MEMBRANE CHARACTERISTICS AND FUNCTIONAL ANALYSIS OF HUMAN T AND B LYMPHOCYTES

A CONTRIBUTION TO THE ANALYSIS OF IMMUNODEFICIENCY IN CHILDREN

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Dedication

The empirical basis of objective science has nothing absolute about it. Science does not rest upon solid bedrock. The bold structure of its theories rises, as it were, above a swamp. It is like a building erected on piles. The piles are driven down from above into the swamp, but not down to any natural or given base; and if we stop driving the piles deeper, it is not because we have reached firm ground. We simply stop when we are satisfied that the piles are firm enough to carry the structure, at least for the time being.
K.R. Popper.
The Logic of Scientific Discovery,
Hutchinson, London, 1972, p. 111.

CONTENTS

ABBREVATIONS

ADA - Adenosine deaminase
AET - Aminoethylisothiouronium bromide
ADCC - Antibody dependent cellular cytotoxicity
ALS - Anti-lymphocyte serum
Aγ - Agammaglobulinemia
B2M - Beta-2-microglobulin
C3-R - Receptor for the third factor of the complement system
CML - Cell mediated lympholysis
ConA - Concanavelin A
CVID - Common variable immunodeficiency disease
E - Sheep red blood cells (SRBC)
E_{AET} - AET treated SRBC
E_{ox} - Ox red blood cells
E^+ - E-rosette-forming (T) lymphocytes
E^- - E-rosette-negative cells
Fc-R - Receptor for the F_c part of immunoglobulins (IgM,IgG)
H-2 - Mouse MHC (regions: K,D,I)
HLA - Human MHC (regions: A,B,C,D/DR)
HTLA - Human thymus lymphocyte antigen
Ia - Immune response associated MHC determinant
cIgM - Cytoplasmatic immunoglobulin (class: M)
sIg - Surface immunoglobulin (M,A,G)
MHC - Major Histocompatibility Complex
MLR - Mixed lymphocyte reaction
NAC - Non adherent cells
NSE - α-Naphthyl esterase (non sepcific esterase)
PBL - Peripheral blood lympho-mononuclear cells
PFC - Hemolytic plaque forming cells
OA-SRBC - Ovalbumin-coated-SRBC
PHA - Phytohemagglutine
PPD - Purified protein derivate of Mycobacterium tuberculosis

PWM - Pokeweed mitogen
SCID - Severe combined immunodeficiency disease
SDS - Sodium dodecylsulfate
SpA - Staphylococcal protein A
STA - Formalinized Staphylococcus aureus Cowan I strain
TCPC - T-colony precursor cells
TdT - Terminal deoxyribonucleotidyl Transferase
TNP-
SRBC - Trinitrophenyl treated SRBC
T-res- Theophylline-resistant T-lymphocytes
T-sens-Theophylline-sensitive T-lymphocytes

1. AIM OF THE INVESTIGATIONS

Concepts of the immune system in man change along with models based on experimental research (52). In the past three decennia the function of human lymphocytes, their differentiation pathways and their disorders have been unraveled by a close collaboration between animal and human immunologists.

The analysis of "experiments of nature", immunodeficiency diseases in man, has not only proven (or rejected) models developed for the human immune system, but also generated new directions for investigation (13). At the same time our understanding is still fragmentary and hence treatment of immunodeficiency diseases in children still in its infancy. Current therapeutic trials are based upon the classical model of the human immune system (15).

In this model lymphocytes are divided into two compartments: T lymphocytes and B lymphocytes. Both originate from bone marrow stem cell(s) (76) and differentiate along separate pathways. Immunodeficiency diseases are analyzed within this framework and are viewed as differentiation blocks within two separate cell-lineages.

A newer concept is based on the central role of T lymphocytes in immuneresponsiveness, either in humoral immunity or in cellular immunity (5,25,33,38,79). T lymphocytes regulate the maturation of B lymphocytes to antibody producing and secreting cells. Within the T lymphocyte population T-helper cells and T-suppressor cells can be distinguished. Recent data indicate the existence of a third subpopulation of T cells, namely amplifier T lymphocytes, which stimulate suppressor T cell precursors to become suppressor effectors and stimulate helper T cell precursors to become helper effector cells (32). Helper-cells for the activation of cytotoxic T cells

("killer" cells) have been demonstrated (38). In this newer concept, still mainly based on experimental data, the immune system is viewed as an intricate regulatory network of cellular and cell-mediated interactions. This implies recognition between cells. Lymphocytes carry cognition and recognition sites on their membrane. These lymphocyte membrane determinants became prime target for investigation and a third concept emerged (6,62).

Gene products of the major histocompatibility complex (MHC), expressed on the cell membrane, were shown to have important roles in the immuneresponse (66). Some of these products are involved in the killing of virus-infected cells (110). Others are involved in the development of the humoral and cellular immuneresponse against certain antigens (62). In the interactions between T lymphocytes and macrophages (69,85,99) the role of MHC products has been demonstrated. The role of MHC products in T-B cell and T-T (e.g., T suppressor cell-T helper cell) interaction has not yet been established, certainly not in human lymphocyte T-B and T-T interaction (46, 53,54,89,95,104,109).

This interest in lymphocyte membrane determinants also stimulated interest in the actual function of the membrane. The capping of surface receptors has been suggested to be involved in lymphocyte activation and possibly so in the interaction between cells (28,80,87).

A picture emerges of a T cell dependent immune system, which is regulated or guided by the MHC products.

Within this framework direct investigation of B lymphocytes necessitates special conditions. Mitogenic (polyclonal) B cell activators are direct probes for B lymphocytes and enable functional studies on B lymphocytes, without the intervention of T lymphocytes (17). The investigations presented are within this scope. The three models described are interwoven, but the fabric is not finished. The development of functional and marker

assays will enable further delineation of the immune system in man. The use of such tools in analysis of immunodeficiency will provide further insight in the normal immuneresponse and the underlying causes of immunodeficiency diseases in children.

