction at lower doses (104,110).

To determine if IFN-g was produced by TDTH clones, their supernatants were tested for IFN-g using a commercial forward sandwich RIA. The frequency distribution of IFN-g production by T-cell clones is shown in Fig 4.4 (levels >75 U/ml were grouped). A large proportion of clones produced <5 U/ml, and this was used as the cut-off for IFN-g production.

As seen in Table 4.12, the majority of CD4+ clones tested produced levels >5 Units/ml following PHA stimulation. In 3 CD4+ clones that produced IFN-g following PHA stimulation, PWM stimulation either failed to induce or induced only 1/10 as much IFN-g production (Fig. 4.3, Table 4.12. In contrast, the one CD8+ clone tested produced more IFN-g with PWM than PHA stimulation. All 5 CD8+ clones tested produced IFN-g following stimulation with PHA.

Linkages between functions of T-cell clones

As mentioned previously, it has been proposed, originally by Mossman *et. al.* (119,120) that a functional dichotomy exists among TH cells in mice. A similar dichotomy has been proposed to exist in humans (118) although its existance is as yet unclear. No evidence has been put forth regarding the presence of these cells at DTH sites, in mice or in humans.

In the murine system, the different TH cells have been shown to present a different array of functional capacities. Many of these functions are unique to each cell type, and can be said to be linked, that is, that if a given cell carries out one function which is specific to the TH1 cell type, it will also carry out a number of other, TH1 functions. The existence of such restricted functional phenotypes among the TDTH clones was examined by comparing the distribution of pairs of functional activities among the panels of clones generated in this investigation.

1: IL-2 & IFN-g production are linked

The production of both IL-2 & IFN-g has been used to help define the TH1 inflammatory T-cell in mice (119). The ability of individual human CD4+ TDTH clones to produce IL-2 & IFN-g were compared. As seen in Fig 4.6A, in Ag-derived CD4+ TDTH cells the production of these two LK was positively associated, with a high degree of significance. A similar association was seen in Ag-derived clones from the blood, however the low number of double-negative clones prevented a statistically significant association. If all the T-cell clones were considered regardless of their origin or the original stimulus used to clone them, the association was also highly significant.

2: IL-2 production is inversely correlated with B-cell Help

IL-2 production and the capacity to give B-cell help have been associated with two independant T-cell populations in the mouse (24,119), The ability of individual clones to produce IL-2 & provide help for Ig synthesis were compared (Fig 4.6B). Ag derived CD4+ TDTH clones showed a significant negative association between IL-2 production & B-cell help. This association could not be seen in Ag-derived CD4+ PBM clones due to the small number of clones that did not produce IL-2. When all the T-cell clones were considered, regardless of their origin or original stimulus, the negative association was also highly significant.

3: IFN-g production and B-cell help

IFN-g production and the capacity to give B-cell help have been associated with two independant T-cell populations in the mouse (24,118), possibly as a result of the potent ability of IFN-g to inhibit Ig synthesis (118,120,123). The ability of individual clones to provide B-cell help and to produce IFN-g was compared (Fig. 4.6c). No correlation could be made for CD4+ clones regardless of origin.

As was shown in Fig. 4.5, PWM induced much lower levels of IFN-g for some CD4+ clones than did PHA, and these clones were capable of providing B-cell help in a PWM driven assay. It has been reported by other authors that the addition of anti-(IFN-g) Ab to similar cultures can confer the ability to provide B-cell help to TH1 clones (118). Since IL-2 & IFN-g transcription are controlled by different regulatory elements, it may be that the production of IFN-g in response to PHA stimulation obscures a negative correlation between IFN-g production and B-cell helper activity.

When the responses of the 3 CD4+ Ag-derived PBM clones that produced IFN-g after PHA but not PWM stimulation were considered as negative for IFN-g production, than IFN-g production and B-cell help were found to be negatively associated ($p=0.03$, data not shown).

4: Clones which produce both IL-2 and IFN-g do not provide B-cell help

CD4+ clones in which IL-2 and IFN-g production is linked correspond to the profile of TH1 helper cells (119). When these clones and the reciprocal set of clones were tested for their capability to provide B-cell help (Fig. 4.6d) it was found that for either TDTH clones or all CD4+ clones, IL-2/IFN-g producers did not provide help, while those that did not produce these LK did. Although a statistically significant association was not seen for Ag-specific PBM clones, owing to the dearth of B-helper clones, 6/7 tested fit the TH1 phenotype.

The fluid from skin window chambers over DTH sites contained IFN-g & TNF

T-cell clones from skin window chambers over DTH sites were found to produce both IFN-g and TNF upon mitogenic stimulation. IFN-g has been hypothesized to be an important mediator of DTH and Issekutz and others have shown that IFN-g alone or IFN-g and either TNF-a or β (in lower doses) produce a DTH-like reaction when injected i.d. (110).

Previous techniques have not permitted the direct demonstration of the production of these mediators at DTH sites. The fluid in the SW chambers could be expected to contain CK produced both by cells activated in the underlying DTH reaction and subsequently delivered to the chambers, and by the cells remaining in the underlying DTH site.

The fluid from the skin window chambers of eight experiments was analysed for IFN-g and TNF. As shown in Table 4.13, all the chambers placed over DTH reactions contained IFN-g. Seven of eight chambers contained TNF. The lone negative sample was obtained from a chambers that had been emptied and refilled at 24 hrs and subsequently re-sampled after a further 24 hrs. The levels of IFN-g seen in this chamber were comparable with those found after the first 24 hrs. Neither the AB serum used to fill the chambers, nor the serum from the subjects themselves contained detectable IFN-g or TNF.

Three of these experiments included windows placed over sites injected with an Ag that did not produce a DTH in the subjects. No TNF was detected in these chambers. Two of the three chambers had no detectable IFN-g and one had minimal levels (1.6% of those seen in the chamber over a DTH reaction in that subject). The mean levels of both IFN-g and TNF were significantly higher in the chambers over DTH reactions than those placed over sites injected with a skin test Ag to which the subject did not give a DTH reaction. The presence of IFN-g or TNF were significantly correlated with the injection of an Ag to which the subject was reactive (Fig. 4.7a & b).

Table 4.1 Surface Phenotypes of Bulk SW T-cell Cultures

Subject	Cells	# Cultures	%CD3+	%CD4+	%CD8+
D	SW(PPD)	9	100	97	2
L	"	2	100	8	75
Po	"	7	100	46	55
Overall*	"	18	100	67	31

*: Mean of % in cultures

Table 4.2: T-Cell Cloning Results for Subject B

Responder Cells	Stimulus	Cells Well*	# Wells	# Clones	% Response
PBM	PHA	3.70	48	47	98
"	"	1.85	48	36	75
"	"	0.370	96	34	35
"	"	0.185	96	6	6.3
"	"	0.037	96	2	2.1
"	CAN	74,000	96	96	100
"	"	7,400	96	96	100
"	"	740	96	59	62
"	"	74	96	7	7.3
"	"	7.4	96	0	0
Skin Window Chamber (over Can DTH SITE)	PHA	1,000	10	10	100
"	"	500	20	19	95
"	"	100	54	25	46
"	"	10	96	13	14
"	CAN	1,000	5	5	100
"	"	500	8	6	75
"	"	100	153	27	17.6
"	"	10	90	1	1.11
"	"	1	89	0	0

* CD3+ cells/well for PBM
Mononuclear cells/well for SW chamber cells

Table 4.3: Frequency Analysis of T-cell Clones From Subjects B, P & S

Subject	Cells	Stimulus	Frequency of Reactive Cells (95% Confidence Int.)		Adjusted Frequency (95% Confidence Int.)		R^2
B	PBM	PHA	1.08	(0.40-1.76)			.978
"	"	CAN	776	(714-838)	719	(662-776)	.999
"	SW(CAN)	PHA	160	(143-176)			.999
"	"	CAN	366	(320-412)	2.29	(2.00-2.56)	.997
P	PBM	PHA	1.05	(0.91-1.19)			.994
"	"	PPD	10.19	(7.33-13.1)	9.75	(7.01-12.5)	.999
"	SW(PPD)	PHA	1257	(262-2252)			.804
"	"	PPD	4915	(4598-5232)	3.74	(3.50-3.98)	.998
S	PBM	PHA	1.06	(0.747-1.36)			.995
"	"	PPD	191	(136-246)	180	(128-232)	.995
"	SW(PPD)	PHA	48.1	(10.5-85.7)			.924
"	"	PPD	996	(664-1328)	20.7	(13.8-27.6)	.945

Table 4.4: Frequency Analysis of T-cell Clones From Subject S

Cells	Stimulus	Frequency of Reactive Cells (95% Confidence Int.)	Adjusted Frequency (95% Confidence Int.)	R^2
PBM	PHA	1.07 (0.935-1.20)		.999
"	PPD	166 (58.9-272.5)	155 (55.2-255)	.957
"	TET	160 (150-171)	150 (140-160)	.999
SW(PPD)	PHA	16.1 (10.5-21.7)		.989
"	PPD	486 (230-742.4)	30.2 (14.3-46.1)	.933
"	TET	2129 (1432-2826)	132 (89-176)	.936
"	MED*	No Growth		
Feeders	PHA	No Growth		
	PPD	"		
	TET	"		

* Cloning medium without PHA or Ag but with IL-2

Table 4.5a: Ag-Specific Proliferation of Ag-Derived clones, Subject B

Clone	Origin	APC#	Culture Duration	Stimulus †	CPM	NEW CPM §	STIMULATION INDEX #
none		PBM	72hrs	–	196	0	
"		"	"	CAN	277	0	
"		"	"	PPD	417	0	
BLTC2.3	PBM	"	"	–	1029	–	
"	"	"	"	CAN	14135	12829	16.6
"	"	"	"	PPD	824	–	–
BLTC2.16	"	"	"	–	1012	–	
"	"	"	"	CAN	11126	9837	13.3
"	"	"	"	PPD	833	–	–
BLTC2.17	"	"	"	–	1301	–	
"	"	"	"	CAN	8794	7216	5.69
"	"	"	"	PPD	1192	–	–
BLTC2.22	"	"	"	–	395	–	
"	"	"	"	CAN	841	169	0.76
"	"	"	"	PPD	815	3	2.00
BLTC4.22	SW(CAN)	"	"	–	1853	–	
"	"	"	"	CAN	9216	7086	5.67
"	"	"	"	PPD	1784	–	–
BLTC4.23	"	"	"	–	1342	–	
"	"	"	"	CAN	14284	12665	13.2
"	"	"	"	PPD	2336	577	1.67
none		PBM	72hrs	–	46	0	
"		"	"	CAN	120	0	
"		"	"	PPD	72	0	
BLTC4.10	SW(CAN)	"	"	–	923	–	
"	"	"	"	CAN	6088	5045	6.81
BLTC4.11	"	"	"	–	757	–	
"	"	"	"	CAN	5921	5044	8.16
"	"	"	"	PPD	1189	360	1.57
BLTC4.12	"	"	"	–	530	–	
"	"	"	"	CAN	4917	4267	9.91
"	"	"	"	PPD	914	312	1.74
BLTC4.14	"	"	"	–	525	–	
"	"	"	"	CAN	8799	8154	18.1
"	"	"	"	PPD	772	175	1.46
BLTC4.20	"	"	"	–	867	–	
"	"	"	"	CAN	601	–	–
"	"	"	"	PPD	680	–	–

#: Antigen Presenting Cell
†: The subject gave a positive skin test & *in vitro* proliferative response of their PBM to both CAN & PPD. All the clones tested were raised by *in vitro* stimulation with CAN.
§: New CPM = CPM –(CPM(of APC) + CPM(of clone without Ag-stimulation))

$$\#: \text{Stimulation Index} = \frac{\text{CPM(Clone + Ag + APC)} - \text{CPM(APC + Ag)}}{\text{CPM(Clone + medium)} - \text{CPM(APC + medium)}}$$

Table 4.5b: Ag-Specific Proliferation of Ag-Derived clones, Subject S

Clone	Origin & stimulus	CELLS/WELL	APC*	Culture Duration †	Stimulus	CPM	NEW CPM §	STIMULATION INDEX θ
none			PBM	72hrs	–	27	0	
"			"	"	PPD	201	0	
"			"	"	TET	246	0	
SATC2A.1	PBL	11200	"	"	–	176		
"	PPD	"	"	"	PPD	5914	5442	38.3
"	"	"	"	"	TET	92	–	–
SATC2A.2	"	19900	"	"	–	271		
"	"	"	"	"	PPD	8202	7730	32.8
"	"	"	"	"	TET	182	–	–
SATC2A.3	"	16400	"	"	–	284		
"	"	"	"	"	PPD	8769	8284	33.3
"	"	"	"	"	TET	526	9	1.09
SATC2A.4	"	7400	"	"	–	234		
"	"	"	"	"	PPD	3921	3486	18.0
"	"	"	"	"	TET	68	–	–
SATC2A.5	"	18200	"	"	–	165		
"	"	"	"	"	PPD	11289	10923	80.4
"	"	"	"	"	TET	391	–	–
SATC2A.6	"	9000	"	"	–	236		
"	"	"	"	"	PPD	5382	4945	24.8
"	"	"	"	"	TET	102	–	–
SATC2A.7	"	15800	"	"	–	1201		
"	"	"	"	"	PPD	10932	9530	9.14
"	"	"	"	"	TET	634	–	–
SATC5A.1	SW(PPD)	7200	"	"	–	98		
"	PPD	"	"	"	PPD	399	100	2.79
"	"	"	"	"	TET	225	–	–
SATC5A.2	"	11200	"	"	–	521		
"	"	"	"	"	PPD	5220	4498	10.2
"	"	"	"	"	TET	84	–	–
SATC5A.3	"	13200	"	"	–	289		
"	"	"	"	"	PPD	4950	4460	18.1
"	"	"	"	"	TET	82	–	–
SATC5A.4	"	5200	"	"	–	2890		
"	"	"	"	"	PPD	5232	2141	1.76
"	"	"	"	"	TET	3920	784	1.28
SATC5A.5	"	7900	"	"	–	890		
"	"	"	"	"	PPD	7230	6139	8.14
"	"	"	"	"	TET	1202	66	1.11
SATC5A.6	"	19800	"	"	–	79		
"	"	"	"	"	PPD	11280	11000	213
"	"	"	"	"	TET	1092	768	16.3
SATC5A.7	"	11200	"	"	–	287		
"	"	"	"	"	PPD	8645	8157	32.5
"	"	"	"	"	TET	186	–	–
SATC5A.8	"	9200	"	"	–	195		
"	"	"	"	"	PPD	7654	7258	44.4
"	"	"	"	"	TET	231	–	–

*: Antigen Presenting Cell

†: The subject gave a positive skin test & *in vitro* proliferative response of their PBM to both TET & PPD. All the clones tested were raised by *in vitro* stimulation with CAN.

§: New CPM = CPM – (CPM(of APC) + CPM(of clone without Ag-stimulation))

θ: Stimulation Index = $\frac{\text{CPM(Clone + Ag + APC)} - \text{CPM(APC + Ag)}}{\text{CPM(Clone + medium)} - \text{CPM(APC + medium)}}$

TABLE 4.6: Surface Phenotypes of T-cell Clones

SUBJECT	ORIGIN	STIMULUS	#TESTED	%CD3+	%CD4+	%CD8+
P	PBM	PPD	13	100	85	15
"	SW(PPD)	PPD	24	100	75	25
"	PBM	PHA	8	100	88	13
"	SW(PPD)	PHA	8	100	87	13
"	PBM *	-	-	100	74	26
B	PBM	CAN	15	100	100	0
"	SW(CAN)	CAN	14	100	57	43
"	PBM	PHA	6	100	83	17
"	SW(CAN)	PHA	6	100	83	17
"	PBM *	-	-	100	69	31
S	PBM	PPD	7	100	100	0
"	SW(PPD)	PPD	5	100	80	20
"	PBM	TET	5	100	60	40
"	SW(PPD)	TET	5	100	60	40
"	PBM	PHA	5	100	100	0
"	SW(PPD)	PHA	6	100	83	17
"	PBM	-	-	100	64	36

Table 4.7a: T-cell Clone Function Test Results, Subject B

Clone	ORIGIN & STIMULUS	T4	T8	CYTOTOXICITY NK	CYTOTOXICITY ALLO	B-CELL HELP MED	B-CELL HELP PWM	IL-2 MED	IL-2 PHA	IL-2 PWM	TNF MED	TNF PHA	TNF PWM	IFN-g MED	IFN-g PHA	IFN-g PWM
				@		§		♦			‡			?		
BLTC2.3	PBL on CAN	+		-	-	125	125	-	1.61		-	53		0	3.2	
														0.314	1.53	
.5	"	+		-	-		399	-	1.39		-	21			14.7	
.6	"	+		-	+	244	1,250		1.20			79				
"	"							-	1.69	-	-	-	-	0	9.45	0
.12	"	+		-	+	190	643	-	2.27		-	-			41.8	
.13	"	+		-	-			-	-		-	8.5			2.37	
.16	"	+		-	-	229	3,544	-	-			67			12.4	
"	"							-	-	-	-	-	-	0	3.23	0.362
.17	"	+		-	-	250	997	-	4.04		-	41			86.5	
.20	"	+					0	-	1.85						27.1	
.22	"	+		-	-		250	-	1.54			43			63.7	
.26	"	+		-	-		0	-	1.16		-	22			20.4	
.15	"		+					-	-		-	86			28.9	
BLTC4.2	SW(CAN)	+		-	-	517	992		2.83						14.0	
.3	on CAN	+		-	+		5,792	-(-)	-(-)		-(-)	-(19)			0	
.10	"	+		-	+	0	257	-	2.2		-	33.7			74.6	
.12	"	+					2,676		1.78			-			32.8	
.14	"	+		-	+				2.86			-			43.2	
.20	"	+		-	+		301		1.37						61.4	
.22	"	+					2,596		-						3.23	
.11	"		+	-	-	2,033	9,618	-(-)	-(-)		-(-)	-(-)		0	7.3,	18.8
.15	"		+	-	+			(-)	(-)		(-)	(-)			56.5	
.23	"		+	-	+				-						123	

@: Specific lysis > or= 10. NK Target: K562. Allo Target: Allogeneic EBVt B-cells + PHA

§: New Ig (ng/ml) produced by allogeneic E- cells.

♦: Results are given in Units/ml. Results from experiments with feeders, either autologous adherent APC or autologous EBV-transformed B-cells are shown in brackets. Supernatants with less than 0.5 Units/ml were considered negative (-).

‡: Results are given in Pg/ml. Results from experiments with feeders (autologous EBV-transformed B-cells) are shown in brackets. Wells with less than 0.5 Units of TNF were considered negative.

?: Results are given in NIH Reference Units/ml. Values greater than 50 U/ml exceeded the std curve and should be considered >50.

Table 4.7b: T-cell Clone Function Tests, Subject P

Clone	ORIGIN & STIMULUS	T4	T8	CYTOTOXICITY NK	CYTOTOXICITY ALLO	CYTOTOXICITY AUTO	B-CELL HELP MED	B-CELL HELP PWM	IL-2 MED	IL-2 PHA	IL-2 PPD	IL-2 PWM	TNF MED	TNF PHA	TNF PWM	IFN-g MED	IFN-g PHA	IFN-g PWM
					@		§		♦				‡				?	
POTC2.11	PBL on PPD	+		-	-	-	24,000	70,000	.55	5.6			-	235			99	
"	"								1.40	1.59		1.56	-	-	-	0	9.9	0
POTC4.1	SW(PPD)	+		-	-			0	-(-)	3.64	(4.78)	(.59)	595	2,045		65	62	
.2	on PPD	+		-	+	+	1,350	2,000	-(-)	-(-)			-	1,670			45.3	
.3	"	+							-(-)	-(-)	(.50)						78.4	
.4	"	+		-	-	-	200	948		-							0.576	
.5	"	+		-	-	+	13,000	14,500	-(-)	-(-)			284	334			0	
.6	"	+		-	+	+												
.7	"	+		-	-					.62	(1.43)						9.78	
.8	"	+		+						-	(1.34)							
.21	"	+		-	-	-	200	5,966		-				-			35.6	
.24	"	+		-	-	-	1,295	14,926	-	-			-	106			2.60	
.25	"	+		+	+	+	500	450	-(-)	3.51	(1.08)	(-)	-	1,170		0	24.8	
.28	"	+		+	+	-	0	0	-	3.04								
.30	"	+		-	-	-			-	.59			209	1,670				
.31	"	+			+				-	-								
.34	"	+							-(-)	(.62)	(-)							
.40	"	+		-	-	-			-	1.07			-	-				
.22	"		+	+	+	+		245		-				-			199	
.23	"		+	-	+	+			-(-)	-(-)			(-)	-(40)			35.6	
.33	"		+				0	0	-	-								

@: Specific lysis > or = 10. NK Target: K562. Allo Target: Allogeneic EBVt B-cell + PHA. Auto Target: Autologous EBVt B-cells + PHA.

§: New Ig (ng/ml) produced by co-cultured allogeneic E- cells.

♦: Results are given in Units/ml. Results from experiments with feeders, either autologous adherent APC or autologous EBV-transformed B-cells are shown in brackets. Supernatants with less than 0.5 Units/ml were considered negative (-).

‡: Results are given in Pg/ml. Results from experiments with feeders (autologous EBV-transformed B-cells) are shown in brackets. Wells with less than 0.5 Units of TNF were considered negative.

?: Results are given in NIH Reference Units/ml. Values greater than 50 U/ml exceeded the std curve and should be considered as >50 U/ml.