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2. INTRODUCTION TO THE IMMUNE SYSTEM AND IMMUNODEFICIENCY DISEASE IN MAN [x]

2.1. The immune system = 2 x 10^{12} lymphocytes

Any biological phenomenon can be explained at many different levels, ranging from its molecular and genetic basis through cellular and tissue events, to effects in the whole animal. Ruling theories in immunology are framed in terms of cells rather than molecules. The central cells in specific immune response are the lymphocytes. The function of lymphocytes was unknown until surprisingly recently. Antibody molecules, present in the blood serum immunoglobulin fraction, had been discovered since 1890, but the cells synthesizing these molecules were unknown. Lymphocytes were described as "phlegmatic spectators watching the turbulent activities of the phagocytes" (81). Although ever since, reports had been published showing immunoglobulins in the cytoplasm of lymphocytes, the evidence was not accepted until the introduction of the immunofluorescence technique. The plasma cells were shown to contain specific antibodies, which had been anticipitated since plasma cells had been shown to secrete immunoglobulins in vitro (31). It was demonstrated, that plasma cells originate from lymphocytes (15). When lymphocytes were drained out the thoracic duct a severe immunodeficiency became manifest (43). By labeling and reinjection of the thoracic duct cells the recirculation of lymphocytes was discovered (42). The route from blood to lymph

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lay in the lymph nodes and Peyer's patches and from there, via lymphatic vessels, back to the blood and again to the lymph nodes and Peyer's patches. They did not enter the thymus, although this organ is crowded with lymphocytes. The number of lymphocytes obtained by thoracic duct drainage is huge. In man, recirculation of lymphocytes has been demonstrated (75). Altogether the human immune system is estimated to encompass 2×10^{12} lymphocytes (52), in volume as large as liver or brain. Lymphocytes are disperses through all organs, tissues and interstitial fluids (except the brain). Large concentrations are found in specialized lymphoid organs. From the developmental and functional viewpoints the lymphoid system can be divided into four compartments:

1. the stem cell pool: bone marrow lymphoid precursor cells (71);
2. the central lymphoid organs: bone marrow and thymus (63,72);
3. the peripheral lymphoid organs or structures: lymph nodes, white pulp of the spleen, gut-associated lymphoid tissue (tonsils, Peyer's patches), bronchus-associated lymphoid tissue (18);
4. the circulating pool: lymphocytes in blood- and lymph (42).

Although the circulating pool contains not more than 5% of all lymphocytes (34), the continuous exchange with lymphoid tissues implies that blood contains virtually all mature lymphocyte subpopulations. This has made investigation of the immune system in man highly accessible, by the use of peripheral blood lymphocytes.

Studies on lymphocytes derived from peripheral lymphoid tissues (tonsil, spleen) and in thoracic duct have shown differences compared with peripheral blood lymphocytes. These differences could still be ex-

plained by different ratios between lymphocyte subpopulations (21). Thusfar no unknown functions of lymphocytes, limited to peripheral lymphoid tissues have been revealed. However, this does not exclude specialized functions in localized environments or rather, important roles of the local environment on lymphocyte function (21,100,101,paper 6.7). Apart from being dispersed and circulating, lymphocytes are also in another sense in a dynamic state: there is a continuous decay of lymphocytes and a differentiation of new lymphocytes from lymphoid progenitors (1,71,76).

2.2. Differentiation of T- and B-lymphocytes

Some thirty years ago it was not only the function of lymphocytes which was unknown, but also of some complete organs: the thymus and the bursa of birds. This lack of knowledge was tested by taking out these organs----------and no function could be demonstrated because no defect became manifest.

Miller, investigating the role of the thymus in mouse leukemia development, started taking out thymuses-----without any effect. However, when the organ was taken out immediately after birth, severe immunodeficiency became manifest (63). Likewise the function of the bursa has been unraveled. When the bursa was taken out immediately after birth or even more evident, when birds were bursectomized in ovo (by treatment with testosteron) the development of plasma cells and the production of antibodies was severely hampered (40,44,65).

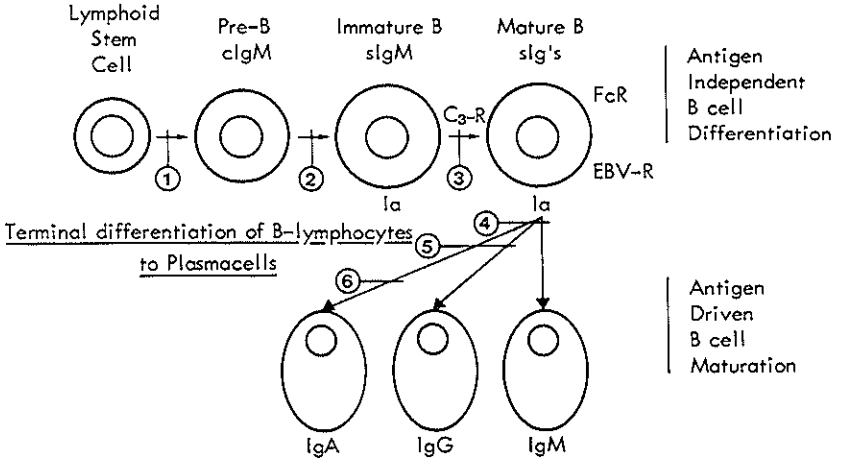
These experiments indicated two separate lymphocyte compartments within the lymphoid system.
a) Cellular (or cell-mediated) immunity, responsible for delayed type hypersensitivity, for the rejection of foreign grafts and for the defense against some

kinds of infections (certain viruses, bacteria, parasites and fungi) which can occur independently of humoral antibody production. Cellular immunity is mediated by the thymus-dependent or T lymphocytes and is transferable by lymphocytes (20,107).
b) The bursa-dependent or B lymphocytes, which mature to plasma cells secreting antibodies. The antibodies have a prime role in the defense against infectious agents. The immunesera which display these characteristics can confer passive immunity to normal non-immune recipients. This broad category of immuneresponse is designated humoral immunity.

Both T and B lymphocytes are descendants of a bone marrow stem cell, which is called pluripotent, because it gives rise to the myeloid, erythroid and megakaryocytic series as well (76). The B and T lymphocyte-axis might already be separated at the bone marrow lymphoid precursor cell stage (1). From that stage on two pathways of differentiation originate one to pre-thymic T precursors, post-thymic T precursors, intrathymic T cells and post-thymic T cells (37,58,97) and the other to pre-B cells, B lymphocytes and finally plasma cells. Mammals have no bursa but they do manufacture B lymphocytes in their bone marrow (hence the designation: bone marrow derived B-lymphocytes). Further maturation of B lymphocytes to plasma cells is induced by antigen and is effectuated in the peripheral lymphoid organs (100,101), but also in the bone marrow (7,49,102). In the spleen this process is initiated in the peripheral, periarteriolar lymphocyt sheaths (101) and in lymph nodes in the superficial cortex (100). The bone marrow plasma cells, hardly recognized as an important site of antibody formation, however prove to be the major source of plasma immunoglobulins (49) and antibodies (7). The hypothetical model of B celldifferentiation

and maturation is presented in Figure 1.

Fig. 1. Hypothetical model of stages in B-cell differentiation



Legend: See text. The numbers refer to differentiation blocks given in Table 1. Modified scheme from E.R. Pearl (73).

Pre-B cells contain small amounts of cytoplasmic IgM (cIgM). Their existence in man has been demonstrated in diseases with a differentiation block after this pre-B cell stage (73). At not yet clearly defined stages of differentiation various determinants and receptors are acquired. The Ia determinant, an HLA-D/DR related common B cell determinant, is probably present from the immature B cell stage on (45). The C3-receptor (C3-R), a receptor for the third factor of the complement system, is expressed on immature and mature B lymphocytes (86). The Fc receptor for the Fc part of immunoglobulins and the EBV receptor is a receptor for Epstein Barr virus.

B lymphocytes, defined as surface-immunoglobulin

bearing lymphocytes, carry first s-IgM (57). Later on all classes of immunoglobulins can occur. Individual B lymphocytes carry one or more classes of immunoglobulin and only one type of light chain (74). After further maturation to plasma cells individual cells secrete antibody molecules of one or more classes, but of only one specificity (48,70). This is fundamental to the understanding of antigen specific hemolytic plaque assays in which amongst many inactive B lymphocytes and amongst cells secreting antibodies with other specificities single antibody secreting cells can be detected. The human antigen specific plaque forming cells (PFC) referred to in paper 6.3 secrete IgM antibody specific for ovalbumin-coated sheep red blood cells (OA-SRBC). Antigenic activation induced in vitro is under strict T cell control (5, 25). Whenever the assay is performed using other antigens than the antigen presented to the lymphocytes in the induction phase no PFC response is detected. Thus a single cell produces antibody of only one specificity and is directed to only one antigenic determinant.

The second type of PFC response described in paper 6.2 is induced by mitogenic activation of lymphocytes. Polyclonally activated B lymphocytes secrete one out of many different antibody specificities. The specificity for trinitrophenylated SRBC (TNP-SRBC) is common. At any rate,in mice,the frequency of TNP reactive B lymphocytes is estimated 1 : 60 - 1 : 230 B lymphocytes after polyclonal activation (3,8). The feasability of in vitro induction of antibody formation stresses another important characteristic of the immune system. The lymphocyte system is autonomous, it does not need a local environment. This is true for mature lymphocytes who have acquired antigen receptors. Depletion of antigen binding lymphocytes

from lymphocytes activated by this antigen has been shown to reduce the responsiveness of the remaining lymphocytes to that antigen (59). At present the surface immunoglobulins on the membrane of B lymphocytes are thought to be antigen receptors.

Antigen reactive cells are also found in the T lymphocyte population and represent a fairly mature T cell stage (14). T cells do not carry immunoglobulin. The nature of the antigen receptor on T cells is still a matter of debate. The HLA encoded determinants, having an immunoglobulin-like part in their structure, have been suggested to be associated with the T cell antigen receptor (9,47,80,91).

In T cell differentiation the local environment of the thymus plays a crucial role (37,78,97). In animals, studies have centered around the athymic nude mice. Nude mice have T precursor cells in their bone marrow (108). Neonatal thymic grafts in nude mice lead to reconstitution: host immunoincompetent pre-T cells will differentiate to immunocompetent T cells (97). The thymus has an "endocrine" function as well. Thymic tissue enclosed in cell impermeable diffusion chambers can reconstitute thymectomized mice but not nude mice (97). Thus it has been suggested that thymic epithelium is obligatory for reconstitution in nude mice.

In man these various factors can be analyzed using bone marrow cells or blood lymphocytes from immunodeficiency patients with differentiation defects in the T cell lineage (37). One can distinguish a prethymic T precursor cell originating from the bone marrow, which can be induced by direct contact with thymic epithelium to differentiate to postthymic T precursor cells (C3 receptor positive, TdT positive, ADA positive, E rosette-negative, mitogen response negative). Factors produced by thymic epithelium and cAMP modu-

lating agents will induce further maturation to intrathymic T cells (TdT positive, ADA positive, E rosette forming at 37°C, theophyllin resistant, mitogen reactive, T helper, MLR-responding) and postthymic T cells (TdT low, ADA-low?, E rosette forming at 4°C, IgM receptor and IgG receptor positive, mitogen reactive, T helper, T suppressor, Ia positive (some); see for a discussion: Gelfand, E.W., Dosch, H.M. et al., 37). "Thymic hormones" induce maturation probably at the intrathymic T cell stage, whereas antigenic activation leads to fully immunocompetent T cells. At present it is unknown whether separate T cell precursor populations follow different differentiation pathways to post thymic T cells. Evidence for two parallel lines of T cell differentiation in the thymus of mice has been presented (61,88). After leaving the thymus, the postthymic T cells migrate into the T cell dependent areas in the peripheral lymphoid organs:(the periarteriolar lymphocyt sheaths of the spleen and paracortical zones of the lymph nodes (7, 11,12,22,24,49). Here, the T cells can be activated by antigen. In vitro, the last phase of T cell differentiation can be induced by antigen and mitogen and can be measured by proliferation assays or assays for helper activity (paper 6.2).