TABLE 4.8: Cytotoxicity of T-cell Clones

Subject Origin		Original Stimulus	% OF CLONES CYTOTOXIC* (#POSITIVE/#TESTED)					
			CD4+			CD8+		
				LDCC			LDCC	
			NK-LIKE	ALLO	AUTO	NK-LIKE	ALLO	AUTO
B	PBM	CAN	0 (0/10)	20 (2/10)				
"	SW(CAN)	"	0 (0/5)	80 (4/5)		0 (0/2)	50 (1/2)	
P	SW(PPD)	PPD	23 (3/13)	36 (5/14)	40 (4/10)	50 (1/2)	100 (2/2)	100 (2/2)
ALL	SW(Ag)	Ag	17 (3/18)	47 (9/19)	40 (4/10)	25 (1/4)	75 (3/4)	100 (2/2)

*: Target cells: NK-LIKE - K562
LDCC, ALLO - K562 + PHA
" , AUTO - autologous EBVt B-cells + PHA

TABLE 4.9: PWM Driven B-Cell Help by CD4+ Clones

Subject	Origin	Original Stimulus	% OF CLONES PROVIDING HELP * (# positive/# tested)	
			MEDIUM	PWM
B	PBM	CAN	0 (0/9)	22 (2/9)
"	SW(CAN)	"	0 (0/3)	50 (3/6)
P	SW(PPD)	PHA	67 (2/3)	50 (2/4)
"	"	PPD	57 (4/7)	63 (5/8)
ALL	SW(Ag)	Ag	40 (4/10)	57 (8/14)

*: ·/= 1,000 ng/ml new Ig

TABLE 4.10: IL-2 Production by CD4+ T-Cell Clones

Subject	Origin	Original Stimulus	% OF CLONES PRODUCING IL-2* (# positive/# tested)			
			MEDIUM	PHA	PPD	PWM
B	PBM	CAN	0 (0/12)	69 (9/13)		0 (0/2)
"	SW(CAN)	"	0 (0/4)	50 (5/10)		
F	PBM	PHA	0 (0/5)	20 (1/5)	0 (0/2)	
"	"	PPD	100 (1/1)	100 (1/1)		100 (1/1)
"	SW(PPD)	PHA	0 (0/4)	40 (2/5)	50 (1/2)	
"	"	PPD	0 (0/13)	39 (7/18)	67 (4/6)	
ALL	SW(Ag)	Ag	0 (0/17)	43 (12/28)	67 (4/6)	

* Supernatants with greater than 0.5 Units/ml were considered positive

TABLE 4.11: TNF Production by CD4+ T-Cell Clones

Subject	Origin	Original Stimulus	% OF CLONES PRODUCING TNF * (# positive/# tested) MEDIUM	PHA	PWM
B	PBM	CAN	0 (0/9)	90 (9/10)	0 (0/2)
"	SW(CAN)	"	0 (0/2)	50 (2/4)	
P	SW(PPD)	PHA	0 (0/2)	50 (1/2)	
"	"	PPD	43 (3/7)	75 (6/8)	
ALL	SW(Ag)	Ag	33 (3/9)	67 (8/12)	

* Wells containing ≥0.5 Units were considered positive

TABLE 4.12: IFN-g Production by T-Cell Clones

Subject	Origin	Original Stimulus	% OF CLONES PRODUCING IFN-g * (# positive/# tested)					
			CD4+			CD8+		
			MED	PHA	PWM	MED	PHA	PWM
B	PBM	CAN	0 (0/3)	80 (8/10)	0 (0/2)		100 (1/1)	
"	SW(CAN)	"		71 (5/7)		0 (0/1)	100 (3/3)	100 (1/1)
P	PBM	PHA		100 (1/1)				
"	"	PPD	0 (0/1)	100 (1/1)	0 (0/1)			
"	SW(PPD)	PHA		100 (2/2)				
"	"	PPD	50 (1/2)	60 (6/10)			100 (2/2)	
ALL	SW(Ag)	Ag	50 (1/2)	65 (11/17)		0 (0/1)	100 (5/5)	100 (1/1)

*: Supernatants with positive 5 NIH reference Units/ml were considered

TABLE 4.13: IFN-g and TNF in Skin Window Chamber Fluid

		Ag(+)		Ag(-)		SERUM	
Experiment	Time	IFN-g*	TNF$	IFN-g	TNF	IFN-g	TNF
1	0-24	73.8	54			0	0
"	24-48	71.9	0				
2	0-48	7.36	278	0	0	0	0
3	"	137.9	194	0	0	0	0
4	"	56.5	201	0.89	0	0	0
5	"	159	833			0	0
6	"	71.4	277			0	0
7	"	11.0	364			0	0
8	"	85.0	523			0	0
AB SERUM						0	0
MEAN	0-48	75.5	381	0.30	0		
±SE		53.6	212	0.42	0		
p ‡				.025	.025	.025	.025

*: IFN-g levels given in NIH Units/ml
$: TNF levels given in Units/ml
‡: p-values determined by the Wilcoxon Rank-Sum test. Values are given for the comparison with the Ag(+) group.

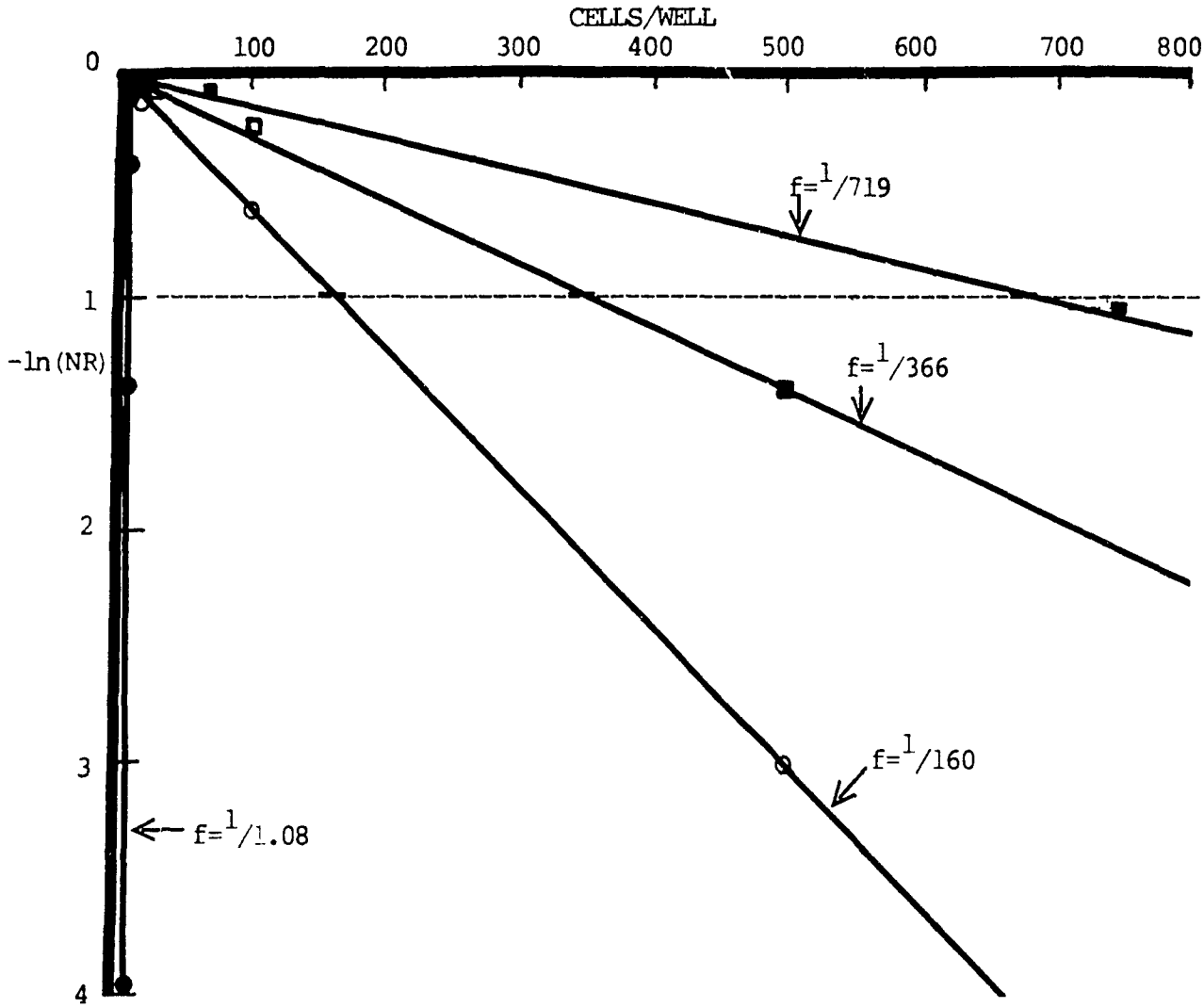

Figure 4.1: Frequency of PHA and CAN reactive T-cells from subject B. PBM and SW chamber cells were cloned in various numbers of cells/well on autologous feeder cells. The -ln of the proportion of non-responding wells was plotted vs the number of CD3+ (PBM) or mononuclear (SW) cells/well and lines fit by a least squares linear regression. The frequency of reactive cells was determined as the # cells/well corresponding to 37% non-responding wells (------ line). Symbols: ● PBM cloned with PHA, ■ PBM cloned with CAN, ○ SW(CAN) cloned with PHA, □ SW(CAN) cloned with CAN.

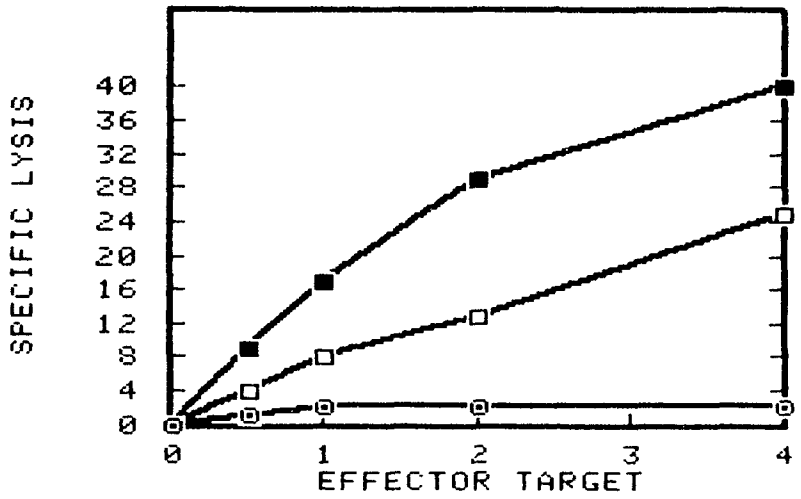

Figure 4.2: Titration of Killer:Target ratio for cytotoxicity screening. To determine the minimum K:T ratio for detecting cytotoxicity (SL</=10) four clones were titrated at various ratios. A K:T ratio of 2:1 was sufficient to detect cytotoxic clones. Symbols ■ POTC4.2, □ CATC1.7, ○ POTC4.24, the fourth clone gave results indistinguishable from those of POTC4.24.

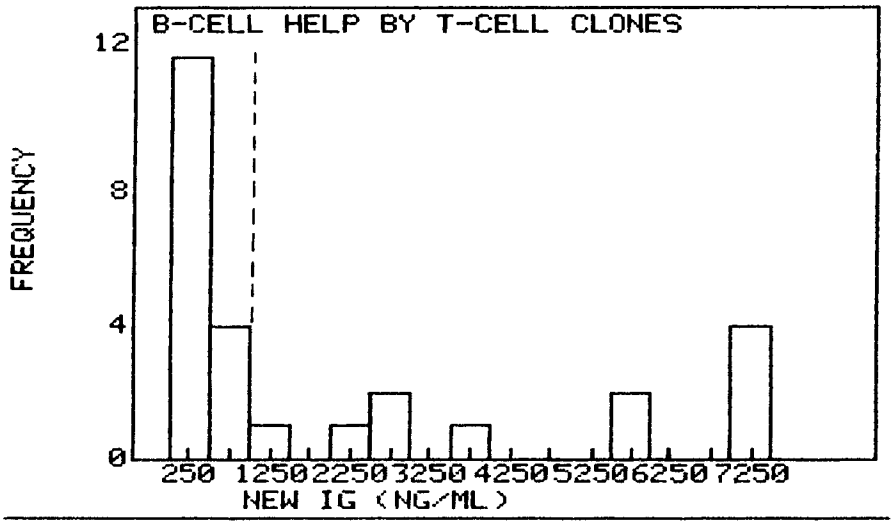

Figure 4.3: Distribution of B-cell helper function in T-cell clones. The frequency of new Ig levels were plotted. Greater than 1,000 ng/ml (------- line) was chosen as the cut-off for helper activity.

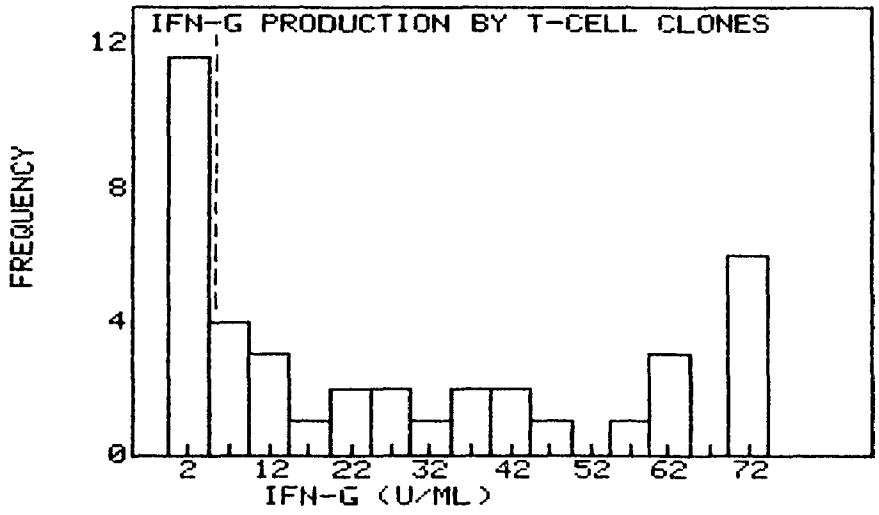

Figure 4.4: Distribution of IFN-g production by T-cell clones. The frequency of IFN-g levels were plotted. Greater than 5 U/ml (------- line) was chosen as the cut-off for IFN-g production.

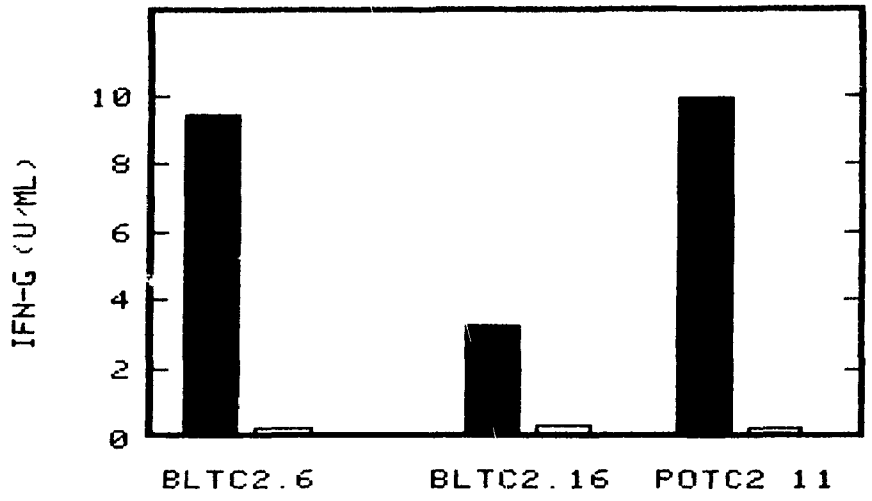

Figure 4.5: Production of IFN-g by CD4+ clones stimulated with different mitogens. Three CD4+ clones, which provided help for Ig-synthesis despite producing IFN-g (upon PHA stimulation) were stimulated by: ■ PHA, or □ PWM, and IFN-g levels measured. Clones BLTC2.6 & POTC2.11 also produced IL-2 upon PHA stimulation (see Tables 4.7a&b). PWM resulted in no detectable IFN-g production or 1/10 the amount produced by PHA. A CD8+ clone (BLTC4.11, data not shown) produced 19 U/ml of IFN-g following PWM stimulation.

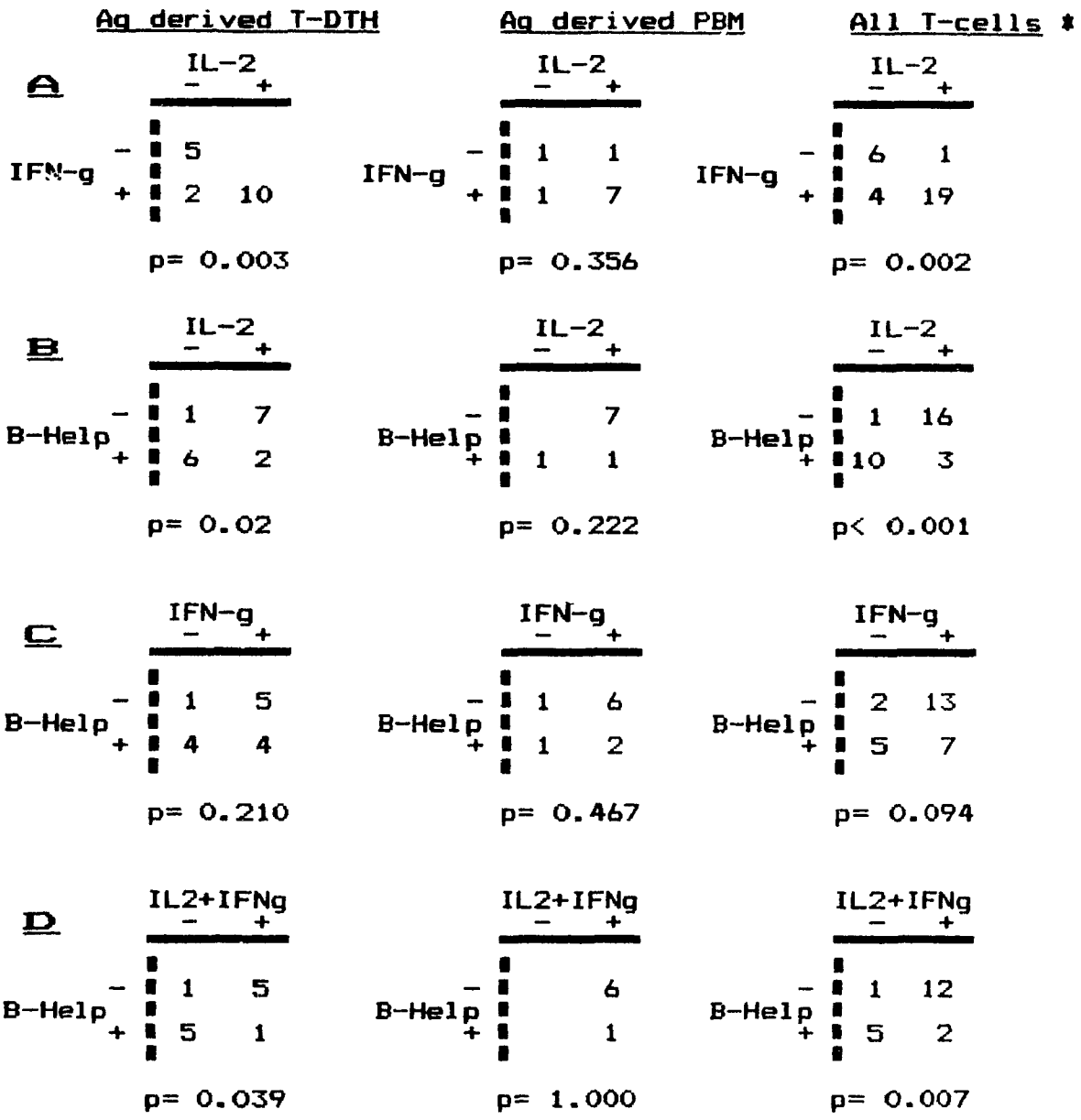

Figure 4.6: Correlations between the production of different CK & B-cell Help in CD4+ T-cell clones. The capacities of individual CD4+ clones were correlated. All T-cells category included 5 PHA derived PBM and 5 PHA derived SW clones. IL-2 or IFN-g production alone was determined following PHA stimulation. IL2+IFNg production was evaluated using PHA stimulation to produce IL-2 and IFNg. Where available, PWM stimulation for IFNg production was used. P-values were determined using Fisher's exact test.

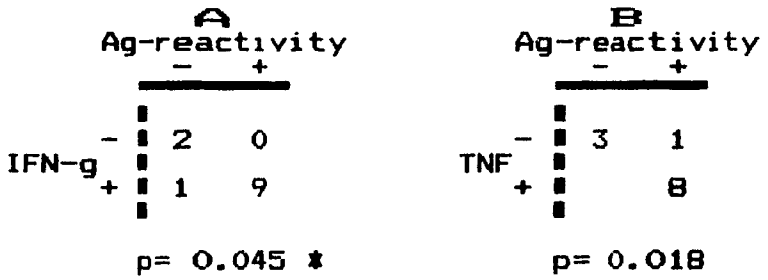

Figure 4.7: Correlations between IFN-g and TNF levels in SW chambers and Antigen-reactivity of DTH sites. SW chambers were placed over skin-test sites injected with Ag to which the subject was either reactive or non-reactive. Fluid was harvested from the chambers after 48 hrs (7 subjects) or 0-24 & 24-48 hrs (1 subject). P-values were determined by Fisher's exact test.

Discussion

The functional repertoire of T-DTH cells has never been precisely established. Numerous *in vitro* functions, demonstrated originally in bulk T-cell populations and more recently in clonal populations have been ascribed to TDTH cells (5,6,118,120). Histological studies by different authors have shown the T-cell infiltrate of DTH sites to consist of conflicting ratios of CD4+ & CD8+ cells, a small number of which express activation surface Ag such as CD25 and OKT10.

Adoptive transfer experiments have identified numerous T-cell subsets involved in DTH, including CD4+, CD8+ and V/V+ cells. Regulatory cells, such as supressor and contra-suppressors have been shown to be involved in some experimental DTH responses, yet adoptive-transfer experiments have also shown that a single TDTH cell may be capable of triggering the entire DTH reaction (75,76). Antibodies to IFN-g have been shown to interfere with DTH (103,104) and recombinant CK such as IL-1, TNF & IFN-g have been shown to synergise to produce local DTH-like reactions (104).