In man T lymphocytes have the characteristic capacity of forming rosettes with sheep red blood cells (E-rosettes) (36). E rosetting enables the isolation of T cells by separation of E rosetting cells from the rest of the cells on density gradients. E rosette formation can be modulated by various substances one of which is theophyllin (60). When T cells are incubated with theophyllin some T cells become unable to bind sheep red blood cells (the theophyllin sensitive T cells: T-sens) and others keep their rosette forming capacity (the theophyllin resistant T cells:

T-res). By theophyllin incubation of E rosetting T cells both populations can be separated on density gradients. Analysis by the OA specific PFC response has revealed distinct functions: T-sens lymphocytes have suppressor activity and T-res have helper activity in the induction phase of the OA-PFC response (90).

The majority of the recirculating cells are T cells. B cells are far more sessile in peripheral lymphoid organs (34). This explains the high percentage of T cells in peripheral blood and lymph, when compared with lymph nodes and spleen (21). Recently, monoclonal antibodies against various T cell subsets and T cell precursors have been developed (78). These monospecific reagents recognize membrane determinants, which are characteristic for certain stages of T cell differentiation. It was already evident that cell surface antigen patterns change gradually during T cell differentiation, although subsets can now clearly be distinguished by these antisera (78). These marker assays used in combination with the effects of various inductive environments or substances have enabled further analysis of immunodeficiency diseases. Within the T and B cell differentiation concept all the assays described are viewed as differentiation markers. Thus an antigenic or mitogenic response, in fact a functional characteristic of lymphocytes, is used as an indicator of maturity of lymphocytes.

This statement is merely made to demonstrate the way of looking at the immune system within this concept. It has already been disclosed that along with their differentiation and maturation lymphocytes acquire different functions.

Table 1: Immunodeficiency diseases classified as presumed differentiation and maturation defects along the B lymphocyte lineage

1.[x] Pre-B cell deficiency:
- Immunodeficiency associated with thymoma
- Severe combined immunodeficiency
- Varied immunodeficiency (hypogammaglobulinemia)
- Kappa chain deficiency
- X-linked agammaglobulinemia

2. B cell deficiency:
- Congenital agammaglobulinemia
- Varied immunodeficiency (hypogammaglobulinemia)
- Severe combined immunodeficiency
- Ig deficiency with IgM production and without $sIgG^+$ - and $sIgA^+$ B cells

3. Mature B cell deficiency:
- Varied hypogammaglobulinemia with or without autoantibodies to B lymphocytes

Failure of terminal differentiation of:

4. All B lymphocytes:
- Transcobalamin II deficiency
- Immunodeficiency with Ataxia teleangiectasia

5. $sIgG^+$ and $sIgA^+$ - lymphocytes
- Ig deficiency with increased IgM

6. $sIgA^+$ lymphocytes
- Selective IgA deficiency

[x] The numbers refer to the differentiation blocks shown in Fig. 1.

The table indicates the presumed level of defect in B cell differentiation. The same type of analysis of immunodeficiencies caused by defects in the T cell axis is shown in Table 2.

2.3. Immunodeficiency diseases in children

In the delineation of T and B cell differentiation "nature's own experiments" have played a major role. In 1952 Bruton described a child suffering from severe bacterial infections, who had no immunoglobulins (12). This disease, agammaglobulinemia, was classified as the analogue of the bursectomized birds disease: both lacked serum immunoglobulins and plasma cells. Agammaglobulinemia can be inherited (X-linked) and the bursectomy experiment settled the idea of a genetic defect of the differentiation of (pre-B cells to) B-lymphocytes. Various antibody deficiencies have now been discovered and classified as differentiation disorders along the B cell axis (Table 1) (83).

Table 2: Immunodeficiency diseases classified as differentiation defects along the T cell lineage

Defect	Clinical designation
Pluripotent stem cell	Reticular dysgenesia
Lymphoid stem cell	Severe combined immunodeficiency
Pre-thymic T precursor	Severe combined immunodeficiency
Thymic T cell	DiGeorge's syndrome
Postthymic T cell	Immunodeficiency with Ataxia teleangiectasia
	Common variable immunodeficiency

The DiGeorge syndrome, an absence of the thymus, stands as a prime example of a T cell differentiation defect by lack of inductive environment (50). It is

evident that most of the T celldifferentiation defects are accompanied by B cell defects. Severe combined immunodeficiency (SCID), a combination of T and B cell deficiency, has been shown to be a heterogeneous group of disorders along with further characterization of various steps in the induction of T cell differentiation (Table 3) (37).

Table 3: Severe combined immunodeficiency and presumed defects

Stage of differentiation	Target for induction of differentiation
bone marrow / bone marrow microenvironment	lymphoid stem cell
thymic epithelium	pre-thymic T precursor
epithelial factors	post-thymic T precursor and intra-thymic T cell
thymic hormones	post-thymic T precursor

Not only external (differentiation induction) factors can be deficient in SCID; a heterogeneous group of intrinsic T cell defects can mimick severe combined immunodeficiency. Examples are the adenosine deaminase deficiency, an enzyme defect in purine metabolism, which is associated with a severe combined immunodeficiency (39), and the membrane capping disorder described in paper 6.6. The designation combined (T plus B cell) defect, has been reevaluated along with this further classification. Some SCID patients do have B lymphocytes; however terminal maturation is nearly always absent. At present only the lymphoid stem cell defect is viewed as a real combined immunodeficiency in the sense of a defect of the anlage of both lympho-

cyte lineages. Two points emerge from these analyses of immunodeficiency diseases. First, the studies on immunodeficiency have a leading role in the delineation of the human immune system. Recently the serendipitous findings of enzyme defects in purine metabolism causing various immunodeficiencies has opened a whole new area of investigation and even promises tools for regulation of the immune response (13,26). Second, the classification of immunodeficiency rests on the basis of the differentiation model of two separate compartments within the immune system: the T cell axis and the B cell axis. However this model lacks one of the most important advancements in understanding immunity namely the central, directive role of T cells in regulating humoral immune responses. For example, the B lymphocyte defect in most of the combined immunodeficiencies is presently seen as the result of the T cell defect and not as a defect in the B cell lineage. The basis of a more functional approach in the classification of immunodeficiencies, in which markers on lymphocytes are seen as indicators of lymphocyte functions, will be discussed.

2.4. Functional properties of T cells

Investigation of lymphocytes derived from various immunodeficiency patients parallel to studies on isolated normal donor lymphocyte subpopulations have revealed many functional properties of human lymphocytes, limited to T cells or T cell subpopulations (Table 4).

Table 4: In vitro functional properties of T lymphocytes

Proliferative responses:

1. to soluble antigens: PPD, Tetanus toxoid
2. to particulate antigens: Candida albicans
3. to cell surface antigens: MLR-response
4. to lectins and other mitogens: PHA, PWM, ConA, spA.

Cytotoxic responses : CML, ADCC
Mediator production : Helper and suppressor factors
Regulatory functions: Helper T cells and suppressor T cells in T-T, T-B and T-macrophage interaction.

Modified after Reinherz, E.L. (79):

PPD = purified protein derivate of mycobacterium tuberculosis
MLR = mixed lymphocyte reaction
PHA = Phytohaemagglutinin
PWM = Pokeweed mitogen
ConA = Concanavalin A
spA = Protein A derived from Staph. aureus
CML = Cell mediated lympholysis
ADCC = Antibody dependent cellular cytotoxicity

In MLR, lymphocytes from two individuals are mixed: the reciprocal aggression of these cells leads to proliferation of the T cells. The stimulating antigenic determinant is encoded by the HLA-D locus. Expression of this structure is largely on B cells, but some T cells also carry this determinant and do stimulate very strongly (paper 6.3). Mitogens such as PHA, ConA, spA and PWM activate a large population of lymphocytescytes, primarily T cells (79). After activation

T cells develop helper activity for B cells leading to B cell proliferation and B cell maturation to plaque forming cells and plasma cells (51,96; paper 6.2). Especially studies on PWM induced activation of lymphocytes have shown that T cells produce helper factors which are obligatory mediators in B lymphocyte activation by PWM (51,96). The mitogens are not selective for either helper T cells or suppressor T cells. By irradiation, however, suppressor T cell activity can be eliminated (paper 6.2). The net effect of the balance between activation of helper T cells and suppressor T cells is dependent on the mitogen used (and the assay used). ConA activation of T cells leads to a suppressive effect on B cell activation, whereas the net effect of PHA, PWM and spA activation is help. Although the mitogens are nonselective, the activated subpopulations of T cells, T helper cells and T suppressor cells, are different. Already before activation they can be distinguished by surface markers. After isolation their different functional instruction can be demonstrated (5,14,25,78,79). The helper T cells involved in the induction of antibody formation are found in the theophyllin resistant T cell (T-res) population (90). The suppressor T cells involved in the prevention or inhibition of B cell maturation are found in the theophyllin sensitive T cell (T-sens) population (90).

In general, B cell triggering by antigen requires help of T cells. The T helper cell activity is conferred to B cells either by direct contact or by the release of helper factors, which stimulate the B cells to respond (22,32,33,68). The immunoregulatory unit consists of an interplay between two antagonists: helper T cells and suppressor T cells. The suppressor T cells act either directly on B lymphocytes or inhibit T helper cell activity (38). In this cellular

interaction model macrophages present the antigen to T cells and probably to B cells (29,69,85,99). Antigen serves as the signal for activation. Thus, the immune system is a functional network of interacting cells which is modulated by external antigen.

Since cell interaction requires recognition of membrane structures, the emphasis on cell interactions has made the cell membrane a main target for structural studies. It has become clear that, apart from antigen receptors at the cell surface, lymphocyte recognition also involves membrane molecules that are products of genes of the major histocompatibility complex.

2.5. Role of gene products of the major histocompatibility complex (MHC) in the immune response

T cells seem to be particularly responsive to antigens that are presented on the surface of macrophages. The interaction of T cells with macrophages however is subject to a crucial restriction: T cells will only be activated if the macrophage, besides presenting the antigen, will carry a certain MHC determinant on its surface (69, 84, 92). Likewise T helper cells will fail to help another lymphocyte in an antigenic response if this lymphocyte carries a foreign MHC determinant (29,46,53,54,62,66,95,104). This so called MHC restriction phenomenon has also been demonstrated in the killing of target cells. Virus infected target cells will only be attacked by cytotoxic T cells if the cytotoxic cell and the target cell share at least one MHC determinant (63,110). T cells are presumed to be educated to this MHC-restricted behaviour when passing through the thymus, although instruction at an earlier stage is debated (111).

In mice, the MHC region contains immune response genes, coding for the level of the immune response

against certain antigens (67) and for the switch from IgM to IgG synthesis (93). Low responses to one antigen can be accompanied by good responses to another antigen. Immune response genes can influence antigen presentation by macrophages and antigen recognition by T helper cells (91). Suppressor genes for immune responsiveness have also been linked to the MHC region (6).

In man, certain MHC alleles or closely linked genes not yet recognized, predispose to a variety of diseases, with or without an immunopathological basis (19). MHC restriction has been demonstrated in cytotoxic reactions to virus infected cells (63) and other targets (41). Macrophage-T cell interaction is also subject to MHC restriction, in this case encoded by the HLA-D region within the MHC (92). The role of the MHC gene products in T-B cell and T-T cell interaction in man is still elusive, mainly because the assays necessary for these investigations are lacking. In mitogenic activation assays no MHC restriction can be demonstrated. In fact, unrestricted interaction between T and B lymphocytes is evident (paper 6.2, 6.7). The human MHC, designated HLA, is a complex of genes located on the short arm of chromosome six (103) and is coding for cell surface glycoproteins common to almost all somatic cells: the HLA-A, B and C antigens. It also includes genes coding for differentiation antigens which are found largely on B lymphocytes: the HLA-D/DR antigens (4). The designation major histocompatibility complex is derived from the fact that incompatibility for HLA is associated with the development of strong immune responses to transplants and with uncontrollable graft versus host responses to bone marrow infusions. Seven major loci can be distinguished within the MHC and dozens of alleles are known to exist. Gene products of the A

and B loci are thought to be analogous to mouse H-2D and H-2K which code for target cell determinants involved in cytotoxic reactions (4,6,41,63). HLA-D/DR determinants are analogous to mouse H-2I region antigens who represent immune response genes. In man, the HLA-A, -B and -C determinants and other possibly closely linked determinants may also represent immune response genes (2). HLA-A and -B determinants are cell surface receptors for certain antigens (47).