These many elegant and often subtle experimental approaches have yielded much information about DTH & related immune phenomena but by virtue of their indirect approach to studying the TDTH cell(s) they have been unable to directly define the T-cell populations involved in DTH.

The skin window chamber technique allowed for the first time direct access to T-cells from DTH sites without contamination with cells from the peripheral circulation. We exploited this new opportunity to clone panels of Ag-reactive TDTH cells, & by carrying out functional tests upon a large number of these clones, to define the TDTH population.

In order to generate a panel of T-cell clones from the chambers two approaches were possible. The first was to establish bulk T-cell lines from the chambers & then to clone the resulting cells. This approach is often employed in situations where the input population of cells does not respond well in culture (171), or when the number of potential

responding cells cannot be estimated in order to set up appropriate limiting dilution cultures. However, this approach suffers from several major drawbacks.

It is not possible to determine the potential cloning efficiency within the original bulk culture. The relative rate of proliferation among the individual T-cells that do respond to the *in vitro* stimulus in the bulk culture may vary greatly, leading to over and under representation of individual clones.

Some functionally related groups, may naturally proliferate more slowly than others, leading to a similar bias. Finally, the activation of certain clones, or functional groups, such as suppressor and/or cytotoxic cells, may slow the proliferation of adjacent cells, or even eliminate them completely.

The effect of any or all of these problems would result in a preferential expansion of some clones relative to others. jeopardising the underlying assumptions that in subsequent cloning, each clone would originate from a different clonal population, and that all such populations in the original bulk culture would have an equal chance of being represented in the cells distributed to the cloning culture wells.

Since the goal of this investigation required the production of representative panel of TDTH clones, it was decided to clone the SW chamber cells directly. This was accomplished using appropriate numbers of cells per well that resulted in cultures adequately covering the range of response from 100% to 0%. This range was critical, since the mathematical transformations of the data require to calculate the frequency of responding cells cannot make use of data from cultures with 100% responses, & the value of cultures with 0% responses is limited.

The cloning technique employed resulted in a frequency of responsiveness to PHA of 1/1.08, 1/1.05, 1.06 and 1/1.07 from PBM, corresponding to cloning efficiencies of 93, 95, 94 and 94% respectively. The near zero values of the Y-intercepts for the resulting frequency plots (Fig 4.1, subject B, other subjects data not shown) indicate that the cloning cultures followed single-hit poisson kinetics, and therefore are the result of cultures with only one cell limiting the response.

Taken together, these indicate that the technique resulted in the cloning of greater than 93% of the input T-cells (171).

Since the cloning conditions were near optimal for PHA responses of the PBM, it can be assumed that the frequency determinations of Ag-reactive cells among the PBM were also valid. The Y-intercepts of the equations for these cultures were also near-zero (Fig. 4.1, subject B, other subjects data not shown) and thus these clones were also derived from single-cell responding cultures (171). All the clones that were typed were CD3+ regardless of origin (Table 4.6) and thus only T-cells responded in these cultures. The PBM preparations were typed by both histological & immunohistochemical techniques. In setting up the original cultures to determine the true number of CD3+ cells/well, the number of lymphocytes/well that had been placed into the cultures was multiplied by the %CD3+ cells among the lymphocytes. Knowing the precise number of CD3+ cells/well, the true value for the frequency of responding cells could be determined for the PBM.

The SW cells presented a different situation. Despite various attempts to determine the precise number of CD3+ cells in the input population, this could not be accomplished due to the small number of cells available for typing, and technical limitations with the typing procedures. To minimize the number of cells required for typing, the multispot immunoperoxidase technique of Bross *et. al.* was adopted. This method can utilise as little as a few hundred cells for analysis, however, since the frequency of response to PHA varied from 1/16 to 1/1257 (Tables 4.3 & 4.4) for the SW chamber cells, if this indicated that there were very few T-cells in the preparations, the use of low cell numbers may have resulted in some cases in the near or total absence of CD3+ cells from the spots being tested. The problem was further compounded by the presence of other non-mononuclear cells, which must also occupy space on the small spots (typical surface areas: 1-50 mm2) used in this method.

These additional cells presented other technical problems. The CD3 & CD4 Ag are very sensitive to fixation. As a result, the multispot method employed a complex immunohistochemical detection proceedure involving a 1o, 2o, and a 3o Ab plus an enzyme-anti-enzyme Ab complex (PAP). The large number of Ab reagents used made background levels a problem, which was exacerbated by two other undesirable facets of the PMN within the SW chambers. Approximately 50% of these PMN were activated and/or disintegrating and tended to non-specifically bind to Ab even when blocking techniques were employed. These cells proved difficult to remove. Density centrifugation, adherence, "panning" with Ab to various PMN surface markers and phagocytosis of iron particles followed by density centrifugation or magnetic seperatation all served to remove the "normal" PMN within the suspension, but did not remove the "activated/exhausted" PMN. All of these techniques resulted in clumping with attendant non-specific loss of some of the total cell population, thus decreasing the number of available mononuclear cells.

The presence of PMN in the SW cells presented another difficulty. PMN contain high levels of peroxidase, the detection enzyme used in the PAP system, which was released upon fixation. This led to large numbers of positively staining cells in the preparation. Attempts to inactivate this endogenous peroxidase resulted in a loss of detection of CD3 & CD4. To counteract this problem, a different detection system based upon glucose-oxidase, which is absent from mammalian cells, was used, however high background levels due to non-specific binding of the reagents remained a problem, and sensitivity to CD3 & CD4 was greatly reduced. As a result the determination of the number of CD3+ cells in the SW chamber cell suspensions was not possible.

Lacking an accurate determination of the number of CD3+ cells/ml in the SW chamber cell suspensions, the cultures were set up using the number of mononuclear cells/ml as determined by Turk's staining. As a result, the frequencies for SW cells shown in Tables 4.3 & 4.4 may be underestimates of the true response

frequencies of these cells. While B-cells would not be expected in the cells from a DTH site (5), monocyte/macrophages from DTH sites would be anticipated (5), and these cells, along with dead PMN, may represent the non-T "mononuclear" cells in the SW cell preparations.

Difficulties in obtaining high cloning efficiencies from cells obtained from immunologic reaction sites are common, even in T-cell preparations with relatively few potential interfering cells (171,172). Cloning of T-cell preparations from human lung tissue (173) and synovial fluid (174) have resulted in PHA response frequencies of 1/6.6 and 1/2.5 respectively, while T-cells cultured from renal allograft biopsies have been reported to yield cloning efficiencies between 1/10 to 1/1000. If non-optimal culture conditions are responsible for these low efficiencies, it is assumed that the bias is non-specific, resulting in a somewhat attenuated but nevertheless representative panel of clones for study (172). Whether the comparatively low response frequencies to PHA of the SW cells represents a low proportion of T-cells in the SW cells or a non-specific bias against the growth of these cells *in vitro* cannot be determined until accurate determination of the numbers of CD3+ cells in the culture input populations is possible.

Notwithstanding these difficulties, the high cloning efficiencies achieved with PBM, the linear nature and near 0 Y-intercept of the regression plots indicates that the resulting panel of clones from SW chambers should be representative of the input population.

The question of how many of the T-cells at DTH sites are reactive with the Ag eliciting the reaction has not been completely resolved. Early adoptive transfer experiments in animals resulted in a concensus opinion that in the order of 1% of infiltrating cells were Ag-reactive, althougth the proportion could rise to 30% in areas of the reaction (5,67-69). These methods were at best semi-quantitative and often suffered from very low levels of detectable events.

In contrast, the present study utilizing the cells from DTH sites is a fully quantitative one. After adjusting for the frequency of PHA reactive cells in the chambers, the proportion of Ag-reactive T-cells from the DTH site was much greater than the previous estimates, varying from 1/2.3 to 1/30. These Ag-derived clones were shown to be Ag-specific *in vitro* in terms of proliferation, and to produce IL-2 in response to the Ag used to clone them. They were not derived from non-specificaly activated cells, nor from feeder cells (Tables 4.4 & 4.5).

The frequencies of Ag-specific cells in the blood of the subjects was also determined (Tables 4.3 & 4). These frequencies varied from 1/10 to 1/180 for PPD, 1/150 for TET and 1/719 for CAN. There was a close correspondance between the PPD-reactive frequencies of subject S obtained in two separate experiments. This, combined with the linearity of the frequency plots (indicated by their high r2 values) and their close passage to the origin demonstrated that the cloning technique was both accurate and reproducible. The generally narrow range of the 95% Confidence Intervals also attests to the reliability of this technique.

The limitations of the classic labelling experiments made the determination of the proportion of Ag-specific cells at DTH sites, relative to the blood, impossible. In determining the frequencies of Ag-reactive cells from both DTH sites and the blood it was discovered that the cells from the DTH sites were enriched for Ag-reactive cells. This enrichment was found in all cases, with no overlapping of the 95% Confidence Intervals, even in Subject P, in whose PBM 1/9.75 T-cells were PPD reactive, 1/3.74 SW cells were Ag-specific. Perhaps most interesting was Subject B. The frequency of CAN specific T-cells in the blood of this subject was 1/719. The frequency of CAN specific T-cells from a CAN SW chamber of this subject, unadjusted for the frequency of PHA responding cells, was 1/366. Thus it is unlikely that the enrichment seen was an artifact resulting from the low numbers of PHA responding cells in the chambers.

The enrichment of Ag-specific cells at DTH sites was limited to cells reactive to the eliciting Ag. The frequency of PPD reactive cells from a SW chamber over a PPD DTH site was 5-fold greater than the corresponding frequency in the blood, while the frequencies of TET reactive cells were equivalent regardless of their origin.

The nature of the mechanism responsible for this enrichment is unknown, but a number of possible explanations exist. Given the number of IL-2 producing cells among the TDTH population, the Ag specific cells may have been more prevalent at the site as a result of *in situ* proliferation. While IL-2 was not detected in the fluid from several SW chambers placed over DTH reactions (data not shown) this may be attributed to its uptake by activated T-cells. If *in situ* proliferation was responsible a cell cycle time of between 6-34 hrs would be required to account for the observed enrichments.

A second explanation could be the selective trafficking into DTH sites of cells activated elsewhere. In this scenario, Ag, either from the DTH site under the chambers or from the previous skin tests that were required to establish reactivity, would have to have been exported to lymph nodes or spleen. At these sites, Ag-specific cells would be activated, multiply and being released into the circulation from which they would exit into any DTH sites they encountered. Such a phenomenon has been demonstrated in animals (69-70).

Alternately, cells activated by Ag *in situ* may be more responsive to migration inhibiting or chemotactic factors. If this were so, then these cells would tend to accumulate *in situ* at DTH sites.

Finally, if the cells that participate in DTH reactions represent a subset of the total circulating T-cell population, than the distribution of Ag-reactive frequencies in these two populations need not be identical. If so, then the "enrichment" for Ag reactivity seen in the TDTH cells may not be the result of a selective process.

In considering the results of previous adoptive-transfer based estimates it must be acknowledged that these histological studies were capable of examining equally cells at all areas of the reaction site. The cells that are delivered into the chambers may be a subset of the total present at the site, and it is conceivable that activation by Ag *in situ* may influence cellular mobility or chemotaxis, resulting in preferential delivery to the chambers.

The surface phenotypes of the clones revealed a problem in the cloning and expansion of CD8+ cells. Based upon the % of CD8+ T-cells in the PBM prparations, the % of CD8+, PHA-derived clones from PBM should have been greater than was observed. CD8+ cells are often difficult to clone and maintain, possibly due to lysis or suppression in the cultures following stimulation, or the use of inadequate or inappropreate growth factors. It is tempting to assume that the difficulties in growing these cells accounted for the 5-8% of cells that did not respond in cloning cultures. While CD8+ cells may have accounted for part of this deficit, this could not account for all the observed discrepancy. The remaining deficit is likely due to the difficulties in expanding and maintaining CD8+ clones to sufficient numbers for typing and analysis. Although growing well in microwells, between 5-25% of PHA derived clones failed to expand sufficiently *in vitro* for typing and analysis to be performed. This was less of a problem when Ag was used to stimulate the cells, and thus was probably due to lysis/suppression within the agglutinated cell clusters.

The Ag-specific SW chamber cells (TDTH) contained both CD4+ and CD8+ cells with CD4+ cells predominating. The number of CD8+ clones among the TDTH was greater than in the PBM, which is interesting, given the discrepancies among many authors regarding the degree of participation of CD8+ cells at DTH sites (44,47). In this study, we concentrated solely upon the Ag-specific cells from DTH sites, which are rare histologicaly and difficult to quantitate.

The presence of a large and reproducable number of CD8+, Ag-specific T-cell clones among the TDTH population should lay to rest any controversy over the participation of these cells *in situ* in DTH.

CD8+ cells have been shown to be required for defence against viral and parasitic infections. *In vitro*, these cells have often demonstrated cytotoxic activity, which has been hypothesised to be their effector function in DTH responses. The CD8+ TDTH clones in this study were predominantly cytotoxic. One showed evidence of NK-like cytotoxicity. Cytotoxic function was not limited to CD8+ cells. Half of the CD4+ clones demonstrated LDCC against both allogeneic and autologous transformed targets, and three lysed K562 in the absence of lectin. All three NK-like CD4+ cytotoxic cells retained the capacity to secrete IL-2 in response to PHA or PPD. These cells therefore did not fit the profile of long-term culture adapted anomalous CD4+ cytotoxic cells as defined by Poulec *et. al.* (179). Only one of these NK-like CD4+ clones was tested for TNF production. It produced high levels of TNF (POTC4.25, Table 4.7b) upon stimulation with lectin. In the absence of PHA, no TNF activity was detected in the supernatants from this clone. Three other CD4+ TDTH clones that produced TNF without lectin and two clones that produced very high levels of TNF after PHA stimulation did not lyse target cells of any sort in the presence of PHA. Thus it would seem that TNF production alone could not account for either NK-like or lectin-dependent cytotoxicity by CD4+ TDTH clones. Cytotoxic capacity was not linked to the ability to produce IL-2 or IFN-g.

There are several mediators by which T-cells can lyse target cells. TNF, especially in concert with IFN-g is one such mediator. Perforin, a C9-like molecule produced by TC and NK cells, is capable of lysing target cells (175), as are serine esterases produced by cytotoxic CD4+ clones (176). While TNF production may play a role in the cytotoxic activities of some TDTH cells, the results presented here suggest that one or more of these alternative mechanisms, or others yet unknown, are likely responsible for the majority of cytotoxic activity at DTH

sites.

The capacity to provide help for Ig synthesis represented the original definition of T-cell help. Recently it has been considered one of the hallmarks of the TH2 cell which does not mediate DTH. No studies have been carried out in humans to determine if these cells are present at DTH sites. Fourty percent of the CD4+ TDTH clones were capable of providing help for Ig synthesis.

Three CD8+ TDTH clones were tested and one provided help for Ig synthesis. This unusual result has been reported previously using anti-CD3 antibody to stimulate T-cell clones (204). The CD8+ "helper" clone was also unique among CD8+ clones in not demonstrating cytotoxicity. The significance of such a CD8+ T-cell is difficult to judge but *in vivo* it may display other regulatory functions such as suppression, not examined in this study.

The proportion of Ig synthesis promoting cells was similar at DTH sites and in the blood. The role of these Ig-synthesis promoting cells at DTH sites is uncertain. They may be unimportant bystanders to the DTH response, producing LK that have no importance in the reaction. Alternatively, they may play a regulatory role, either for DTH *in situ* or after trafficking out of the DTH reaction site they may direct Ig synthesis at a distant site. Whatever their role if any *in situ* in DTH, their presence may simply be a reflection of the tendancy of recently activated T-cells to accumulate at DTH sites.

IL-2 production has been considered to play a role in DTH, but IL-2 production by human TDTH cells has not been previously demonstrated. In this study, one half of the CD4+ TDTH clones produced IL-2 upon PHA stimulation and the majority produced IL-2 upon Ag stimulation. None of the CD8+ clones tested produced IL-2. Other workers have reported that up to 70% of circulating CD4+ cells produce IL-2 upon mitogenic stimulation (118) and in this study, 71% of CD4+ Ag-derived PBM clones produced IL-2, while 43% of TDTH clones produced IL-2.

The observation that a greater proportion of circulating Ag-derived CD4+ clones produced IL-2 than TDTH clones was not statistically significant (by X2 with Yates's correction) despite the large number of clones assayed (28 SW, 14 PBM). Thus it is possible that there is no real difference in the proportion of IL-2 producing cells in the two populations.

The results of the present study are largely consistant with previous *in vitro* studies, and prove that TDTH cells produce IL-2 following antigenic stimulation.

TNF was produced by 2/3 of all CD4+ TDTH clones, while it was an uncommon feature of the CD8+ clones. Since the bioassay for TNF measures cell death it is possible that some of the TNF activity was attributable to other cytotoxic mediators produced by T-cells, such as perforin or serine esterase.

A more attractive explanation for the high incidence of TNF production may reside in the ability of TNF to promote DTH-like cell influx, as described by Issekutz and co-workers (110). TNF was also detected in the fluid from SW chambers over skin test sites when the Ag injected provoked a DTH reaction. In one experiment in which the chamber was removed after 24 hrs and refilled with serum for another 24 hrs, TNF was only detected in the SW fluid from the first 24 hr period. This implies that the majority of TNF production occurs early in the DTH reaction, possibly *in situ*, although production by those cells recruited into the chamber in the first 24 hrs may also account for the presence of TNF in the chambers early in the reaction.

The L929 bioassay is not specific for either form of TNF (180). Since T-cell clones have been shown to produce both TNF-a and TNF-β, while monocytes produce TNF-a exclusively (6,181) it cannot be stated which form of TNF was produced by the clones *in vitro*, or which form was present in the SW chamber fluids. Possibly both forms would be expected in the chambers, since TNF-β production is more likely from the TDTH cells, while the monocytes in the chamber and *in situ* may produce TNF-a. The use of appropriate specific Ab can render the L929 assay specific for either form of TNF. When such Ab become available, the answers to these qestions will be easily determined.

IFN-g has long been presumed to be involved in CMI, principally because of it's *in vitro* capacity to stimulate macrophages to kill intracellular organisms. The demonstration by Issekutz and co-workers that IFN-g, injected i.d. can induce a DTH-like response and that Ab to IFN-g blocks the expression of DTH (103,104) was strong evidence that this CK was involved in DTH. In this study, the majority of TDTH cells produced substantial levels of IFN-g upon PHA stimulation. PWM was a less potent inducer of IFN-g production. All CD8+ TDTH clones produced IFN-g. IFN-g was also detected in the fluid from SW chambers placed over skin test sites that evoked DTH reactions. The fluid recovered from Ag-'ve sites contained negligible IFN-g or no IFN-g at all. Thus the capacity of TDTH cells to produce IFN-g *in vitro* was confirmed *in vivo*.

In one experiment, the levels of IFN-g in the chamber fluid was the same from 0-24 hrs and 24-48 hrs, unlike TNF. It is interesting to speculate that the majority of the TNF activity detected in the chambers is an early, possibly macrophage associated event, while the production of IFN-g, a T-cell mediated event, continues throughout the reaction.

Issekutz *et.al.* found that while IFN-g and TNF (both forms) were capable of provoking a DTH-like response when injected i.d., IFN-g exhibited a potent synergy with TNF. The levels of IFN-g found in the chambers were capable of evoking the skin response in rats (103) and much greater than those neccessary when TNF is present (110). Thus the presence of IFN-g in the chambers at the observed levels supports the hypothesis that it is involved in directing the cellular influx to DTH sites.

The capacity of many of the TDTH clones to produce IFN-g and TNF *in vitro* is strong evidence of their involvement in DTH, and the presence of these CK in the SW chamber fluid represents the first direct evidence for their production *in situ* at DTH sites.

The definition of the TH1 "inflammatory" T-cells by Mossman *et. al.* included both the pattern of LK production as well as functional capacities. Among their claims was that TH1 clones secrete both IL-2 and IFN-g, while the TH2 "helper" T-cell did not.

In this study, the majority of CD4+ TDTH IL-2 producing clones also produced IFN-g. Among the Ag-derived PBM clones there were insufficient IL-2, IFN-g non-producing clones to establish a statistically significant link between the production of these two LK, although 80% of the clones tested met the IL2-IFNg linked production profile.

The IL-2 & IFN-g producing subset was reciprocal with the B-helper subset of Ag-specific CD4+ TDTH clones. The dearth of Ag-derived B-helper clones from the blood made it impossible to draw similar conclusions regarding the PBM. However from this evidence it can be concluded that the Ag-specific CD4+ cell populations recovered from DTH sites correspond to the TH1 and TH2 profiles of Mossman, although the production of IL-4 and IL-5 by this population has yet to be determined.

The differential stimulation capacities of the mitogens PHA and PWM raise frequently asked questions regarding the relationship between T-cell functions produced by polyspecific stimuli *in vitro* and the functions evoked by Ag stimulus *in vivo*. Generally, the TH1/TH2 dichotomy among T-cells/clones begins to break down when powerful non-antigenic stimuli are used. The use of calcium ionophores + TPA results in the simultaneous production of IL-2, INF-g & IL-4 (123). Immobilized anti-CD3 antibody results in IL-2, IFN-g, TNF production and B-helper activity from all CD4+ and >90% of CD8+ clones (204).

A similar pattern with regards to IL-2 and IL-4 production has been observed in normal CD4+ cells in response to various mitogens. In decending order of potency, ConA, PHA and PWM induce different levels of IL-2, while the sequence is reversed for IL-4 production (118). An additional complication may arise when testing for the net production of LK, as is done when measuring the levels in culture supernatants. LK such as IL-2 and IL-4 may be utilized by the same cells that produce them.