HLA-D/DR related immune response associated (Ia-like) structures are found on a small percentage of circulating T lymphocytes (paper 6.3). After activation of T lymphocytes (by mitogens or antigens) almost all T lymphoblasts carry the Ia determinant (30, 56). Also, when T cells are grown in colonies, a large percentage expresses the Ia determinant (paper 6.4). The Ia-positive T lymphocytes belong to the T-sens population (paper 6.3) shown to have a suppressor function in the development of the in vitro PFC response (90).

Thus a picture emerges of a possible feed back mechanism in the development of the immune response in man: along with activation and proliferation, suppressor T cells increase in number, which is reflected by the increase in Ia positive T cells and which may indicate a prevention of overshoot of the immune response.

2.6. Functional properties of B lymphocytes

As has been stated the T lymphocytes play a central role in the immune system: "Man is a T dependent animal". The T cell regulates the B lymphocyte maturation to antibody producing cells. This is true for almost all known antigens and mitogens. Some exceptions do exist: Nocardia-water-soluble-mitogen (11), Epstein-Barr virus (55) and (Protein A carrying) Sta-

phylococcus aureus (35,82, paper 6.1). They activate a large population of B lymphocytes, hence the designation: polyclonal B cell activators (17). Although B cells are directly activated by these mitogens a secondary regulatory influence of T cells on the B cell proliferation is likely and actually demonstrated (98). However this does not exclude the possibility to investigate human B lymphocyte functions of normal donors and in immunodeficiency patients, using these B cell mitogens.

2.7. Reevaluation of immunodeficiency diseases

The discovery of the regulatory role of T lymphocytes in B cell activation and maturation has changed the model by which immunodeficiency diseases are analysed. The "classical" concept of inborn errors of differentiation of the T cell and/or B cell lineage is now intermingled with a concept of dysregulation. Waldmann has described some patients with hypogammaglobulinemia in which T cells were responsible for suppression of B cell maturation (105). These studies have been extended and a whole set of B cell disorders are now distinguished, ranging from severe suppression by T cells to the originally postulated intrinsic B cell differentiation defect (106). In these studies almost exclusively a T dependent mitogen (PWM) has been utilized.

Antigen specific immune response assays in vitro provide a more (patho)physiological picture of the in vivo situation. In the OA-SRBC in vitro PFC response, Dosch has shown that congenital agammaglobulinemia patients (who had no circulating B lymphocytes) were able to produce and secrete specific antibody (23,25). Excessive suppressor cell activity in these patients was shown to inhibit B cell antibody secretion (24). Thus, these patient do have pre-B cells

with a full maturation capacity which is however suppressed by T lymphocytes. The balance between helper T cell activity and suppressor T cell activity is tipped to excessive suppressor activity.

This implies that if this suppressor activity can be modulated in the sense that excess activity is prevented, patients must be able to develop B lymphocytes and to secrete antibodies. This has indeed been demonstrated (27).

These findings would imply that the neonatally bursectomized chickens are no longer the animal model for at least part of the agammaglobulinemias. This is not true. Following bursectomy, suppressor T cells develop, which can be transferred to syngeneic chickens, which then become rapidly agammaglobulinemic (10,44). These findings suggest that the crucial question of whether excessive suppressor T cell activity is cause or effect of agammaglobulinemia, can be investigated by animal experiments.

To summarize, during the past thirty years close collaboration between mice immunologists and human immunodeficiency investigators has led to a concept of the human immune system which anticipates that immunoprophylaxis and immunotherapy may become reality in the near future.

3. INTRODUCTION TO THE INVESTIGATIONS

The investigation of immunodeficiency diseases in man parallel to animal experiments enables the delineation of human lymphocyte subpopulations, their characteristics and their functional properties (chapter 1). Chapter 2 contains an introduction to basic aspects of specific immunity in man. Changing concepts of lymphocyte differentiation, lymphocyte interactions, and lymphocyte activation are emphasized. Along with new concepts, immunodeficiency diseases need to be re-analysed. The analysis in its turn is of value to proof (or reject) the concepts. A widely used method of investigation of lymphocytes, the mitogenic lymphocyte transformation test, is presented in paper 6.1. Many mitogens primarily activate T lymphocytes. Staphylococcus aureus Cowan-1 strain (STA) is a T cell independent polyclonal B lymphocyte activator. Studies in various immunodeficiency diseases and on various lymphoid tissues are reported. The STA responsive B cell is further delineated. The investigations were performed in comparison with protein A (a soluble (membrane) protein derived from Staph. aureus) and other known T cell mitogens.

These studies have led to a reevaluation of some functional properties of T and B lymphocytes in mitogenic lymphocyte transformation assays, described in paper 6.2. In the presence of T cell mitogens, helper T cells are generated which enable cocultured B lymphocytes to respond to these mitogens and to differentiate into IgM secreting (direct) hemolytic plaque forming cells (PFC). The tissue distribution of helper cell activity and the occurrence of helper T cells in various immunodeficiency diseases is analysed. Suppressor T cell activity induced by mitogens is demonstrated in the PFC assay.

Suppressor T cells in mice have been shown to carry

immune response locus associated determinants (Ia) (62). In paper 6.3 Ia^+-positive human T cells are demonstrated within the suppressor T cell population (designated as theophyllin-sensitive, because of their inability to bind sheep red blood cells in the presence of theophyllin). The presence of Ia^+-T-cells, detected by indirect immunofluorescence, is confirmed by studies showing that the theophyllin sensitive T cell subpopulation stimulated allogeneic lymphocytes (MLR). The stimulatory capacity of this T cell subpopulation is ten times stronger than the stimulatory capacity of B cells, when calculated on the basis of Ia^+-lymphocytes.

When T cells are activated by mitogens the number of Ia^+-T cells increases rapidly. After some days in culture a large part of the T lymphoclasts carry the Ia determinant (56). In paper 6.4 T cell growth in semisolid microcultures, containing mitogen (PHA) conditioned medium, is described. These colony-forming T-lymphoblasts originate from the theophyllin sensitive T cell population in peripheral blood. A large fraction of the colony-forming T-lymphoblasts carry the Ia determinant and they stimulate in MLR.

The Ia determinants are products of gene loci, inserted in the major histocompatibility complex (MHC). The human MHC, designated HLA, contains seven major loci. HLA-A and B determinants are recognition structures, associated with antigen recognition either in the induction phase of the immune response or in the effector phase of cytotoxic reactions. The chance observation of lack of expression of HLA-A and B determinants on lymphocytes associated with a combined immunodeficiency is described in paper 6.5.

Another membrane defect of lymphocytes associated with severe combined immunodeficiency is presented in paper 6.6. Abnormal lymphocyte capping in a immunodeficient patient supports the theory that plasma membrane

cytoskeleton interactions have a role in the expression of specific immunity.

In paper 6.7 other HLA deficient patients, with a type of immunodeficiency remarkably like the one described in paper 6.5, are presented. Furthermore, the HLA deficient lymphocytes are compared with the abnormally capping lymphocytes with regard to their functional properties in vitro. Possible implications for their in vivo behaviour are discussed.

Chapter 4 summarizes the studies and discusses their contricution to the analysis of immunodeficiency in children.

4. DISCUSSION AND SUMMARY

The recirculation of lymphocytes to and from various lymphoid organs and the autonomy of lymphocytes have made the immune system highly accessible for investigation. Many functions of lymphocytes and a large part of lymphocyte differentiation and maturation can be studied on peripheral blood lymphocytes. Since the discovery of the central role of T-lymphocytes in immunity, the understanding of the regulatory circuits behind specific immune responses has rapidly expanded. The analysis of immunodeficiency diseases in man has played, and still plays, an important role in the dissection of the human immune system. These "experiments of nature" not only confirm (or disprove) the models developed by animal research, but also lead to new ways of looking at the immune system. A recent example is the discovery of purine metabolism enzyme defects associated with immunodeficiency, which initiated studies on the molecular machinery in the lymphocyte. In this thesis the immune system is still framed in terms of cells, their functions and membrane characteristics. In chapter 2 the changing concepts of the immune system and the basis of the current classification of immunodeficiency diseases are outlined. Recent modifications of the classification of immunodeficiencies are indicated. The immune system is divided into two compartments: T lymphocytes and B lymphocytes, both originating from bone marrow stem cells. Immunodeficiency diseases are analysed in terms of differentiation defects along the T cell lineage, the B cell lineage or both. This model, although still usefull, is now incomplete, and is in fact only part of

the truth. For instance, what was originally presumed to be a differentiation defect of B lymphocytes, agammaglobulinemia, turned out to be sometimes due to an imbalance between T helper and T suppressor cells. Excessive T suppressor activity can be eliminated so that B lymphocytes can differentiate and mature into antibody secreting cells.

In paper 6.1 this is further substantiated. A polyclonal B cell mitogen, Staph. aureus Cowan I strain (STA) can induce proliferation of human B lymphocytes which have a deficiency of one of the steps of terminal B cell maturation. The presence of sIg^+ B lymphocytes is requisite. Lymphocytes of patients with congenital agammaglobulinemia (without B cells) had no response to STA, whereas lymphocytes from hypogammaglobulinemic patients (with B cells) had a normal response to STA. Another example, severe combined immunodeficiency (SCID), was originally thought to represent a deficiency of lymphoid stem cells, leading to combined absence of T and B lymphocytes. However, some SCID patients do have sIg^+ B which do not mature into plasma cells because of the lack of T cell help. In these cases the defect probably resides in one of the thymic stages of T cell differentiation. The positive response to STA activation (except in the cases with a inborn defect of purine metabolism) indicates a normal maturation capacity of B lymphocytes in SCID. Paper 6.1 further describes the preparation of the polyclonal B cell activator STA, the culture conditions for human B cell activation, the tissue distribution of STA responsive cells throughout the immune system, and the delineation of the STA responsive lymphocyte subset within the B lymphocyte population. Leakage of protein A (spA), the major cell wall protein carried by STA, disturbs the selectivity of the mitogen for B lymphocytes. Protein A was shown to acti-

vate T cells, to the same extent as other T cell mitogens (PHA, ConA). Culture conditions are important: human IgG, especially IgG1, but not IgG3, inhibited the STA response in a dose-dependent fashion, by blocking the mitogenic sites on the STA-bacterium. STA responsive lymphocytes were found in the circulating pool and in all peripheral lymphoid tissues tested (human tonsil cells, spleen cells), but not in the thymus. The height of response appears to be determined by the absolute numbers of B lymphocytes per culture. Lymphocyte subpopulations were purified by isolation techniques and analysed for STA responsiveness. The lymphocytes stimulated by STA were characterized: E rosette negative, C3 receptor positive, STA receptor positive, Ia positive and likely sIg positive. Taken together, the STA responsive cell fulfills the criteria of a fairly mature B lymphocyte. The investigations in various immunodeficiency diseases, either of the B cell lineage or the T cell lineage, confirmed this conclusion.

In these investigations the STA mitogenicity was compared with lymphocyte activation induced by T cell mitogens (spA, PHA, ConA). Responses were measured at the B cell level, in a proliferation assay as well as by a plaque forming cell (PFC) assay. The interaction between T and B lymphocytes was target of the studies described in paper 6.2. Normally, the mitogenic transformation of B lymphocytes is hidden behind redundant T cell responses. By irradiation of T lymphocytes, transformation of T cells is blocked and radioresistant T helper activity can be demonstrated as follows: T and B lymphocytes are isolated, the T cells are irradiated, mixed with B lymphocytes and subsequently a T cell mitogen is added. After a culture period of 80 hours the activation of the B lymphocytes is measured. All T cell mitogens induced (irradiated) T cells to provide help to the B cells, which enabled the B cells to proliferate and differenti-