Thus they may reach levels in the supernatant that are artificial if the stimulant utilized induces receptors for these LK in a pattern that is different from that induced by Ag.

In contrast to the generalized LK production induced by many non-specific stimuli, alloantigen-specific stimulation of human PBM-derived clones results in TH2 type clones and a second population of IL-2, IFN-g and IL-4 producing human CD4+ clones (125).

Thus antigenic stimulation of human CD4+ cells and clones appears to initiate production of a more restricted subset of LK than highly non-specific stimuli. Unfortunately, the use of antigens to stimulate large panels of clones during extensive long-term study is difficult, due to the repeated requirement for APC from the same individual.

In the present study, PHA and PWM were used. These represent the least potent mitogens known to be capable of inducing the LK and functions tested. This approach resulted in the detection of TH1 and TH2 like responses from the majority of clones. It would appear therefore that these mitogens did not induce a wide degree of artifactual polyfunctional clones, a conclusion supported by the results of an experiment involving stimulation with Ag, in which 2/3 of the clones retained the capacity to produce IL-2, previously demonstrated by PHA stimulation.

The SW chamber technique has been utilized to extract both cells and CK directly from human DTH sites. *In vitro* assays were then used to define the TDTH population.

The T-cells from DTH sites were enriched for Ag reactive cells, relative to the blood. The Ag-specific TDTH clones were found to consist of both CD4+ and CD8+ clones. Cytotoxic function and TNF production were broadly distributed. Half of the clones produced IL-2 and IFN-g, but did not give help for Ig synthesis. The reciprocal population were capable of providing B-cell help and did not produce IL-2 or IFN-g. These two clonotypes roughly correspond to the TH1 and TH2 populations defined in the murine system. The production of both TNF and IFN-g *in situ* at DTH sites was confirmed by the high levels of

these CK in the fluid from SW chambers placed over DTH reactions.

Chapter 5: The Restoration by Cytokines of DTH in Anergics

Introductory comments

Anergy to recall skin test antigens has been shown to be predictive of the incidence of both sepsis and septic-related death in surgical patients (127). A DTH-like reaction can be restored in these patients when antigen is injected along with cytokines, released by either soluble or allo-antigen stimulated mononuclear cells (140). These cytokines provoke an initial rapid non-specific inflammatory response which fades rapidly (139). In order for a subsequent DTH reaction to appear it is necessary for the patient to have been previously sensitized to the antigen, i.e. have lymphocytes reactive to the antigen in the circulation (139,140,143). Injection of the cytokines alone without antigen results in the initial "flare" reaction but no subsequent DTH-like response is seen, regardless of previous sensitivity (139). This argues that the cytokines do not themselves produce or provoke the DTH-like reaction, but rather that they permit the Ag-specific TDTH cells to function normally, if a suitable antigen is present.

Despite the similarities between the reaction restored in anergic patients by cytokine co-injection and a normal "unassisted" DTH response it is not known whether the the T-cells delivered to sites of antigen+cytokines injection in anergic patients represent a population capable of carrying out the same functions as T-cells from normal DTH reactions.

In the skin window chamber technique, we have for the first time a method which permits the recovery of cells from DTH sites without contamination with cells coming directly from the circulation. It was demonstrated that the expression of DTH is accompanied by a quantitative increase in mononuclear cell delivery to the chamber.

The T-cells recovered from the chambers were cloned and studied to provide for the first time a description of the functional capacities of human TDTH cells. TDTH cells were found to be enriched for cells specific for the Ag eliciting the

DTH reaction. Both CD4+ and CD8+ cells were found at DTH sites. Cytotoxicity and TNF production was broadly distributed across both phenotypes. TDTH cells were found to produce IL-2, TNF and IFN-g and some also gave help for Ig synthesis, with distinct patterns of these function being distributed among the CD4+ population.

Accordingly, using the SW chamber technique, we sought to determine: if anergy was associated with alterations in the delivery of mononuclear cells to DTH sites, if the restoration of DTH by cytokines was accompanied by a change in mononuclear cell delivery, and to examine the antigen recognition and functional capacities of the lymphocytes delivered to sites injected with antigen plus cytokines, in order to compare them with the capacities of normal TDTH cells with a view towards assessing their potential for involvement in augmented anti-pathogen responses.

Materials & methods

Patients and patient classification

Nineteen surgical patients were classified as either reactive or anergic on the basis of their response to a battery of 5 recall skin test antigens as described in chapter 3. Those with a sum of the diameters of all 5 tests (Skin Test Score or STS) less than 5mm were classified as anergic. All reactive patients were reactive to two or more Ag and had an STS >10mm except #3, who as a result of their reactivity to PPD (alone) was classified as reactive. In addition, all patients were subsequently re-tested with PPD alone at the same time as the application of the skin window chambers. Reactive patients all gave positive (>5mm) responses to this skin test. Healthy control subjects were young volunteers who were skin tested and had chambers placed over sites injected with an antigen to which they were reactive (data from these subjects has already been shown in chapter 3). Patients were subclassified on the basis of their *in vitro* response to PPD to give four groups:

Hospital reactives		PPD reactive	*in vitro*	(HR PPD+'ve)
"	"	PPD non-reactive	"	(HR PPD-'ve)
"	anergics	PPD reactive	"	(HA PPD+'ve)
"	"	PPD non-reactive	"	(HA PPD-'ve)

Those patients that had been operated on were initially skin tested at least 72 hrs following surgery. SW chambers & the subsequent re-skin testing were performed at least 5 days post-op. The entire protocol was approved by the human experimentation committee of the Royal Victoria Hospital and informed consent was obtained from all participants.

Assessment of Innate PPD Reactivity

The determination of the *in vitro* reactivity to PPD was carried out as described in chapter 3. Briefly: one hundred thousand peripheral blood mononuclear cells were cultured in flat-bottomed microtitre plates in RPMI 1640 supplemented with glutamine, .01M HEPES and 20% pooled normal human serum in triplicate wells. PPD (Connaught) was added to give a final concentration of 20 µg/ml. The cultures were incubated for 7 days @ 37t in a 5% CO2 atmosphere.

One μCi of 3H-Thymidine was added to each well for the last 6hrs. The cultures were harvested with a MASH apparatus and the amount of 3H-Thymidine incorporated into DNA counted in a scintillation counter.

Recovery of Cells from DTH Sites

The cells from DTH sites were recovered using the Skin Window Chamber technique as detailed in Chapter 3. No Tisseal sealant was used in these experiments, including the healthy control subjects. A standard skin test dose of PPD±CK (see below) was injected intradermally in 0.1ml into each site. The chambers were filled with Type AB serum. 48 hrs later the chamber fluid, containing cells delivered to the site, was collected and the cells enumerated with Turk's stain and confirmed by differential counting with Wright's stain when numbers were sufficient.

Production of Cytokines

The CK utilized for restoration of the DTH reaction were prepared by culturing together equal numbers of peripheral blood lymphocytes from two healthy volunteers (blood type AB) at 2x106/ml in RPMI + glutamine and 8% pooled human (AB) serum in disposable plastic flasks (Falcon) for 48 hrs. The supernatants were collected by centrifugation and concentrated 10-fold using either a Filtron (Fisher) or Amicon (Amicon) ultrafiltration apparatus fitted with membranes with either a 3kD (Grey Filtron) or 1kD (YM-2 Amicon) exclusion size. The supernatants and donor sera were tested for sterility and exposure to hepatitis, HIV and CMV. The concentrated preparations were mixed with PPD so that each intradermal skin test dose of 100Hl contained 5 TTU of PPD and the equivalent of 500Hl of supernatant. This preparation is referred to as PPD+CK.

Bulk culture of lymphocytes

250,000 mononuclear cells from skin window chambers placed over sites injected with PPD+CK were cultured in 24-well plates (Linbro) with 106 irradiated allogeneic stimulator cells in T-cell growth medium (see chapter 4) and 0.5 μg/ml PHA.

Cloning & Frequency Analysis

Cloning was performed as previously described (see chapter 4) with 10S irradiated autologous PBM as feeders. Wells were fed weekly and clones picked at 14 & 21 days for expansion in 24-well plates as above. Limiting Dilution cultures were set up using various numbers of cells/well as described previously (see chapter 4). Medium was replaced weekly and clones were picked after three weeks and expanded in 24 well plates as above. CD3+ cells were enumerated in PBM preparations & the number of CD3+ cells/well used in a least-squares regression analysis of the results to obtain the frequency of responding cells. This technique resulted in a cloning efficiency > 95%. The SW cells from subject C were cloned directly from the chamber. The SW cells from Subject T were first cultured in bulk as above using PHA as the stimulus and then cloned in limiting dilution.

Frequency of PPD-Reactive Cells

Determination of the frequency of PPD-reactive cells was assessed as previously described (see chapter 4).

Surface Phenotype of Cells

The surface markers of cultures and clones were determined using an Immunoperoxidase staining technique as previously described (see chapter 4).

NK-like Cellular Cytotoxicity

The potential of T-cell clones to lyse K562 target cells in the absence of lectin was assessed as described previously detailed (see chapter 4).

Lectin-Dependent Cellular Cytotoxicity (LDCC)

The cytotoxic potential of T-cell clones in the presence of lectin (PHA) was assessed against K562, U937, autologous or allogeneic EBV-transformed B-cell targets as previously described (see chapter 4).

Production of Lymphokines by Skin Window Clones

T-cell clones derived from the cells recovered from skin window chambers over PPD+CK sites and their PBM were tested. These clones were established in limiting dilution using PPD as

the stimulant. They were grown and stimulated with PHA, and their supernatants collected as described previously (see chapter 4).

Detection of Interleukin-2, TNF & IFN-g

The presence of LK in the supernatants from PHA stimulated T-cell clones were detected as previously described (see chapter 4).

B-cell Help

The ability of T-cell clones to provide help for Ig synthesis was assessed in a mitogen-driven allogeneic system as described previously (see chapter 4).

Statistical Analysis

Mean values are given alone or ± standard error. Comparisons of cell delivery between subject groups were carried out using the Wilcoxon Rank Sum Test. Comparisons of cell delivery with/without CK were analysed with a one-tailed Wilcoxan Signed Rank Sum Test and also by the Wilcoxon Rank Sum Test where specified. These tests were carried out upon a microcomputer using a statistics program (Stats-Plus, Human Systems Dynamics) to generate the rank sums. The p-values were then determined using the tables of Choi (166).

Results

The patient groups used in the study were similar in age and sex (Table 5.1). The skin test score was significantly lower in the anergic group, regardless of *in vitro* PPD reactivity.

In Vitro PPD reactivity of subjects.

Anergic patients by definition do not give DTH reactions. The ability of their PBM to respond to antigen *in vitro* remains intact and this can be utilized to assess their potential for an *in vivo* DTH response (140). Among those reactive *in vitro* to PPD, the *in vitro* response of both the HR and HA groups was not different (Table 5.1) (minimum response: new CPM=11,728 stimulation index=48, data not shown) indicating their potential to respond to a PPD skin test. Non-reactive subjects all gave less than 700 new CPM in response to PPD. Their PBM did respond *in vitro* to PHA (17,214-42,615 new CPM, all stimulation indices > 20, data not shown) indicating that the cells were capable of responding *in vitro*, given the appropriate stimulus.

The delivery of mononuclear cells to DTH sites is reduced in anergy

The presence of a DTH reaction in the skin below a SW chamber results in an increased mononuclear cell delivery to the chamber. In order to determine if the lack of a visible DTH reaction that occurs in anergy was reflected in mononuclear cell delivery, the number of mononuclear cells delivered into skin window chambers placed over PPD sites of PPD+'ve subjects was assessed.

As shown in Fig. 5.1, PPD +'ve anergic patients delivered significantly fewer mononuclear cells to PPD injection sites than either PPD +'ve HR patients or healthy controls.

Mononuclear Cell Delivery in Anergics is Restored by CK.

The co-injection of CK with PPD results in a DTH-like reaction in patients reactive *in vitro* to PPD (140). Since the lack of an observable reaction to injected Ag was found to be

reflected in a decreased mononuclear cell delivery, the delivery of these cells to Ag+CK sites was evaluated.

The co-injection of cytokines with PPD significantly increased the delivery of mononuclear cells both in terms of the net increase ($p<0.01$) and the means of the two populations ($p<.005$, one-sided Wilcoxon Rank Sum Test) (mean increment: 107-fold (Fig. 5.2). The number of cells delivered approached that of the reactive patients in response to PPD.

Mononuclear cell delivery in HR is slightly improved by CK

PPD+'ve HR patients delivered similar numbers of mononuclear cells to PPD sites as healthy controls (Fig. 5.1). The co-injection of CK along with PPD significantly increased mononuclear cell delivery (Fig. 5.3, mean increment: 38-fold, $p<.025$). The magnitude of this increase was approximately 1/3 that seen in the Anergic patients, and did not result in a delivery significantly different from that found in healthy control subjects.

Restoration of mononuclear cell delivery by CK is dependent upon previous sensitivity to the test Ag

The ability of CK to restore an observable DTH reaction has been found to be dependent upon a previous sensitization to the test Ag (161). Co-injection of PPD+CK restored the delivery of mononuclear cells in anergic patients reactive *in vitro* to PPD (Fig. 5.2) and substantially improved, although to a much lesser degree, in PPD responsive HR patients (Fig. 5.3).

SW chambers were placed over Ag & Ag+CK sites of 2 Reactive and 4 Anergic patients non-responsive to PPD *in vitro*. There was a doubling of mononuclear cell delivery to Ag+CK sites in these patients (Fig. 5.4) which was consistent, but much smaller than the 38 and 107-fold increment seen in their PPD-reactive counterparts (Fig 5.2).

Phenotypic & functional analysis of clones from PPD+CK sites of Anergics

Using the SW chambers to obtain TDTH cells from normal individuals, the functional characteristics of this cell population were defined in the previous chapter. To determine if

the reaction seen when PPD+'ve anergic patients are co-injected i.d. with PPD+CK results in the delivery of a cellular population capable of participating in a DTH response, the same approach was used to examine the cells delivered to such sites.

T-cells from skin window chambers over PPD+CK sites of Anergics are enriched for cells reactive to PPD

It had previously been observed that the T-cells from SW chambers over DTH sites were enriched, relative to the circulating population, for cells reactive to the Ag used to elicit the underlying DTH reaction. If the reaction seen at Ag+CK injection sites of sensitized anergic patients was indeed a reflection of a DTH reaction, then this enrichment should also be present in the SW cells from these sites.

Both the PBM and the cells from a SW chamber placed over a PPD+CK injection site of one anergic subject (C) were cloned directly in limiting dilution as previously described. The frequency of PPD-reactive T-cells in the blood was 1/153. The SW cells were enriched 20-fold for PPD-reactive cells, with a frequency of 1/7.20 (Table 5.2).

A persistent observation in the cloning experiments related in this thesis was the relatively low frequency of PHA responding cells among the "mononuclear" cell preparations taken from the chambers. As has been discussed, there are several possible explanations for this, many of which have been controlled for in the experiments detailed in chapter 4. Without FACS analysis of the cell population input into the limiting dilution cultures it was not possible to precisely enumerate the % CD3+ve cells/well that were used in the cultures & thus the cloning efficiency of the SW cells. One approach to answering this problem is the use of primary bulk cultures, followed by cloning & limiting dilution analysis. While this technique has the potential for bias, chiefly due to preferential expansion of clones within the bulk culture, it was decided to utilize it for anergic subject T to determine if the enrichment that was observed in subject C was due solely to the frequency of PHA responding cells from the skin window chamber.

As can be seen in Table 5.2, the frequency of PPD-reactive cells in PHA-stimulated bulk cultures of PBM was 1/460. This was similar to the PPD-reactive frequency found in fresh uncultured PBM (1/438) and the two values had overlapping 95% confidence intervals. This indicates that little, if any, preferential expansion had occurred in the 1° bulk culture. PPD-reactive cells were 2.8-fold more frequent in PHA-stimulated bulk cultured SW cells (f=1/165) than in the bulk cultured PBM, and 2.6-fold more frequent than in freshly drawn PBM.

Surface phenotypes of T-cell clones from Ag+CK sites of Anergics

T-cell clones were established from PPD+CK SW chamber contents & the PBM of three PPD+'ve HA. For two subjects (M & T) both the SW cells and the PBM were initially cultured in bulk using PHA and then clones subsequently derived by limiting dilution with either PHA or PPD. One subject's (C) cells were cloned directly in limiting dilution. The results, shown in Table 5.3, indicate that both CD4+ & CD8+ cells were present in the SW chambers. CD4+ cells predominated in the chambers to a slightly higher extent than in the PBM. These results were found in both the general (PHA-stimulated) population and in those cells specific for the inducing Ag (PPD-stimulated).

Cellular cytotoxicity of PPD-reactive clones from PPD+CK sites of Anergics

The analysis of a panel of Ag-reactive TDTH clones from normal subjects has shown that the majority of CD8+ and one-half of the CD4+ clones were capable of cytotoxicity.

The cytotoxic capacities of 1 CD8+ and 4 CD4+ SW clones of two anergic subjects were tested (Table 5.4). The CD8+ clone lysed the target cells both with and without lectin. One of the four CD4+ clones lysed target cells in the presence of lectin.

B-cell helper function of PPD-reactive clones from PPD+CK sites of Anergics

T-cell clones capable of providing help for Ig-synthesis were found in the PPD-reactive TDTH population. Fourty percent of the PPD-reactive CD4+ clones from PPD+CK SW chambers were

also capable of providing help for Ig-synthesis (Table 5.5)

IL-2 production by PPD-reactive clones from PPD+CK sites of Anergics

The study of CD4+ TDTH clones found that 43% of the clones produced IL-2 following stimulation. When a similar panel of clones from PPD+CK sites of anergics was tested, 5 of 7 (71%) of them produced IL-2 (Table 5.6). One CD8+ clone tested did not produce IL-2.

TNF production by PPD-reactive clones from PPD+CK sites of Anergics

TNF has been implicated in DTH as potentially capable of producing DTH-like indurations alone and in synergy with IFN-g (110,112), and as an effector CK lysing target cells (6). TNF was found in the fluid from SW chambers placed over DTH sites, and the majority (2/3) of TDTH clones tested produced TNF. If the cells delivered to Ag+CK sites of Anergics are indeed typical TDTH cells, then they to could be expected to produce TNF.

Upon testing, all of the seven CD4+ clones did produce TNF following stimulation (Table 5.7). One CD8+ clone was also tested, and it was found to produce TNF upon PHA stimulation, but only in the presence of autologous irradiated EBV-transformed B-cells (data not shown).

IFN-g production by PPD-reactive clones from PPD+CK sites of Anergics

IFN-g is an important promotor of both intracellular killing by monocytes and the induction of DTH-like reactions (110,112). IFN-g was detected in the fluid from SW chambers over DTH reactions. Sixty-five percent of CD4+ TDTH clones from normal individuals produced >5 NIH Units/ml of IFN-g following PHA stimulation. A higher proportion, 5 of 6 (83%), of the PPD-reactive CD4+ clones from PPD+CK sites of anergic patients also produced >5 Units/ml of IFN-g following stimulation (Table 5.8).

Linkages between LK production and B-cell help

All 6 CD4+ clones tested produced either both or neither of iL-2 and IFN-g while 4 of 5 clones tested showed a negative linkage between IL-2/IFN-g co-production and B-cell help. Thus while the numbers were too low to make statistical inferences, the majority of clones fit either the TH1 or TH2 profile.

Table 5.1. - Patient Data

ID	CLASS	AGE	SEX	DIAGNOSIS †	SURGICAL STATUS	STS	PPD SKIN TEST	PPD NEW CPM #	PPD STATUS ¤
1	HR	60	M	COLON CA	POST-OP	16	11	28,879	PPD+'ve
2	"	75	F	GASTRIC CA	PRE-OP	35	6	50,972	"
3	"	74	M	COLON CA	POST-OP	6	9	40,220	"
4	"	59	M	RECTAL CA	PRE-OP	31	12	11,485	"
5	"	78	M	BILIARY CA	PRE-OP	31	6	42,058	"
6	"	59	M	CIRRHOSIS	NO-OP	12	12	26,893	"
mean		68+/-8				22+/-11	9+/-3	33,418	
7	"	27	M	E.C.FIST $	NO-OP	29	0	0	PPD-'ve !
8	"	47	M	PANC. CA $	NO-OP	32	0	77	"
mean		37+/-10				31+/-2	0	39	
9	HA	72	M	ULCER	NO-OP	0	0	0	PPD-'ve !
10	"	23	M	TRAUMA $	POST-OP	0	0	0	"
11	"	64	M	CHOLY $	POST-OP	3	0	608	"
12	"	82	F	CHOLY $	POST-OP	0	0	0	"
mean		60+/-22				0.8+/-1.3	0	152	
13	HA	73	M	AORT. ST $	POST-OP	0	0	16,517	PPD+'ve
14	"	71	M	RECTAL CA	POST-OP	9	0	26,510	"
15	"	63	M	PANC. CA	NO-OP	0	0	27,939	"
16	"	70	M	GASTRIC CA	POST-OP	0	0	23,329	"
17(M)	"	76	M	ESOPHAGITIS	NO-OP	3	0	15,567	"
18(C)	"	38	F	GASTRITIS	NO-OP	0	0	23,275	"
19(T)	"	63	M	GASTRIC OBS$	NO-OP	0	0	12,259	"
mean		65+/-13 ns				1.7+/-3.1‡	0‡	20,770	

† : CA = Cancer
# : New CPM = CPM (PPD stimulated) - CPM (Medium alone)
¤ : Reactivity to PPD was defined as >5,000 New CPM & Stimulation Index > 5
$
Patient #7 - Entercutaneous fistula + bowel obstruction
Patient #8 - Pancreatic Cancer
Patient #10 - Post-trauma patient, failure to heal wound (non-septic)
Patient #11 - Cholycistitis
Patient #12 - Cholycistitis
Patient #13 - Aortic stenosis
Patient #15 - Pancreatic Cancer
Patient #19 - Gastric obstruction
! : PPD-'ve subjects had positive *in virto* responses to PHA
‡ : p<.004 relative to Reactive group
ns: not significant

Table 5.2: Frequency Analysis of T-cell Clones From Anergics

Subject	Cells Cloning Stimulus		Frequency of Reactive Cells (95% C.I.)		Adjusted Frequency (95% C.I.)	
C	PBM	PHA	1.09	(.009-2.18)		
"	"	PPD	398	(241-555)	153	(92.4-213)
"	SW(PPD+CK)	PHA	346	(325-366)		
"	"	PPD	2488	(2078-2900)	7.20	(6.01-8.34)
T	PBM	PPD *	438	(397-481)		N/A
"	"	PPD †	460	(393-528)		N/A
"	SW(PPD+CK)	PPD †	165	(153-176)		N/A

* Freshly drawn PBM
† Subcloning of bulk cultures, originaly stimulated with PHA

Table 5.3: Surface Markers of T-cell Clones From Anergics †

Cloning Stimulus	SW(PPD+CK) %CD4+ ‡	SW(PPD+CK) %CD8+	PBM %CD4+	PBM %CD8+
PHA	94 (15/16)	6 (1/16)	75 (9/12)	25 (3/12)
PPD	91 (10/11)	9 (1/11)	86 (6/7)	14 (1/7)

† Data from three subjects (C,T & M)

‡ Actual numbers of clones are shown in brackets beneath the percentages

TABLE 5.4: Cytotoxicity of PPD Reactive Clones From PPD+CK Sites

Subject	Origin	Cloning Stimulus	CYTOTOXIC CLONES (# positive/# tested)			
			CD4+		CD8+	
			NK*	LDCC#	NK	LDCC
C	SW(PPD+CK)	PPD	0/2	1/3		
T	"	"	0/1	0/1	1/1	1/1
ALL	"	"	0/3	1/4	1/1	1/1

*: NK = NK-like killing of K562 without lectin
#: LDCC = killing of K562/U937/autologous and or allogeneic EBV-transformed B-cells with lectin

TABLE 5.5: B-Cell Help by Ag-specific CD4+ Clones from PPD+CK Sites

Subject	Origin	Cloning Stimulus	CLONES PROVIDING HELP (# positive/# tested)	
			MEDIUM	PWM
C	SW(PPD+CK)	PPD	0/3	1/3
T	"	"		1/2
ALL	"	"	0/3	2/5

TABLE 5.6: IL-2 Prod'n by PPD-Reactive CD4+ Clones from PPD+Cl Sites

Subject	Origin	Cloning Stimulus	IL-2 PRODUCING CLONES* (# positive/# tested)	
			MEDIUM	PHA
C	SW(PPD+Cl)	PPD	0/3	3/3
T	"	"	0/4	2/4
ALL	"	"	0/7	5/7

* Supernatants with greater than 0.5 Units/ml were considered positive

TABLE 5.7: TNF Prod'n by PPD-Reactive CD4+ T-Cell Clones from PPD+Cl Sites

Subject	Origin	Cloning Stimulus	TNF PRODUCING CLONES * (# positive/# tested)	
			MEDIUM	PHA
C	SW(PPD+Cl)	PPD	0/3	3/3
T	"	"	0/4	4/4
ALL	"	"	0/7	7/7

* Wells containing ≥0.5 Units/ml were considered positive

TABLE 5.8: INF-g Prod'n by PPD-Reactive CD4+ Clones from PPD+Cl Sites

Subject	Origin	Cloning Stimulus	IFN-g PRODUCING CLONES* (# positive/# tested)	
			MEDIUM	PHA
C	SW(PPD+Cl')	PPD	0/3	3/3
T	"	"	0/3	2/3
ALL	"	"	0/6	5/6

* Supernatants with . 5 NIH reference Units/ml were considered positive

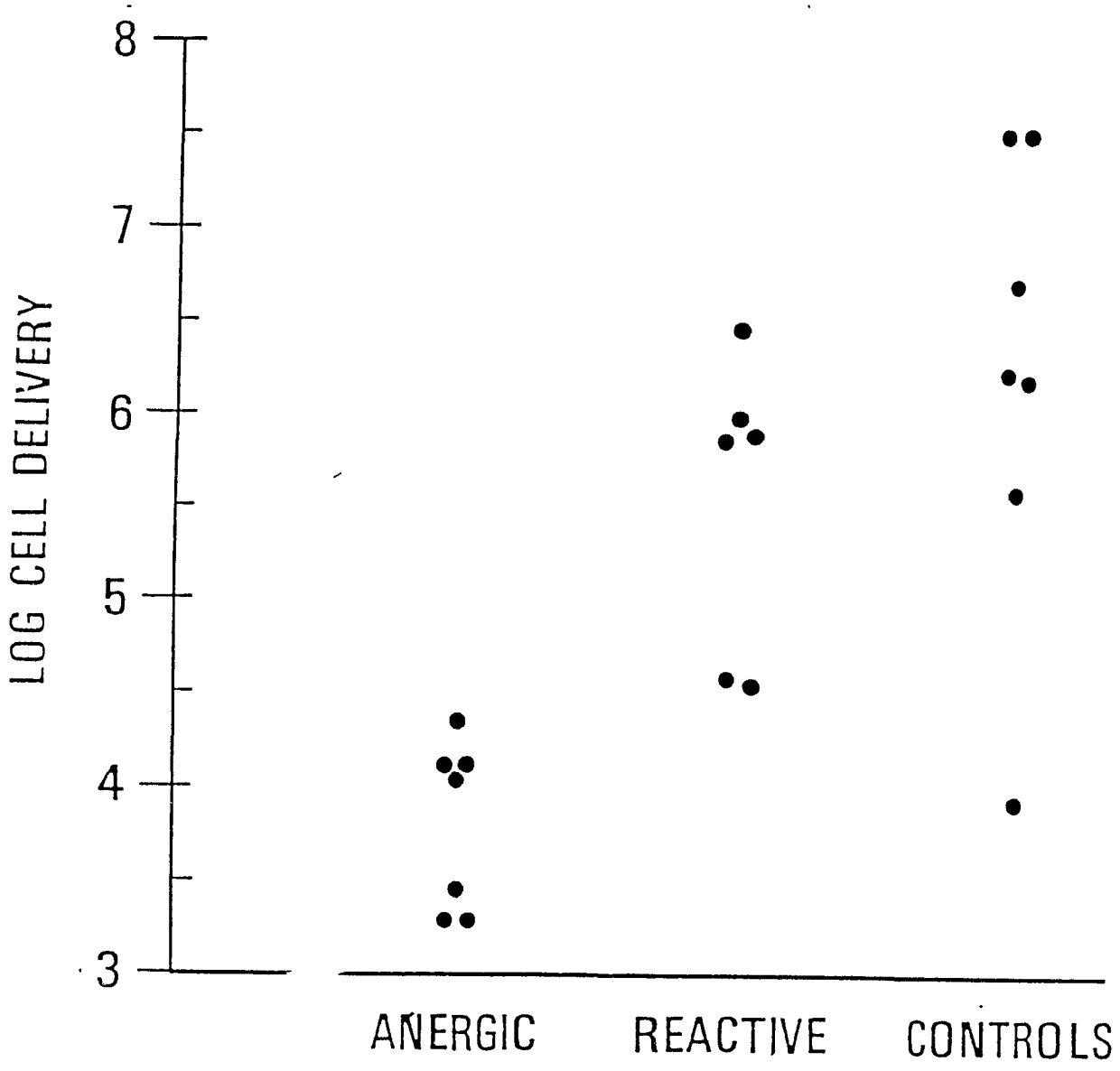

Fig 5.1 - Deficient mononuclear cell delivery to DTH sites in anergic patients. Anergic patients gave a significantly reduced mononuclear cell delivery as compared to *in vivo* reactive patients (median values 0.0122 and 0.751x106 respectively, $p<.01$). Reactive patients were similar to healthy control subjects ($p<.528$).

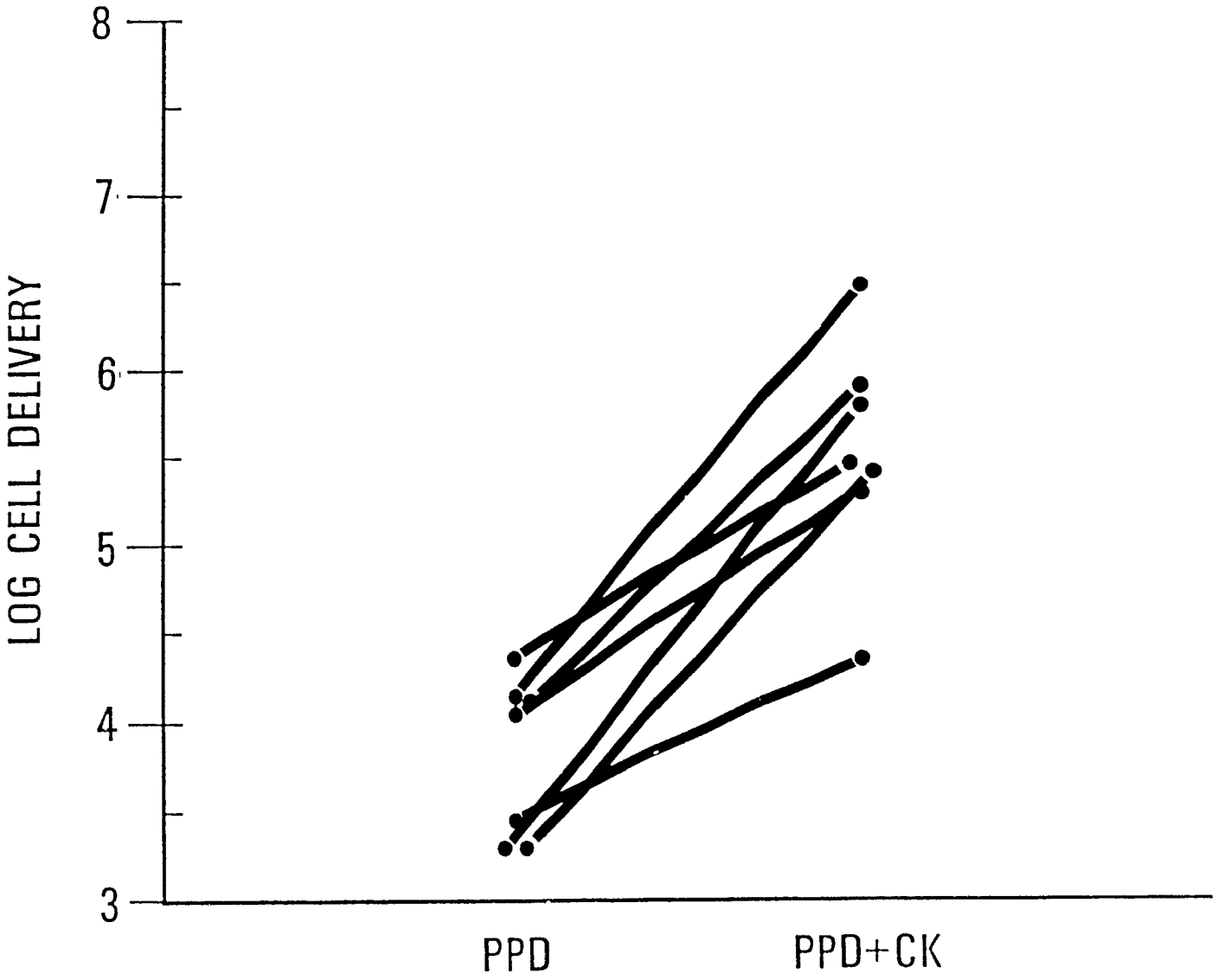

Fig 5.2 - Restoration by cytokines of mononuclear cell delivery to DTH sites in PPD+'ve anergic patients. The diminished delivery of anergic patients was increased an average of 114-fold by the co-injection of cytokine preparations along with antigen. This increase was significant ($p < .01$) and resulted in levels comparable to those seen in the PPD responses of reactive patients.

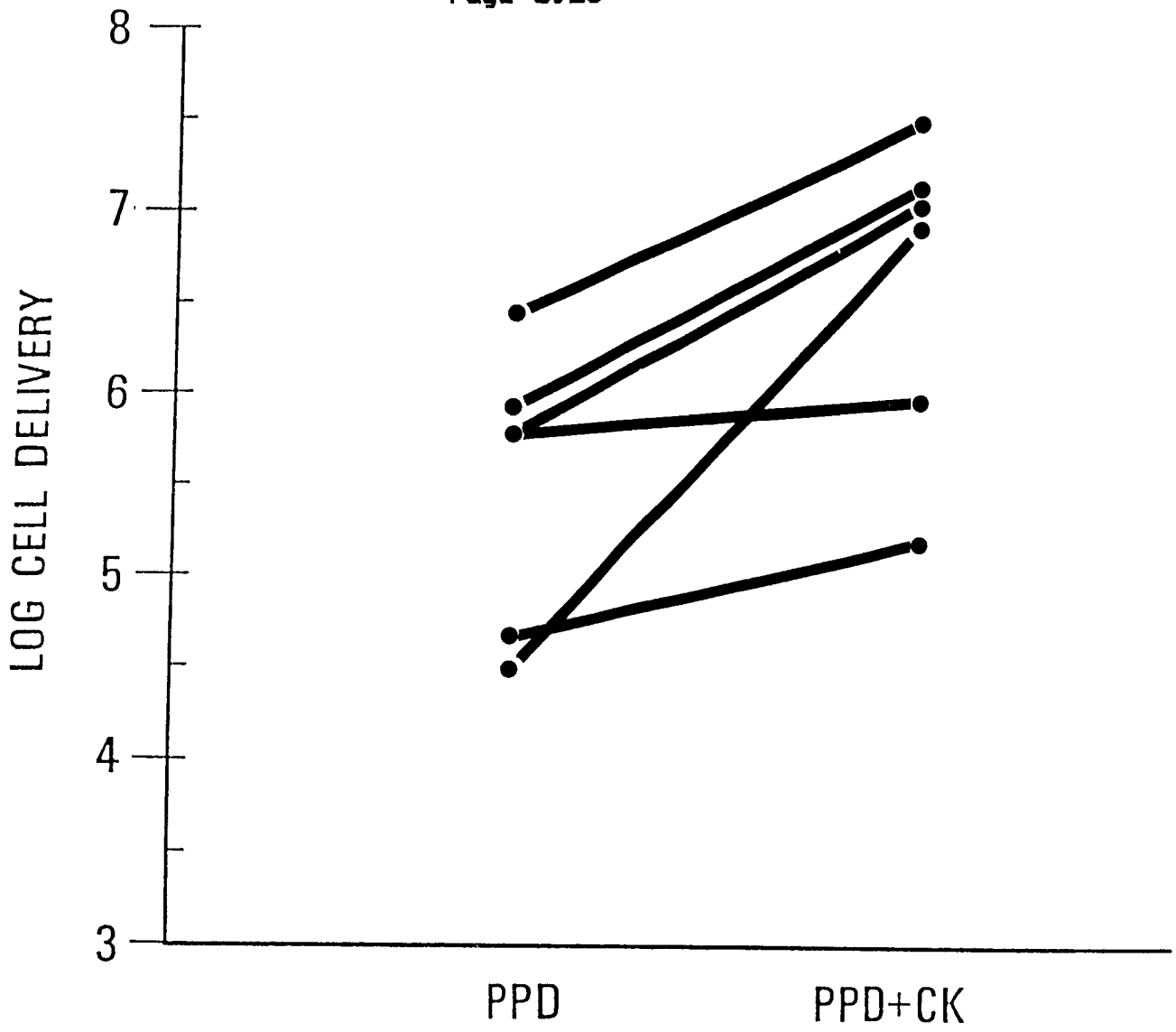

Fig. 5.3 - Improvement by cytokines of mononuclear cell delivery to DTH sites in PPD+'ve reactive patients. Hospital patients, reactive both *in vivo* and *in vitro* (to PPD) delivered similar levels of mononuclear cells to Ag injection sites as normal controls. When Ag was co-injected with CK, the delivery was increased an average of 38-fold. This increase was statistically significant ($p<.025$) and resulted in a delivery indistinguishable from normal subjects.

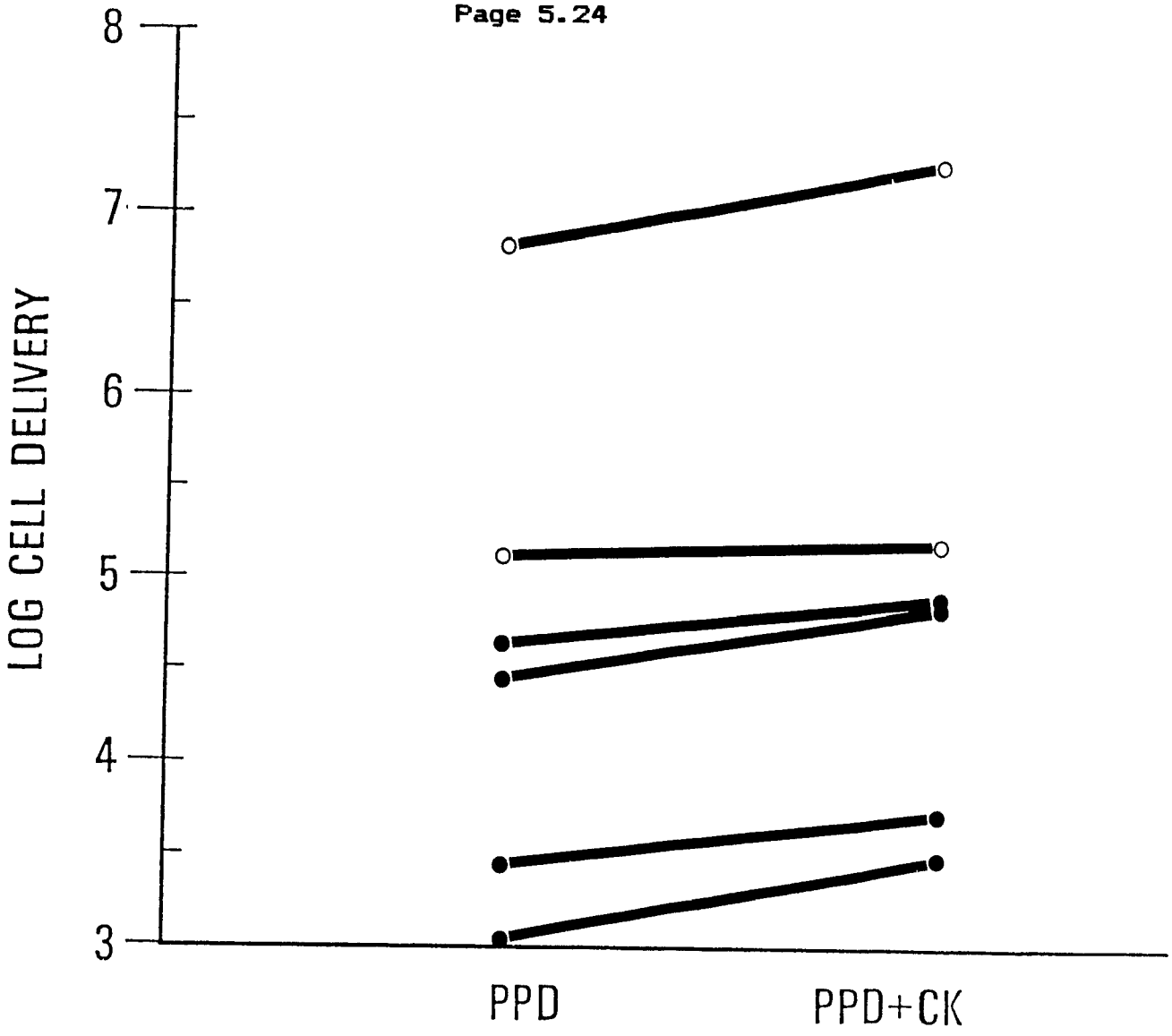

Fig 5.4 - Effect of CK on mononuclear cell delivery to DTH sites in PPD-'ve hospital patients. The delivery of mononuclear cells to PPD injection sites of both anergic (◆) and reactive (o) patients, non-responsive to PPD *in vitro* to PPD was doubled by co-injection with CK.

Discussion

Although there are many alterations in non-specific host defence associated with it, the anergic state is defined by a loss of the specific immune reaction, DTH (127,140). By employing the SW chamber technique described in chapter 3, the loss of DTH seen in anergic patients was shown to be reflected in a significantly lower number of mononuclear cells delivered to sites injected with antigen as compared with reactive patients and healthy controls. This occurred despite the fact that all these patients had lymphocytes sensitive to PPD in the circulation, indicating that they had the potential to generate a DTH reaction to PPD but that it had been masked by the anergic state.

It has been previously shown that the co-injection into anergics of cytokines from cultures of activated mononuclear cells along with an antigen to which the patient has sensitized lymphocytes will restore the DTH response in the majority of patients (140). Both the mechanism responsible for this change, and the relation between the cells delivered to the restored reaction and those at a normal unassisted DTH reaction were unknown.

In this investigation, it was found that co-injection of these cytokines with PPD increased the delivery of mononuclear cells 107-fold, resulting in levels of cell delivery indistinguishable from those seen in reactive patients. Thus the CK not only restored the visible components of the DTH reaction but also reversed the defect in mononuclear cell delivery present in anergy.

The relationship between the action of the CK and the anergic state was probed by conducting the same tests in a population of reactive surgical patients. These reactive patients, sensitive to PPD both *in vitro* and *in vivo*, also showed a significant increment in mononuclear cell delivery when PPD was co-injected with CK. The magnitude of this increase was approximately 1/3 of that seen in the anergic population, and if one reactive patient, whose increase was an order of

magnitude greater than any of the other reactive subjects, is discounted, the increase was only 7.5% of that seen in the anergic population. Thus while the CK did act to enhance the overall numbers of cells delivered to DTH sites in reactive patients, the magnitude of the effect was clearly linked to the state of the patients immune system.

Using the SW chambers it was found that the delivery of mononuclear cells to sites injected with Ag to which anergic subjects were non-reactive *in vitro* was in all cases lower than the delivery to Ag-'ve sites of two reactive patients, and significantly lower than the delivery to Ag-'ve sites of healthy controls ($p<0.025$, data shown in Chapter 3, Fig. 3.5). This data suggests that the Ag non-specific portion of the DTH reaction that occurs prior to T-cell activation was deficient in anergic patients.

Rode *et. al.* demonstrated the need for a prior sensitization to the co-injected Ag for the CK to restore the DTH reaction (140). In contrast with the the 107-fold and 38-fold increases in mononuclear cell delivery seen in Ag+'ve anergic and reactive patients co-injected with CK, the Ag-'ve patients merely doubled the numbers of mononuclear cells delivered when Ag was co-injected with CK.

The restoration by CK of an observable reaction to a sterile antigen in anergic patients represents an important step in restoring their immune function. In light of the significantly diminished delivery of mononuclear cells seen in anergy, it would be desirable to restore this delivery to normal levels at sites of infection. We have shown that the CK preparations used in this study are capable of restoring these levels at sites of sterile Ag deposition when specific cells exist in the circulation. Yet cell delivery and the visible appearance of a DTH reaction is not sufficient to ensure that the attracted cells will participate in a cell mediated immune response to an infecting pathogen. To do this there must be cells responsive to the Ag not only in the circulation but also at the site of infection, and these cells must carry out the functions required for cell mediated immunity.

The Ag-specific T-cell population delivered to DTH sites has been characterized earlier in this thesis by the examination of panels of Ag-reactive T-cell clones from SW chambers over normal DTH sites. These cells were enriched, relative to the PBM, for cells reactive to the Ag eliciting the DTH reaction. This population was found to consist of both CD4+ and CD8+ cells, with CD4+ clones predominating. Cytotoxicity and TNF production were broadly distributed throughout the population. Substantial portions of the CD4+ population produced both IL-2 and IFN-g and did not provide help for Ig synthesis in a PWM driven assay. These cells corresponded to the TH1 population described by Mossman *et. al.*, believed to mediate DTH while non-IL-2 producing, B-helper clones found at the DTH sites were believed to correspond roughly to the TH2 helper cell population. Both TNF and IFN-g were found in the SW chamber fluid over DTH sites, indicating that these cytokines <u>are</u> produced *in vivo* at DTH reactions.

When the same analysis was performed on the cells delivered to PPD+CH sites of two PPD+'ve anergic patients, very similar results were found. The frequency of Ag-reactive cells in the SW cells was enriched by 3 to 21-fold. Both CD4+ and CD8+ clones were found, with CD4+ clones in the majority.

One quarter of the CD4+ clones demonstrated LDCC. The only CD8+ clone tested demonstrated both LDCC and NK-like killing of K562 cells in the absence of lectin. All the CD4+ clones produced TNF. Two of the CD4+ clones gave help for Ig synthesis. Seventy-one percent of the CD4+ clones produced IL-2 and 83% produced IFN-g upon stimulation with mitogen.

In concert, these results reported in this chapter indicate that the anergic state is accompanied by a quantitative deficiency in mononuclear cell extravasation, both non-specific and (perhaps consequentially) specific, and that normal levels of cell delivery are restored by cytokines, if there are previously sensitized cells present in the circulation. This would indicate that the role of these CK is to act early in the DTH response to permit the delivery and subsequent activation of a relatively small number of specific cells and not to directly

deliver to the site the large numbers of mononuclear cells seen when Ck restores the DTH reaction.

The co-injection into anergics of Ck with Ag to which they were reactive resulted in the delivery of a population of cells comparable to those found at normal DTH sites. The T-cells delivered in response to a Ck+Ag injection were enriched for cells reactive to antigens at the site. These Ag-specific cells were cytotoxic, produced IL-2, IFN-g and TNF in a manner similar to the Ag-reactive cells from normal DTH sites. Other functions not investigated in this study may also be required for CMI reactions, but such a population of cells could be expected to participate in T-cell directed, CMI anti-pathogen responses.

It has been shown that the anergic state is reflected in a deficient delivery of cells to sites of sterile Ag deposition and that the delivery of mononuclear cells, including those specific for the antigen at the site and capable of participating in DTH, can be restored by the appropriate use of cytokines. If this is also true at sites of infection, such mediators would appear to have potential for site-directed treatment of infections in anergic patients.

Chapter 6: The Use of Cytokines to Treat Infection in Anergy

Introductory comments

The increased incidence of sepsis and septic related mortality, often including peritonitis, in anergic surgical patients implies that the defects seen in anergy may result in a reduced capacity to successfully mount an immune response to infecting organisms.

Rapidly evolving, diffuse peritonitis is a serious problem involved in septic episodes of anergic patients having undergone abdominal surgery. This form of infection in anergic patients is largely refractory to antibiotic therapy (148). Therefore we chose to use peritonitis as the infectious challenge in this investigation.

Previously it was shown that the lack of a visible DTH reaction in anergic patients is reflected in an abnormally low mononuclear cell delivery to sites of sterile Ag deposition. It was also demonstrated that the co-injection of CK along with Ag restored the delivery of normal numbers of mononuclear cells, and that the T-cells from these sites are capable of the same functions as those from normal DTH sites.

The ability of the cytokine preparations to restore a normal cell mediated immune response to sites of sterile Ag deposition in anergics led us to ask whether these same cytokine preparations might restore the cell-mediated immune response to infecting organisms. In order to examine this hypothesis, rats were rendered anergic with a thermal trauma procedure, and then infected, to produce a model of anergy with subsequent peritonitis.

We sought to determine, using this model, if injection at the site of infection, of the same cytokines that restored DTH in anergic patients, could restore the immune responsiveness of the animals and lead to increased survival.

Materials and Methods

Animals

Male and female Sprague-Dawley and Lu/Lewis F1 hybrid rats between 160-400gm were used. Within each experiment rats of one sex were utilized and their weights were normally within a 60 gm range. The animals were housed communally until thermal trauma and/or infection. Subsequently they were housed individually. All animals had free access to food and water throughout.

Immunization

For DTH skin testing, rats were immunized with a 1mg of KLH (Calbiochem) in CFA (Gibco) injected s.c. in 0.1ml. After 14-21 days, skin testing was carried out by shaving a small area of the flank or abdomen and injecting 0.3mg of KLH i.d., suspended in 0.1ml of saline (Travenol).

The thermal trauma model chosen for the studies in this chapter is an ethical procedure, since it is painless, and minimizes, for the animals, the duration of the experimental process. All the experiments in this chapter were approved by the animal care committee of McGill University.

Ethical considerations

From an ethical standpoint, a properly conducted experiment involves the production, in a fully anesthetized animal, of a full thickness (3rd degree) scald injury. The nerve endings in the skin are destroyed in a 3rd degree injury, thus the procedure is painless when properly carried out since the animal is unconscious during the process (144). The resulting injury does not result in distressed behaviour or alteration in feeding patterns or mobility (144), which indicates that the animals are not in pain. To insure that no individual animals have partial or secondary injuries, insensitivity in the affected area was assessed, and animals in which there was any possibility of retained sensation euthanized.

Thermal trauma

Rats were injected i.m. with 5 mg/Kg of Atropine (Astra). Ten minutes later they were anesthetized with 50 mg/Kg of Pentabarbitol (Somnitol, MTC Pharmaceuticals). When deep anesthesia was attained (assessed by the absence of the eyeblink, startle and toe-pinch(pain) reflexes, approximately 10-15 min post-induction) the back of the animals were shaved and the animals given fluid support in the form of 5ml/100gm of saline, injected I.P. The rats were then placed in a mould which exposed the clipped area of the back. This area was then exposed to boiling water for 11 sec. The animals were then removed from the mold and dryed. They were then inspected visually for evidence of incomplete (1st or 2nd degree) injury. The animals were kept warm and upon regaining consciousness they were assessed for any signs of pain (autonomy, cowering, inappropriate orientation, vocalization, aggressiveness). The extent of the third degree injury was determined by insensitivity to the pinprick test. Recovered animals were observed for several hours and then rechecked several times daily thereafter for any signs of distress.

Infection

The day of infection was considered Day 0. Anergic animals were traumatized on day -3. The rats were infected with an i.p. injection (2ml/100gm) of three enteric bacteria (see below) suspended in 25% BHI (McGill University, Dept. of Microbiology) + 25% prereduced thioglycolate broth (McGill University, Dept. of Microbiology) + 50% saline (or test substances) + 10% Ba2SO4 (w/v) (Royal Victoria Hospital Pharmacy). Various numbers of bacteria were used, however following dosage studies, the doses were standardized as follows:

Anergic rats - *E. coli*/*S. fecalis* 1.25x108/100gm, *B. fragilis* 2.5x108/100gm

Normal rats - *E. coli* 1.25x109/100gm, *S. fecalis* 1.25x108/100gm, *B. fragilis* 2.5x108/100gm.

Preparation of bacteria

B. fragilis (ATCC strain 25285) was grown under anaerobic conditions in prereduced thioglycolate broth. *E. coli* (National Collection of Type Cultures (England) strain 9001) and *S. fecalis* (ATCC strain 8043) were grown in BHI. Initial growth curves and concentrations were derived from spectrophotometry (O.D. at 600Hm). Bacteria were used while in the log phase of growth. Precise quantitation of bacterial numbers was accomplished by determining the number of colony forming units (CFU)/ml by triplicate platings of serial 10-fold dilutions of the bacterial suspensions on blood agar (Institute Armand Frappier).

Preparation of test substances

CF were prepared and fractionated as described in chapter 5. Single donor cultures (SDC) were prepared in the same manner except that the PBM from each donor were cultured seperately and the supernatants combined. Zymosan activated serum (ZAS) was prepared by mixing fresh human serum with 10mg/ml of zymosan (Sigma) and agitating for 1 hr at 37t. The zymosan was then removed by centrifugation followed by sterile filtration.

Zone of inhibition tests

The ability of test substances to inhibit the growth of *E. coli* and *B. fragilis* was assessed in a standard zone of inhibition test. Bacteria in the log phase of growth were used to "seed" a plate of the appropriate growth agar (see above). cardboard discs soaked in the test substances or the positive control, ampicillin (B-D), were then pressed into the plate, which was inverted and incubated for 24 hrs at 37t. The radius of the circle around the discs not showing growth was measured. This distance, minus the radius of the disc itself was reported as the zone of inhibition.

Cytotoxicity/growth inhibition test

All three bacteria were used in the log phase of growth, at the same concentrations as were used to infect the animals. In the same fashion as was used to prepare the injections, equal volumes of the bacterial suspensions and the test substances

were mixed in test tubes. The tubes were then placed in a 37℃ waterbath for 30 min. An aliquot of the suspension was then taken and serial 10-fold dilutions plated as described above to determine the CFU/ml.

Results

Thermal trauma results in anergy in rats

Christou *et al* defined anergy in rats sensitized with KLH as a DTH reaction less than 5mm in diameter (153). To determine if the thermal trauma resulted in anergy, sensitized rats were split into two groups, one of which was anesthetized but not traumatized (shams) while the other was anesthetized and approximately 30% of their skin exposed to boiling water for 11 seconds. Three days later these animals were skin tested. The results of these tests are shown in Table 6.1. The traumatized group gave significantly smaller skin tests then the sham group with the majority (15 of 18, data not shown) giving no reponse.

Thermal injury results in increased succeptability to infection

To determine if the thermal trauma resulted in increased succeptability to infection as well as decreased DTH, anergic (3 days post-trauma) and normal rats were infected i.p. with a constant number of *S. fecalis* and *B. fragilis*, and since it had previously been shown that *E. coli* was responsible for the lethality in this model of peritonitis (151), a varying number of *E. coli*. At the lowest dose of *E. coli* (Fig 6.1) 6 of 7 normal rats survived the first 48 hrs, while only 2 of 6 anergic animals survived. This increased succeptability to infection was also seen at higher levels of innoculation. The dose of *E. coli* given in Fig 4.1 was adopted for the subsequent experiments with anergic rats (this is the value given in the Materials and Methods).

Co-injection of CK from MLC supernatants with bacteria affords protection from lethal peritonitis

We tested the hypothesis that the increased succeptability of anergic rats to peritonitis could be reversed by co-injecting CK with the bacteria. Anergic rats were infected i.p. with a lethal mixture of enteric bacteria and either saline or CK. As seen in Fig 6.2, the co-injection of CK (in this case the equivalent of 5ml of MLC supernatant/100gm) afforded significant

protection from peritonitis which resulted in increased survival for as long as 11 days post infection.

Supernatants from unactivated PBM do not confer protection

To confirm that the protection the MLC supernatants confer was due to CK and not the culture medium (which contains human serum) or the products of non-activated cells, control cultures containing the supernatants from the cells of the same donors as used to generate the MLC supernatants, but cultured separately, were tested for their ability to confer protection.

These preparations, referred to as single-donor culture (SDC) supernatant were co-injected i.p. with the same bacterial suspension as the CK. For these experiments, dermoclysis was performed for the first three days post-infection, to provide additional fluid support for the animals.

As shown in Fig. 6.3, the effects of the dermoclysis were apparent in the 15-20% increase in survival in both the CK group and the SDC group (relative to the saline group from Fig 6.1). Nevertheless the CK afforded significantly greater protection than the SDC.

Zymosan activated serum does not afford protection

The presence of serum in the MLC culture medium made it possible that the protection was related to the presence of complement components, modified during the MLC reaction. The fragments that result when complement is activated, most notably C3b and C5a, are powerful chemotactic and activating agents for PMN. To explore whether these or other complement components played a prominant role in the protection by CK of anergic rats, fresh human serum was exposed to the complement activating agent, zymosan, and then injected in the same fashion as the CK. As shown in Fig. 6.4, the zymosan activated serum was no more effective than SDC or saline. MLC supernatant afforded significant protection from peritonitis in the anergic rats.

The protecting factor has a molecular weight less than 10kD

The MLC supernatant was passed through molecular sieves with preogressively smaller pore sizes, followed by dialysis, and

then tested for its ability to protect anergic rats from peritonitis to determine the approximate molecular weight of the protecting factor. As seen in Fig 6.5, protection from infection was afforded by the molecular weight fraction from 14-12kD to 1kD. Additional experiments utilizing ultradiafiltration further established that the protecting factor had a molecular weight between 10-1kD (data not shown).

The protecting factor is not bacteriocidal or bacteriostatic

One obvious explanation for the mechanism by which the factor conferred protection from peritonitis would be that it was directly toxic to the infecting bacteria in the innoculum. This possibility was evaluated in three different experiments. In the first, small fiber discs were soaked in CK and SDC preparations and used in a standard zone of inhibition test for 24hrs. The CK preparations, and the <100kD fraction used in these experiments conferred protection to anergic rats. The results of four such experiments are given in Table 6.2. In two there was a minor inhibition zone, much smaller than that seen around a standard antibiotic disc, while two other experiments showed no inhibition whatsoever.

The small size of the zones seen in half of the experiments related above could have been the result of excess fluid from the discs displacing the seeded bacteria immediately surrounding them. To determine if the CK did kill or inhibit their growth, the bacteria, in growth medium, were mixed with saline, SDC or CK preparations and incubated at 37t for 30 min. The bacteria were then enumerated by plating. The results of this experiment, in table 6.3, demonstrated that the CK neither killed nor inhibited the growth of the bacteria.

Further confirmation that the CK had no effect upon the survival of the bacteria was obtained under experimental conditions. Samples were drawn from the flasks (kept on ice) containing the mixture of bacteria + saline/SCD/CK + Ba2SO4 used to innoculate the rats after all the injections were performed (60 min) (survival data is shown in Table 6.5). The number of viable *E. coli*/ml in each flask was determined by plating. The results (Table 6.4) indicated that there was no

substantial difference between the four groups, none of which showed growth during the period they were kept on ice.

The level of protective activity found in MLC supernatants is high

Prior to carrying out further experiments to identify and characterize the active factor within the MLC supernatant it was necessary to determine the potency of the preparations. The potency of preparations from several supernatant pools was also assessed. Typically, preparations retained the capacity to confer protection at dilutions between 1:20 to 1:50. Diminished potency was usually observed at higher dilutions (data not shown).

One <100kD CK preparation was diluted with saline and assessed for its ability to protect anergic rats. The CK preparation used was a pool of supernatants from more than 15 MLC reactions. The experiment was performed with dilutions of 1:2, 1:25 & 1:100, all of which conferred equal protection (Table 6.5) Thus the protection could be achieved with as little as 0.1ml of supernatant per 100 gm of body weight.

MLC supernatants provide transient protection for normal rats

The <10kD fraction of MLC supernatants protects anergic rats from a lethal peritonitis. This protection is evident by 12-24 hrs and results in between 30-60% of the infected animals surviving long-term. To determine whether this protective capacity was restricted to animals with a pre-existing immunosuppression, similar experiments were carried out using normal (non-traumatized) animals.

As previously mentioned, non-traumatized animals require a higher dose of bacteria in order for the peritoneal infection to be lethal. In all other respects the experimental design was the same as that used in the previous experiments, except for the absence of the thermal-trauma procedure. The results are shown in Fig. 6.7. The normal rats were protected to a significant degree only during the first 24 hrs. After that period, the increased survival among the CK treated group declined to a level that was not statisticaly significant and was much smaller

than the final survival advantage seen in traumatized animals.

The rate at which the normal rats died was much greater than in the anergic rats. The much larger innoculation (10-fold greater) of *E. coli* necessary to result in mortality in the normal rats may have had a large influence on the mortality rate. If there was a time delay between the injection and the onset of the maximum effect of the factor, the earlier deaths in the normal rats may have accounted for the decreased effectiveness of the factor. To test for this, the factor was administered prior to infection under various experimental conditions.

Non-traumatized rats were pre-treated with a standard dose of the <10kD fraction of CK supernatants 2 hrs prior to co-injection with another equal dose of the same fraction plus the bacterial mixture. The results indicated that the combination of early administration plus a doubling of dosage resulted in no improvement in the overall survival as compared with the results from the previous experiment (data not shown). To further explore the efficacy of early administration, rats were given a single pre-treatment with the <10kD fraction either 6 or 30 hrs prior to infection. As can be seen from the results in Table 6.6, the pre-treatment 6hrs prior to infection afforded significant protection for the 24 hrs following infection, while the pre-treatment 30 hrs before infection had a lesser effect. As in the previous experiments, neither treatment improved the eventual percentage of surviving animals to a significant degree (% survival of all groups on Day 7: 0%). In an additional experiment (not shown) it was verified that the increased survival afforded normal rats by the MLC was due to a factor with MW <10kD.

The efficacy of pre-administration of the factor was also examined in anergic animals. From the previous experiments it appeared that the degree of protection provided by pre-administration of the <10kD fraction was equivalent to that seen when given simultaneously with infection to anergic rats. It was possible that this represented the maximum effect of the CK. Therefore to determine if pre-administration was more

effective in anergic rats, a CK preparation was diluted until it did not afford protection to anergic rats when given with the bacteria, it was found that pre-administration of the CK both 6 & 24 hrs prior to infection afforded significant protection (Fig 6.6). Thus when pre-administered, the CK was more potent than if given simultaneously with the innoculum.

MLC supernatants that provide protection do not restore local DTH

The original observation of the effect of MLC supernatants in anergy was their ability to restore local DTH. The ability of these same supernatants, which confer protection to peritonitis in anergic rats, to restore the DTH of anergic rats was examined. Rats sensitized to KLH were rendered anergic and skin tested with various fractions of MLC supernatants (that had been shown to provide protection) ± KLH. In two experiments involving 5 & 9 rats and the co-injection of two different KLH + CK preparations, the CK restored the DTH response although individual animals often responded to one, but not both supernatant pools (data not shown). Subsequent experiments with these pools plus one other which restored the DTH reaction in anergic patients, and using both intact and fractionated MLC supernatants were unable to replicate these results, leading to the conclusion that these CK protect anergic animals but do not restore their DTH response locally.

Treatment of peritonitis in anergic rats with the CK, although affording protection, did not restore the ability to mount a DTH response at a distant site. As shown in Table 6.7, the effects of the thermal trauma on Day -3 was detectable as early as Day -2. On day 0 (at the time of infection) the traumatized and infected animals were anergic when skin tested regardless of the treatment utilized. Surviving animals in all groups gave positive skin tests by Day 12 although the magnitude of their responses remained lower than their pre-trauma values.

The restoration of DTH in anergic patients and the protection of anergic rats from peritonitis are due to different factors

MLC supernatants from human cells restored the DTH response

and mononuclear cell delivery in anergic surgical patients and this led to the hypothesis that they might also influence the response to infecting organisms. This hypothesis was found to be correct in anergic rats, with the factor responsible for protection having a molecular weight less than 10kD. These same supernatants and fraction were not successfull in restoring the DTH reaction in anergic rats. The apparent contradiction between these two effects could be explained if they were due to different factors, one species specific (restoring DTH) and the other non-species specific (protecting from peritonitis).

The factor affording protection for anergic rats had a molecular weight less than 10kD. Anergic surgical patients were skin tested with PPD±CK or CK fractions. The results, (in Table 6.8) showed that *in vitro* PPD reactive anergic patients responded with a DTH-like reaction when PPD was co-injected with intact CK. All five showed the same response when the PPD was co-injected with the >50kD fraction of the CK preparation. Only one of five subjects reacted when the Ag was co-injected with the CK fraction between 10-3kD in molecular weight. The 10-1kD fraction of this same CK pool provided protection for anergic rats. The 10-1kD, but not the 100-10kD fraction also provided transient protection for normal rats (data not shown). Thus the two effects of MLC supernatants were shown to be due to factors with different molecular weights.

Table 6.1: Effect of thermal injury on DTH

Thermal Injury	N	DTH (mm) Day 0
Sham	10	7.9
Y	18	0.6

Table 6.2: Zone of inhibition tests

Test Substance	Organism #	Zone of Inhibition (mm)†
Saline	E	0
"	S	0
CK*	E	1
"	S	0
SDC	E	0
CK>50kD	"	0
CK<100kD*	"	0
SDC	E	0
CK**	"	0
CK<100kD*	"	0
SDC	E	0
"	S	0
CK>100kD	E	0
"	S	0
CK<100kD*	E	3
"	S	0
Ampicillin	E	6
"	S	12

*: CK preparations that protected anergic rats from peritonitis
#: E = *E. coli*
S = *S. fecalis*
†: Zone of inhibition =
radius of zone without growth - radius of disc

Table 6.3: Direct cytotoxicity tests

Test Substance †	Organism #	Bacteria/ml (x10⁸)
None(pre-incubation)	E	3.4
Saline	"	7.3
SDC	"	6.4
CK*	"	7.9
CK (<100kD, >50kD)	"	7.9
CK (<50kD, >1kD)*	"	6.1
None(pre-incubation)	S	5.2
Saline	"	9.7
SDC	"	12
CK*	"	9.0
CK (<100kD, >50kD)	"	10
CK (>50kD, >1kD)*	"	11
None(pre-incubation)	B	7
Saline	"	12
SDC	"	11
CK*	"	11
CK (<100kD, >50kD)	"	9
CK (>50kD, >1kD)*	"	11

*: CK preparations that protected anergic rats from peritonitis
#: E = *E. coli*
S = *S. fecalis*
B = *B. fragilis*
†: All SDC & CK prepararations were used at the same concentrations used in the i.p. injections in the rats

Table 6.4: Recovery of *E. coli* from injection mixtures

Sample	*E. coli*/ml (x108)
Input	5.1
Saline	6.5
MLC(1:2)*	4.8
MLC(1:25)	6.3
MLC(1:100)	6.7

*: Represents the dilution relative to the standard dose (10ml equivalent of supernatant/100gm. All three dilutions afforded significant protection (see Table 6.6 for survival data).

Table 6.5: Protection afforded by differing dilutions of MLC

Treatment	Equivalent volume of MLC injected* (ml/100gm)	Dilution relative to previous experiments	% Survival Day 1	Day 3	Day 7
Saline	n/a	n/a	62	62	46
MLC<100kD	5	1:2	90	90	90 (<.035)#
"	0.4	1:25	100 (<.035)	100 (<.035)	90 (<.035)
"	0.1	1:100	100 (<.035)	100 (<.035)	90 (<.035)

*: Volumes represent the equivalent volume of unprocessed MLC supernatant in each i.p. injection. Previous experiments had utilized the equivalent of 10ml/100gm. All injections were given in a volume of 2ml/100gm.

#: p-value relative to saline group

Table 6.6: Protection of normal rats afforded by MLC given prior to the administration of bacteria

Treatment	Time of Administration relative to infection	N*	% Survival (p-value relative to saline#) 3 Hrs	6 Hrs	12 Hrs	18 Hrs	24 Hrs	72 Hrs
Saline		16	94	94	19	0	0	0
MLC< 10kD	6 hrs prior	16	94	94	50 (<.056)	31 (<.022)	31 (<.022)	19
MLC< 10kD	30 hrs prior	16	94	88	44 (<.09)	19	19	6

*: N = number of animals/group
#: p-values by Fisher's exact test

Table 6.7: The effects of thermal injury and infection on DTH

Time of Skin Test*	Treatment Group			
	Saline	ZAS	SDC	MLC
Day -5	13#	13	12	12
Day -2	9	9	8	9
Day 0	0	0	0	0
Day 12	7	8	6	5
N† (S)'	20 (4)	30 (5)	30 (7)	30 (16)

*: Day 0 = day of infection (thermal injury took place on day -3)
#: % Survival
†: N = number of animals/group
': S = number of survivors

Table 6.8: Skin test results of anergic patients

ID	CLASS	AGE	Maximum Skin Test result(mm), 0-48 hrs PPD #	CK	CK +PPD	CK (>50kD) +PPD	CK (50-10kD) +PPD	CK (10-3kD) +PPD	NEW CPM #	STATUS ∞
1	HR	73	0	0	8	8	0	0	15,980	PPD+'ve
2	"	68	0	0	12	11	0	0	44,930	"
3	"	74	0	0	11	10	0	0	31,234	"
4	"	78	0	0	0	10	0	0	12,358	"
5	"	66	0	0	17	10	0	6	11,078	"
mean		72+/-4			10+/-6	10+/-1				

: New CPM = CPM (PPD stimulated) - CPM (Medium alone)
∞ : Reactivity to PPD was defined as >5,000 New CPM & Stimulation Index > 5

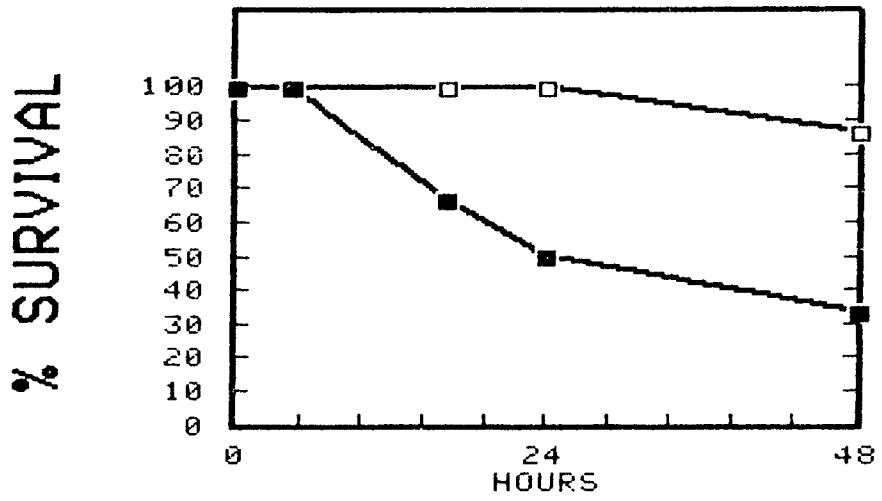

Figure 6.1: Increased succeptability to infection in anergy. Normal (□) and anergic (■) rats were infected i.p. with the standard innoculum of *E. coli*, *B. fragilis*, *S. fecalis* and Ba2SO4. The anergic rats had a greater mortality than the normal rats. This was also seen when higher numbers of *E. coli* were used in the innoculum (data not shown).

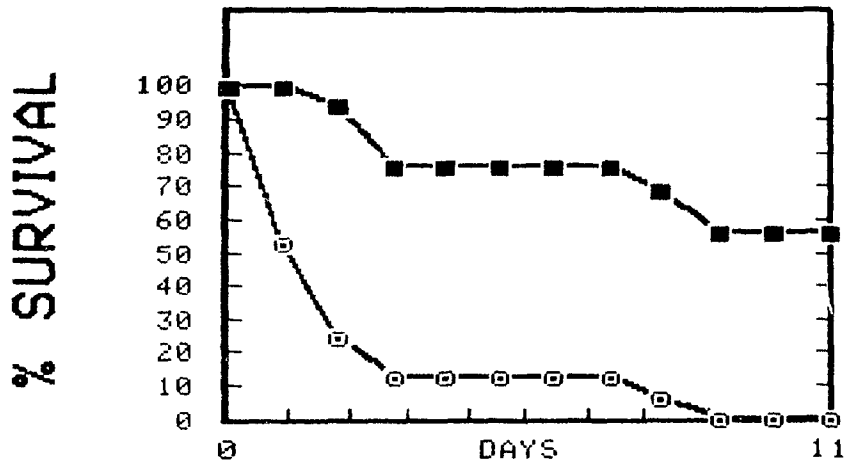

Figure 6.2: Protection from peritonitis afforded by MLC supernatants. Anergic rats were co-injected with bacteria and either saline (o) or MLC supernatants (■) The CK treated animals showed a significantly higher survival ($p < .005$, Fisher's exact test) from day 1 onwards.

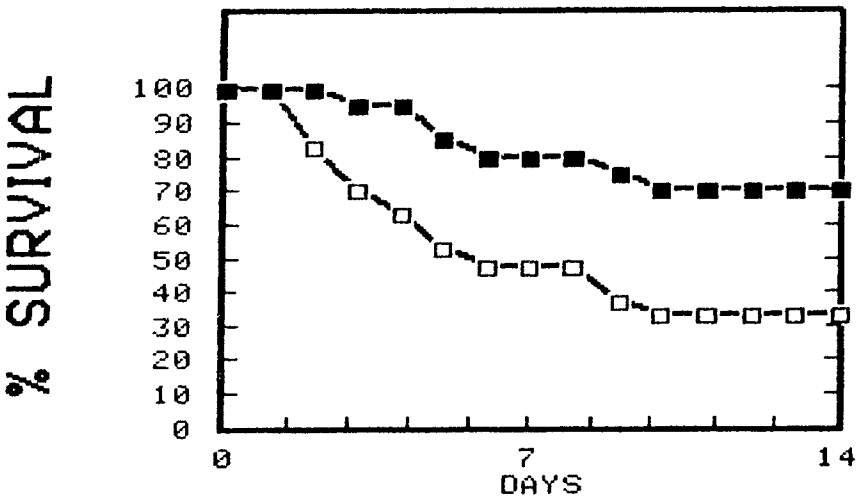

Figure 6.3: Control culture supernatants do not protect from peritonitis. Anergic rats were co-injected with bacteria and the supernatants from either MLC (■) or SDC (□). The rats were treated for dehydration by dermoclysis each day for the first 72 hrs post-infection. From day 3 onwards, animals treated with supernatants from SDC had a significantly higher mortality ($p<.03$) than the Ck treated rats.

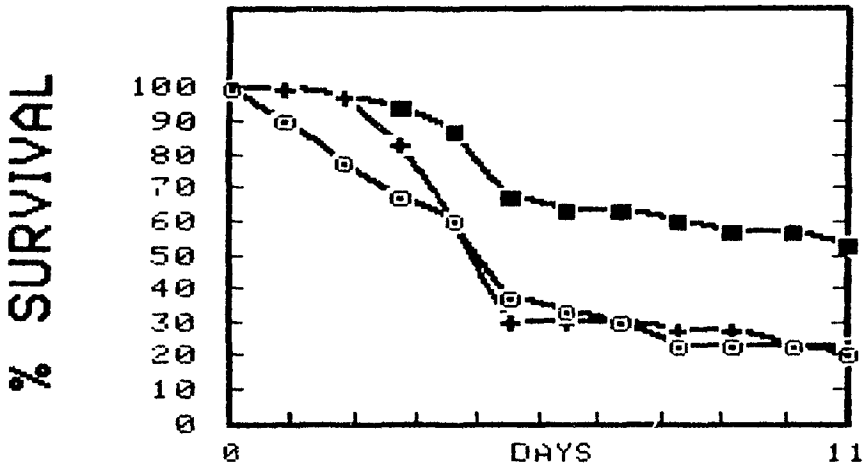

Figure 6.4: SDC and zymosan activated serum do not afford protection. Anergic rats were co-injected with bacteria and either CK (■), zymosan activated serum (+), saline (□) or SDC (not shown). Only the CK provided significant protection from infection, relative to the saline group ($p<.05$ from day 1 onwards).

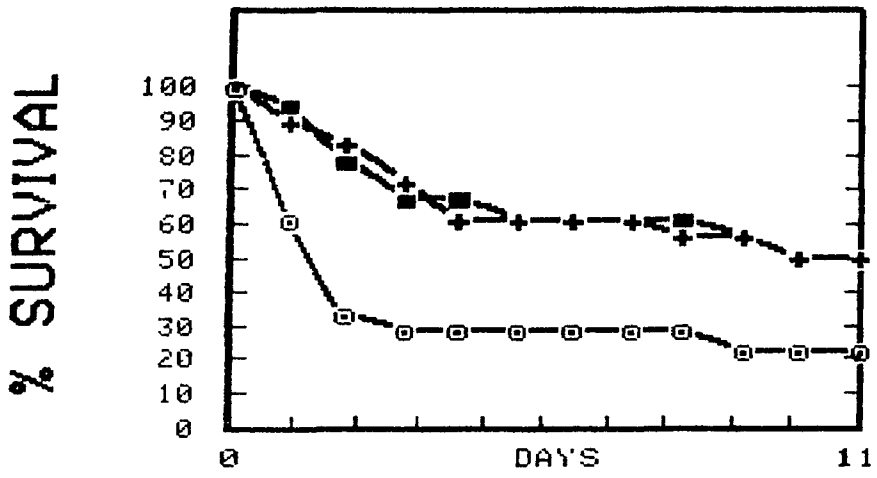

Figure 6.5: The factor affording protection from sepsis has a molecular weight less than 14-12kD. Anergic rats were co-injected i.p. with bacteria and intact CF (■), a CF fraction (molecular weight 14-12kD to 1kD, +) or saline (o). Only the intact supernatant and the fraction within the molecular weight range of 14-12 to 1kD afforded significant protection ($p<.05$).

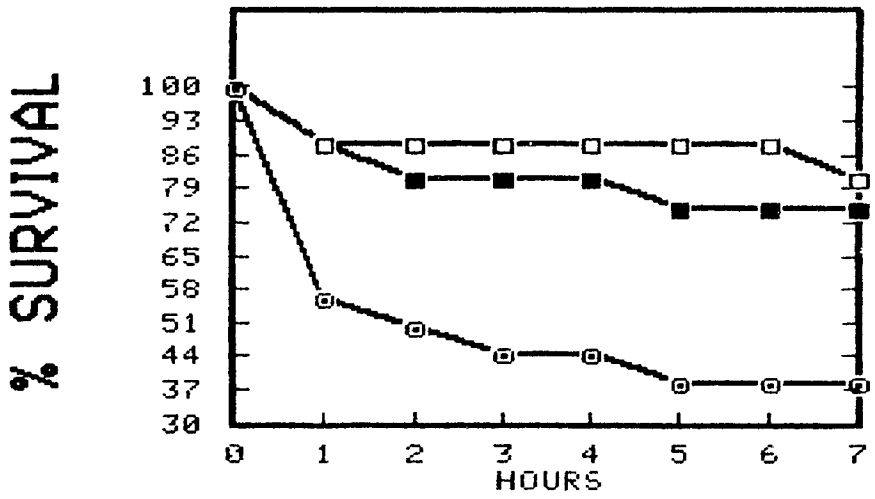

Figure 6.6: Pre-administration of the <10kD fraction of MLC supernatants enhances their ability to protect anergic animals from peritonitis. Using a pool of MLC supernatants that did not afford significant protection when diluted 1:20 with normal saline, anergic rats were pre-treated 6 (■) or 24 (□) hrs prior to infection. The pre-treated groups had significantly greater survival than the saline control group (○) from 48 hrs onwards (6hr group: $p<0.024$, 24 hr group: $p<0.028$, Fisher's exact test). The group co-injected with diluted CK and bacteria was not protected (not shown).

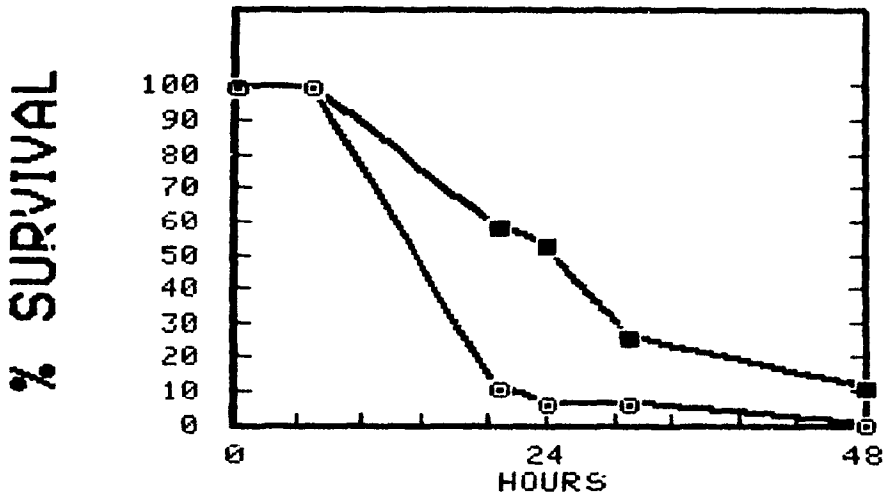

Figure 6.7: CK provides transient protection for non-traumatized animals. Normal (non-traumatized) rats were co-injected i.p. with bacteria and either CK (■) (molecular weight <100kD) or Saline (o). The CK afforded significant protection ($p<.005$) for the first 20 hrs. Survival percentages remained constant for both groups from 48 hrs to 14 days post infection (data not shown).

Discussion

The ability of MLC supernatants present at the site of Ag deposition to restore cell delivery and CMI to sterile Ag led us to question whether the same supernatants could modify the CMI responses at sites of infection.

The results presented in this chapter demonstrated that MLC supernatants do contain a factor or factors capable of protecting anergic animals from peritonitis when injected at the site of infection. The inability of control cultures prepared from the same cell preparations used to produce CK that did confer protection demonstrated that this factor(s) is the product of the activation of mononuclear cells. Furthermore, the inability of serum treated with zymosan to afford protection makes the involvement of complement components and antibody in this protection unlikely. The involvement of antibody and intact complement components was ruled out by the establishment that the molecular weight of the protecting factor was less than 10 kD (189). This also rules out the majority, but not all of the products of complement activation most notably C3a (189).

The question of whether the factor is effective only in improving the survival of anergic animals was addressed using non-traumatized rats. Co-injection of the active fraction of MLC supernatants resulted in a reproducible, significant but transient improvement in survival. The much greater effect, both in terms of the percentage improvement in survival and the long-term nature of improved survival of the anergic vs normal rats implies that the mechanism which is effected by the factor(s) is strongly down-regulated in anergy. Seen in this light, the smaller, transient effect seen in normal animals may be due to an increase in the tempo of the response to infection, or by a more modest increase in the already "normal" levels of the affected host-defence function than is produced when a "subnormal" anergic response is upregulated to "normal" or "near-normal" levels by the protecting factor. It is interesting to note that Christou *et. al.* demonstrated that with a similar

innoculum, including Ba2SO4, half the infected "normal" rats became anergic within 24 hours of infection (153). Thus the transient nature of the effectiveness of the factor in normals may indicate that it indeed may only have a significant effect upon an anergic host-defence response, and that in normal animals, the factor has no effect until the animals were sufficiently immunosuppressed by the infection itself.

Certainly some important conclusions regarding the mechanism by which the factor functions can be drawn from these experiments. The most important of these is that the factor is neither bacteriocidal nor bacteriostatic. The potential of the factor to kill the infecting organisms was evaluated in three different systems, both at 37℃ and on ice, alone and in conjunction with all the components of the innoculum.

In only one, the zone of inhibition tests, was any bacteriocidal/bacteriostatic activity detected. The effect however was both marginal and unreliable. The size of the zones detected were consistent with the possibility that the absorbant discs may have released some liquid onto the plate when pressed into the surface of the agar (necessary to ensure the adherence to the agar when the plate was inverted for growth) and merely displaced the relatively small numbers of bacteria present in the immediate vicinity prior to incubation and growth. The size of the areas of growth inhibition relative to the zone produced by a conventional antibiotic, ampicillin were not impressive, and the absence of any zone of inhibition in half of these experiments also supports the attribution of these results to experimental error.

In the direct cytotoxicity experiments the bacteria not only survived exposure to the MLC supernatants (at 37℃) but also grew at rates comparable to those seen in saline controls, as a result these experiments are more convincing. Finally, the possibility that the factor was bacteriocidal but required the presence of some component(s) of the two different growth media used for the bacteria or the Ba2SO4, or that it functioned only at the reduced temperatures found during the pre-injection period when the mixture was kept on ice, was eliminated (for *E.*

coli). By culturing the mixtures after all injections had been completed it was shown that the number of *E. coli*/ml remaining did not differ substantially between saline and MLC groups which did afford protection. Thus it must be concluded that the MLC supernatants neither kill nor inhibit the growth of the bacteria.

Although the factor may make the bacteria more succeptible to host defence mechanisms, it seems more reasonable to assume that it acts rather on the host, an hypothesis bolstered by the observation that hosts with different immune status respond to the factor in differing degrees.

The ability of the factor to significantly improve survival in both normal and anergic rats if given as much as 24 hrs prior to infection also supports the host-modifying hypothesis. Even when given as much as 30 hrs pre-infection the factor had an effect, although not statisticaly significant. It is unlikely that much of the original injection would remain in the peritoneum after 24 hrs to affect directly the bacteria subsequently injected. A more plausible explanation would be that the factor was absorbed, either into the surrounding tissue or systemically, where it could affect cells to improve their capacity to participate in the response to infection.

The high degree of potency found in the MLC supernatants is worthy of note. Most previous attempts to use known cytokines to effect the outcome of infections have required the injection of large quantities, on the order of tens of thousands of units or milligram doses per hundred grams of body weight in order to effect survival (157-160). Most cytokines such as IL-1 & 2, TNF & IFN-g are found in the order of tens of units/ml of stimulated normal cells cultures. The ability of as little as 0.1ml/100gm of an MLC supernatant to confer a significant improval in survival is unusual. Even this figure may be too conservative since the MLC was more effective if used to pre-treat anergic rats.

The identity of the protecting factor has not yet been fully established. From these experiments it is possible to state what the factor is not. The low molecular weight of the factor indicates that it is not: IL-1,2,3,4,5, or 6 (6,185,187) or IFN-a,β or g (184,200). It also rules out the possibility that the protection is due to TNF-a or β, ETAF, MIF or LIF (6,182,183,186,201,202). The low molecular weight of the factor raises the possibility that it may be related to the T-Cell chemotactic/neutrophil-activating protein described by Oppenheim *et. al.* (190) or C3a (189). In addition, the ability of a human factor to effect the survival of rats likely eliminates a number of cytokines, such as the Interferons and IL-4 which are species restricted in their known effects (6,188,203). Purification and sequencing of the factor will be required to determine its true identity.

Most surprising was the discovery that this protecting factor was not the same as the factor responsible for restoring DTH in anergic patients. This was the activity of the MLC supernatants that led to the experiments described herein, thus it was unexpected that the two activities were attributable to different factors. This does however offer an explanation for the finding that the MLC supernatants did not restore the DTH of the anergic rats. Many of the factors involved in DTH show species restriction that results in little or no activity of human cytokines in rodents. While it is possible that the differential effects represents a different underlying defect responsible for the anergic states in patients vs traumatized rats, a simpler explanation would be that unlike the protection from peritonitis, the restoration of DTH is due to the action of a factor(s) that are species restricted.

The experiments detailed in this chapter are not the end of the efforts necessary to identify and characterize the factor(s) affording protection from infection in anergic animals. The discovery of this protecting factor in MLC supernatants was an extension of the experiments in the three preceeding chapters.

As such it has opened up an entirely new area of investigation and the results presented here represent the first steps in isolating this intriguing factor, determining its mode of action(s) and utilizing its full theraputic potential.

Chapter 7: Discussion

The cells and mediators involved in DTH reactions have been poorly characterized largely due to their inaccessibility. The skin window chamber technique was developed to solve this problem. It was shown that the delivery of mononuclear cells to these chambers follows DTH-like kinetics with only a minor non-specific component, and is a faithful quantitative reflection of the underlying DTH reaction. The technique is a general one which can be used in virtually any subject population with a variety of antigens, and which delivers sufficient numbers of cells to permit the cloning and examination of the T-cell population involved in DTH and the recovery of mediators from DTH sites.

The initial objective of these experiments was to identify and characterize the cells and mediators involved in DTH and to illuminate the alteration(s) in these that occured in anergy.

The study of the SW chamber clones confirmed and expanded upon a number of the findings rom previous, less direct techniques. The classical autoradiological studies of Ag-specificity indicated that less than 5% of the cells at DTH sites were specific for the eliciting Ag (For convenience, these cells will be referred to as "Ag-specific" and other cells as "non-specific". This relates only to the Ag eliciting the DTH reaction, and is not meant to imply that the other T-cells are not in fact specific for Ag, but rather that their specificity is irrelevant to the reaction being investigated). The SW chamber technique is presumed to be more precise, relying as it does upon a greater sample size. Using this method, it was found that Ag-specific cells represented between 3-44% of the T-cells in the chambers. The source of the variation between subjects is unknown but not unprecedented. Some of the autoradiological studies of McCluskey *et. al.* (69) reported that Ag-specific cells were 10-fold more common in the epidermis than in the perivascular regions, suggesting that some compartmentalization of Ag-specific cells into the upper regions of the skin may

occur in DTH. Since the chambers are placed over the skin, in some cases they may have received a disproportionate number of these cells.

The most striking aspect of the proportion of Ag-specific cells in the chambers was their consistent enrichment relative to the blood. Ag-specific cells were 2.5 to 300-fold more frequent in the chambers than the blood and this enrichment was evident even when the frequency of Ag-reactive cells in the blood was very high. Evidence that this phenomenon is fundamental to DTH is contained in a recent report of a similar enrichment for PPD reactivity seen in the pleural effusions of TB patients (194).

The enrichment may be a result of the proliferation of Ag-activated cells *in situ* in response to growth factors such as IL-2/4 produced during the DTH reaction. If this were true, one would expect the appearance of Ag-specific cells in the chambers to follow exponential kinetics. The alteration of these kinetics by the co-injection of neutralizing Ab to IL-2/4 along with Ag could be used to probe the involvement of these growth factors in this phenomenon.

Another hypothesis that could account for this observation is that the prior skin tests received by the subjects might have resulted in the activation of Ag-specific cells *in situ* and possibly in the draining lymph nodes, which could then have entered the circulation where they would tend to be delivered to any subsequent DTH site. If such traffic occurred, it could account for the sometimes quite high frequency of Ag-reactive cells both in the chambers and also in the blood, such as was seen in subject P.

This hypothesis could be tested by placing SW chambers over sequential skin tests with the same Ag, on a subject who had not been skin tested recently. and determining the frequency of cells reactive to the Ag in the chambers and in the blood.

Another plausible hypothesis is that the cells activated by Ag *in situ* have different chemotactic or chemokinetic responses than non-activated cells, leading to a

disproportionate delivery to the chambers. Such a differential responsiveness would serve to enhance the delivery of CMI-promoting cells to sites of infection distant from the perivascular areas. Alternately, these cells may be more responsive to migration inhibitory factors, which would facilitate their "trapping" at sites of Ag presentation, while permitting a constant immigration of new cells from the circulation, and the emigration of non-activated cells, resulting in a progressive accumulation of Ag-reactive cells at the site. Testing freshly isolated SW cells in chemotaxis experiments and determining the Ag-specificity of motile and non-motile cells would reveal if either of these hypotheses are correct.

While Ag-specific T-cells are an absolute requirement for DTH reactions, it is obvious that they do not represent the majority of cells at DTH sites. The role of the non-specific cells present in the majority at DTH sites has never been determined. Classically, these cells have been thought to be recently proliferating T-cells attracted to the site through a non-specific mechanism. As a result of their inappropriate specificity, it was believed that these cells played no role in the DTH response, and were unimportant, inert bystanders.

Nevertheless, the possibility exists that these cells may respond to signals other than Ag, such as CF produced by Ag-specific cells, and thereafter exhibit some effector function. While such behavior would be unusual in unactivated circulating T-cells, the resident cells in the skin, or those attracted to the skin from the circulation, may differ from "typical" circulating T-cells. It is known that murine skin contains a large number of epithelial dendritic cells that express both CD3 and the Tgδ T-cell receptor (196). Intra-epithelial lymphocytes have also been shown to largely consist of similar cells that also express CD8 (197). These cells both produce and respond to CF (196,198) and respond to mitogens (196). Interestingly, these cells may also exhibit MHC-unrestricted cytotoxicity. If these cells are also present

in large numbers in human skin, it is quite conceivable that they may respond to the CK produced in DTH reactions and subsequently play a role in the reaction.

It has not been possible to determine the potential of the Ag-nonspecific cells at DTH sites to produce, or respond to Ck produced by other cells. Using cells from the SW chambers, these cells could be examined, both directly and by studying Ag-nonspecific SW clones. The presence or absence of Tgδ cells in the SW chambers, and the clones derived from the chambers, both Ag-specific and non-specific could also be determined. Exposure of the resulting populations to purified CK, or supernatants from Ag-specific TDTH clones, and to the SW chamber fluid could determine what role, if any, these cel's play in DTH.

The separation of SW chamber cells into subsets will likely require the use of flow cytometry and sorting. At the time the thesis work originated there was limited access to FACS machines on campus and objections were raised regarding the tendency of SW chamber preparations to stick or aggregate owing to the number of activated PMN in them. The efforts to remove these cells and the difficulties encountered have been described previously, however it should be noted that in recent experiments by utilizing substitutes for the 100% serum that was used to fill the chambers in these experiments up to 90% reductions in the PMN influx to the chambers was achieved. The use of such samples in FACS apparatus is feasible, and thus SW chamber cells could now be run and sorted for use in experiments.

It has been proposed that, in the mouse, there are two distinct forms of CD4+ cells. The TH1 "inflammatory" and the TH2 (B-cell) "helper" cell. It has remained unclear whether there are human counterparts to these cells, and also whether the TH1 cells would preferentially accumulate at Ag-specific inflammatory sites. While some functions were broadly distributed in human clones, in these experiments, the Ag-reactive T-cell clones from human DTH sites could for the

most part be divided into two groups analogous to the murine system in terms of IL-2/IFN-g production and B-cell help. Thus it appears that a similar functional dichotomy exists in human TDTH cells.

It should be noted however that while Mossman *et. al.* have claimed that all TH1 clones mediate DTH (25) Clark *et. al.* have reported that only 1 of 5 MBP specific murine clones could mediate DTH when injected with Ag into the footpad (175) and attributed the previous findings to the non-specific swelling that occurs when large numbers of cloned cells are injected, a phenomenon also noted by Marchal (75,76). This implies that there may be other functions than those used to define the TH1 population, that are required for the generation of DTH.

Thus the current definition of TH1 cells may be incomplete. There are a large number of Ck that are proposed to play a role in DTH, based largely upon their *in vitro* activities (6). The cells from SW chambers, and particularly the Ag-specific cells, are presumed to take part in DTH reactions, and the same for the Ck in the chamber fluid. By determining which Ck of the numerous possible candidates are actually produced by TDTH cells and those that are detected in the chamber fluid, the rather bewildering array of Ck thought to play a role in DTH by virtue of their *in vitro* activity might be narrowed down to a list of those demonstrably involved.

Using this approach, we were able to demonstrate conclusively that both TNF and IFN-g are produced *in situ* and also by TDTH clones. Examples of likely candidates for involvement in DTH that could be easily be confirmed would be LIF, MIF and the induction of fibrin deposition via MPIF as described by Geczy (6). Novel activities might also be sought. The ability of SW chamber cells and their supernatants to induce diapedesis through artificial vascular models could be a profitable way to identify Ck that are important in this part of DTH. From these approaches, a better definition of the functions carried out by DTH cells could be developed.

It has been asserted that TH2 cells would not play a role in inflammatory processes. Yet Ag-specific TH2-type cells are present in large numbers at DTH sites and it must be assumed that they are as likely to be activated as the Ag-specific TH1-type cells. What role, if any, is played by these cells in providing the afore-mentioned "missing" activity(s) involved in DTH, or other regulatory functions, should be as vigorously pursued as the studies of the TH1-type cells.

Gaining access to the cells directly from human DTH sites also allows an *in vivo* approach to be used in defining the functional phenotypes of DTH cells. The xenogeneic model of Gordon *et. al.* (195) may be used to evaluate the capacity of human "DTH" clones of varying phenotypes to induce Ag-driven inflammation. Additionally, sub-populations of SW chamber cells could be tested by adding purified APC and pulsing the cells briefly with Ag *ex vivo* and then washing the activated cells free of residual Ag and injecting them i.d. The CK purified from SW chamber fluids may also be injected i.d. into the same subjects from which they originated in order to determine their participation in, or regulation of, DTH reactions.

The availability of relatively large numbers of T-cells directly from DTH lesions, as well as functionally phenotyped clones of these cells will provide an opportunity to develop new MoAbs that might define the "TDTH" cell population. Obviously, currently available Ab may also be screened to determine their relevance to the cells involved in DTH. A large library of both Ag-specific PBM and SW clones from numerous individuals has already been established in the course of these investigations and these may prove invaluable in screening potential Ab candidates.

It has recently been shown that CD4+, CD45r+ cells do not provide help for B-cells but produce IL-2 and IFN-g, while the CD45r- population has the reverse phenotype (118). It would appear therefore that these correspond roughly to the TH1 and TH2 profiles. It is possible then that anti-CD45r Ab may delineate TH1 cells. If so then they should label the

population of SW cells capable of evoking DTH reactions. This could be tested in the xenogenic and possibly the autologous human model.

It has also been shown that in some instances the activation of CD45r+ cells *in vitro* causes them to differentiate into CD45-, TH2 - like cells (124). There is as yet no *in vivo* evidence of a similar phenomenon (118). It should be possible to determine if an activation by Ag *in vivo* induces the differentiation of CD45r+ cells to CD45r-, by placing freshly isolated SW chamber cells into culture with appropriate growth factors, and enumerating the changes, if any, in the proportions of CD45r+ and CD45r- cells.

While these experiments have focussed upon the study of T-cells, macrophages/monocytes also play a role in DTH, and these cells, and perhaps their CK are found in SW chambers in greater abundance than T-cells. The functional capacities of these cells could be investigated in a fashion very similar to the T-cells, and their role(s) in DTH defined, with particular attention to the interactions via CK between them and TDTH cells.

In addition to expanding our understanding of the cells and mediators involved in DTH, one of the goals of this work was to accomplish a similar investigation of anergy, with a view towards both describing the immunological events that do or do not occur in this condition, and possibly to use it as an "experiment of nature" to learn more about the immune system.

It was found that anergic patients had an abnormally low mononuclear cell delivery to recall DTH sites than both normal and hospitalized controls. The co-injection of CK with Ag restored delivery to the levels seen in response to Ag alone in reactive patients. Reactive patients also showed an improvement in cell delivery when Ag was injected with CK, although it was much smaller in magnitude.

Christou has developed the concept of anergy as a continuum of depression of DTH (and other host defences) leading from a normal response to zero (anergy) with progressively greater

probability of post-operative infection and septic-related mortality (129). This concept may explain the different magnitude of the effect of CK on the cell delivery of anergic vs reactive patients by implying that among the reactive patients, some, or even most, were partially immunosuppressed. If so, since the co-injection of CK with Ag in reactive patients did not result in as great an increase, relative to their response to Ag alone, as in anergic patients, then the magnitude of delivery to PPD+CK sites in reactive patients may be close to the maximum number of cells that can be delivered to DTH sites.

To prove such a conclusion requires data from a large patient population, varying somewhat continuously from a normal to totally deficient immune response. Obviously, the modest numbers of patients used in this study were drawn from the two extremes of the population. However, it can be concluded that on a population level, when injecting recall Ag, the magnitude of response to the co-injection of CK is inversely related to the state of immunocompetence.

Previous investigations of the restoration of DTH by CK in anergic patients resulted in the conclusion that these CK functioned to overcome some aspect of the anergic state which blocks T-cell activation *in vivo*. Marchal has reported that a small number of Ag-reactive cells *in situ* are required to generate a DTH response (75,76). In these experiments it was discovered that anergic patients have very low mononuclear cell deliveries that are restored to normal levels by the co-injection of Ag with CK. As a result, an alternative explanation that was consistent both with the previous observations by Rode *et. al.*, and the SW chamber data was proposed. This was the hypothesis that the early non-specific component of DTH is deficient in anergy, to the extent that insufficient numbers of T-cells from the circulation enter the skin to permit a cell of the appropriate Ag specificity to become activated. In this hypothesis, the action of the CK that restores DTH, is to increase the early non-specific delivery to sufficient levels to allow appropriate Ag-specific T-cells to enter the site.

In relation to this hypothesis, it should be noted that PPD-'ve (ie non-reactive *in vitro*) anergic subjects delivered lower numbers of cells to PPD injection sites than their two PPD-'ve reactive counterparts and also significantly lower numbers than normal subjects injected with an Ag to which they were not sensitized. This would imply that there is a defect in the response to, or production of, non-specific inflammatory mediators in anergic subjects.

Perhaps the most interesting observation was that in contrast to the responses of Ag sensitized patients the relative magnitude of the increase in cell delivery upon co-injection of Ag wit CK was insensitive to the underlying state of the immune system, when the subjects were <u>not</u> sensitized to the test Ag. Although patient numbers were small, both anergic and reactive patients responded by a doubling of cell delivery.

Thus one effect of the CK may be to mimic the effect of non-specific mediators in producing the early cell delivery. If reactive patients were as responsive to these mediators, as to those produced in the site, they would deliver more cells with the co-injection of CK. This is what was observed. If the anergic subjects remained fully responsive to the mediators, but simply failed to produce the initial mediators *in vivo*, one would expect that they should have increased their cell delivery to the levels seen in the Ag non-responsive reactive patients. This was not found to be the case. Since co-injection of CK with Ag restores the cell delivery in Ag responsive anergic patients to the levels seen in Ag sites of reactive subjects, it cannot be argued that patients in the anergic state are intrinsically incapable of normal cell deliveries. The differences would seem rather to be a result of a non-responsiveness to the early inflammatory factors normally produced in the Ag non-specific portion of DTH. Thus in the the anergic patients, their innate responsiveness to the additional factors provided by the CK was as defective as it was to those that should have been produced by their own cells.

There is additional evidence to support this hypothesis of a progressive loss of responsiveness to early non-specific inflammatory mediators in the work of Puyana et. al. (199). These investigators found that some anergic surgical patients, injected i.d. with the same CK preparations used in the experiments in this thesis developed a non-specific "flare" reaction which was predictive of individual post-operative survival. The authors interpreted this erythematous fluid-filled reaction as reflecting the ability or inability of the individual anergic patients to respond to an as yet unidentified non-specific inflammatory mediator(s) in the CK preparation.

Overall the available data would support the hypothesis that the anergic subjects were deficient in their responsiveness to inflammatory factors produced early in the non-specific portion of DTH.

Data upon which to base a judgement regarding the importance of the change in mononuclear cell delivery afforded by CK is scanty. One observation however, may serve to put the question within a frame of reference. The variation in mononuclear cell delivery to Ag-'ve sites among anergic patients was on the order of 10-fold. In contrast, the CK afforded only a doubling of mononuclear cell delivery to these same sites. In light of this, it would seem unlikely that each anergic patient would deliver exactly one half the number of cells required to trigger a successful Ag-specific T-cell - APC interaction. This is especially so when one considers the variation in Ag-specific frequencies one would expect both among the patients and between different Ag in the same patient. The fact that anergic patients still deliver several thousands of cells to Ag injection sites, and that only a single Ag-specific cell *in situ* may be required in order to initiate DTH make the likelihood that the observed non-specific doubling of cell delivery afforded by CK is alone responsible for the restoration of DTH improbable.

On balance it would seem more likely that these results indicate that there are two separate defects in anergy, possibly of a common origin, that are affected by the injection of CK.

One being the refractory response to inflammatory mediators, and the second, the inability of T-cells to be activated *in situ*. This hypothesis is entirely compatible with the results of Rode *et. al.*, in which PBM were cultured ± Ag and then re-injected i.d. (140). In these experiments, the re-injection of cells that had been cultured with PPD, or the supernatants from such cultures, restored DTH in anergic patients who were reactive to PPD *in vitro*, while cells cultured without Ag had no effect when injected i.d. The addition of a group in which the PBM were cultured without Ag and then re-injected along with Ag would serve to test the hypothesis and determine if the delivery of sufficient numbers of Ag specific cells into the skin suffices, in the absence of injected CK, to overcome the effects of anergy and mount a DTH response.

To verify that the early non-specific component of DTH is indeed defective in the anergic state, the response to small quantities of irritants could be used to determine if the non-specific production of inflammatory mediators is defective in anergy, and the injection of such mediators could suffice to establish if it is the response to such mediators that is faulty, without altering the local potential for T-cell activation.

The ability of CK to restore a DTH-like skin response has been known for some time. It has not been clear whether this reaction represents a true DTH response, involving the delivery of cells capable of carrying out the functions involved in DTH, or simply a partial replica of DTH. Following the description of the cells delivered to SW chambers over normal DTH sites, the same cells from anergics injected with Ag+CK were cloned and their functional capacities determined. In every function assessed, including the enrichment of Ag-specific cells, cytotoxicity and LK production, the T-cells from Ag+CK sites of anergic patients were comparable to those from normal DTH reactions. Thus the results indicate that the co-injection of Ag with CK results in a true restoration of DTH.

The failure of human MLC supernatants to restore DTH *in situ* in rats may indicate that the responsible CK is species-restricted. Rat MLC supernatants should restore DTH in anergic rats and if so, the rat may serve as a convenient system in which to isolate and describe the mode of action of this CK.

The importance of T-cell mediated immune responses in host defence against infection is well known. These results provide reason to be optimistic that the deposition of CK at sites of infection in anergic patients may serve to improve their immune response to the infecting organism(s). The ability to affect such an improvement in the immune response of anergic patients would be of obvious theraputic value, particularly since these patients are the most at risk for lethal outcome of post-operative infections.

It was for this reason that the experiments with anergic rats were carried out. The ability of miniscule amounts of human MLC supernatant to afford protection from lethal infection in these animals is further cause for optimism regarding the application of these results to patient therapy. The unexpected finding that the restoration of DTH and the protection from infection were due to two different factors may mean that eventually two distinct modes of immunotherapy may be applied to the treatment of such infections, and the effects of the CK on normal rats may signal that these benefits need not be confined to the anergic population.

At the present time, there is little evidence from which insights into the mechanism by which the factor affords protection from lethal peritonitis can be drawn. Some informed speculation however, may be carried out.

The initial hypothesis that the MLC supernatants would restore local host defences led to the decision to inject it directly at the site of the infection, i.e. intraperitonealy. If the factor functions only when introduced in such a local fashion, then this may simply indicate that it has a short half-life in the circulation. Alternately, it might reveal something more fundamental about its mode of action.

The factor may work by activating resident phagocytic cells, or by altering the local endothelium in such a way as to promote the recruitment of cells into the site, possibly by promoting adherence, gap formation or some other function vital to diapedesis. The factor may simply be highly chemotactic, and thus recruit cells, either specific or non-specific, into the site. The factor could act in some fashion, that rather than altering the type of reaction that occurs, simply accelerates the response that would take place without it, and thus protects the anergic animals by allowing their weakened host defences to "get a jump" on the infecting bacteria.

Naturally, a more attractive explanation would be that the factor represents an essential component of normal local host defences that is not produced in the anergic state, possibly the factor whose non-production results in anergy. Experiments to identify the factor and its mode of action would also determine the cell or cells that respond to it. Several possible candidates have been mentioned above, if the action of the factor is not restricted to the locale at which it is injected, a probable candidate for the target of the factor would be a phagocytic cell. Systemic non-specific activation of this type of cell could account for the protection of the anergic rats. The possibility of a non-specific activation of specific cells, possibly Tgδ cells, can not be ruled out, although such an activation would be unprecedented.

The factor is produced in MLC reactions and may therefore be a normal component of cell mediated immune responses. Should this be so, then anergy will indeed have served as a useful "experiment of nature" for investigating fundamental aspects of immunity.

In summary, the development, and application of, the SW chamber technique to the examination of DTH and anergy has led to a substantial refinement in our knowledge of the cellular and soluble mediators involved in these two facets of the immune system. This knowledge has in turn resulted in many new questions that may now be addressed, and also to the discovery

of a factor that may be of substantial theraputic value in the treatment of anergic patients.

Appendix 1: References

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