ate into IgM PFC. Irradiation not only allows the measurement of B cell proliferation but also blocks T suppressor cell activity. Thus the balance between T cell help and T cell suppression, which regulates the B lymphocyte response, is predominated by T helper activity, probably because suppression becomes manifest after proliferation only. In the PFC response the balance could be investigated using non-irradiated T cells. With all mitogens the induced helper T cell activity outweighed suppressor cell activity, except after ConA stimulation. Helper cell activity was found in the T cell fraction of the circulating pool, in thymus and in peripheral lymphoid tissues. Bone marrow cells had no helper activity. SCID patients lacked the mitogen-induced helper activity. Their B cells responded when T helper cells from normal individuals were added. This again shows that these patients have B cells which can respond normally. The opposite situation was found in patients with various humoral immunodeficiencies: helper cell activity was present. The data indicate that the helper T cells belong to a fairly mature T cell population. Helper cell function is acquired at the intrathymic stage. Cell-cell interaction in T cell-mitogen, but not in B cell-mitogen stimulated cultures was demonstrated in mixing experiments of irradiated T cells and normal B cells. This was performed by varying the numbers of cells in one of the lymphocyte populations (ratios from 1 : 50 to 1 : 1) and by linear regression analysis of cellnumber: mitogen relationships. This confirmed the T cell independence of STA responses. Two general conclusions can be made: 1) polyclonal B activators provide a valuable tool for further analysis of immunodeficiency diseases as well as for the analysis of the normal immune response and 2) the studies exemplify the cellular network regulating the immune response in man, and show that helper T cell activity is lacking in some combined

immunodeficiencies.

The T suppressor cells analysed in an antigen specific human PFC response belong to a subset of T lymphocytes, designated as theophyllin sensitive (T-sens) because they are unable to bind sheep red blood cells in the presence of theophyllin. This characteristic enables the isolation of this T cell subpopulation. In paper 6.3 the expression of Ia on T-sens lymphocytes is demonstrated. First the isolation procedure of T-sens lymphocytes is described. Peripheral blood mononuclear cells (PBL) were depleted of adherent cells. The non-adherent lymphocytes were incubated with theophyllin and sheep red blood cells, which enabled the isolation of the theophyllin resistant T cells (T-res). The left-over cells were depleted of C3 receptor positive cells (B-lymphocytes). The lymphocytes left over after this second depletion were analyzed by marker tests and shown to be E rosette positive, sIg negative, C3 receptor negative, and NSE negative (NSE is a stain for monocytes). This T-sens population contained 25 to 45% Ia positive cells, demonstrated by immunofluorescence with a heterologous antiserum. It was calculated that about 5% of PBL-T cells carried the Ia determinant. The presence of Ia determinants on T-sens lymphocytes was further demonstrated after radioactive labeling of T-sens and immunoprecipitation of the solubilized membrane structures by the anti Ia-serum. A double marker study on T-sens lymphocytes visualized the Ia determinant on E-rosetting cells. When the T-sens lymphocytes were used as stimulator cells in a mixed lymphocyte culture (MLR) with unrelated donor PBL as responder cells, the T-sens were shown to be potent stimulators, 3-5 times stronger than equal numbers of B lymphocytes. The T-res lymphocytes did not stimulate. When calculated on the basis of the numbers of Ia positive cells in the B cell and T-sens population, the stimulatory capacity of Ia^+-T-sens was shown to be

10 times stronger than those of (Ia^+) B lymphocytes.

The MLR studies, detecting the HLA-D determinant, and the immunofluorescent studies, detecting an HLA-D related Ia determinant both indicate the expression of MHC encoded regulatory gene products on a human T lymphocyte subpopulation. When T lymphocytes are activated Ia^+ T cells rapidly expand. Within some days of culture almost all T lymphoblasts carry the Ia determinant. This was further analyzed by the T cell colony assay described in paper 6.4. T-colony precursor cells in PBL were detected in a microculture system using PHA conditioned medium dissolved in semisolid culture medium, containing fetal calf serum. The T colony precursor cells were theophyllin sensitive T lymphocytes and could also be found in peripheral lymphoid tissues. Thymus and thoracic duct cells did not contain T colony precursors, while bone marrow had very low numbers. This tissue distribution suggested that T colony precursor cells are fairly mature T lymphocytes. The T colony cells were identified by surface marker studies: they were E rosette positive (theophyllin resistant and stable rosetting at 37°C), had no sIg and did not have receptors for IgM, IgG or C3. When whole cultures (T colony cells plus other lymphocytes) were analyzed, 40-80% of the cells were Ia^+ positive. The cultured lymphocytes did stimulate in MLR as well. Some SCID patients were investigated and shown to have a deficiency in T colony precursor cells, even after reconstitution with thymic epithelial tissue implants. The studies of the T cell Ia marker on circulating T lymphocytes and of the growth of Ia^+ T cells in the colony culture system clearly indicate that the Ia determinant is a differentiation marker. The response of allogeneic lymphocytes to this T cell subpopulation also indicates that the T cell Ia determinant serves as a cognition structure in cellular interactions.

Products of genes of the major histocompatibility

complex (MHC) are, in various ways, involved in lymphocyte recognition. The human MHC, the HLA complex, encodes two classes of determinants. The Ia-related or HLA-D/DR antigens are involved in co-recognition whereby an antigen is "seen" along with an accompanying HLA-D/DR antigen on the macrophage surface. The Ia-related HLA antigens do not have Beta-2 microglobulin (B2M) as their common molecular subunit. The other class of HLA determinants, HLA-A, -B and -C, anchors to B2M on the cell surface and relates primarily to T cell antigen recognition either in the induction phase of the immune response or in the effector phase in target cell killing. In paper 6.5 an "experiment of nature" is presented, namely the association between a failure of lymphocyte membrane HLA-A and -B determinants expression and combined immunodeficiency. The investigations indicate a possible role of the HLA-A and -B determinants in the recognition of antigen by T lymphocytes. The role of B2M in the anchorage of HLA-A and -B determinants on the lymphocyte membrane was substantiated. Circulating T cells lacked B2M whereas T cells in lymphoid tissues showed B2M, but more in between the T cells than really fixed to the membrane. It was speculated that the basic defect lay in B2M fixation to the membrane, leading to failure of expression of HLA-A and B antigens, in its turn leading to a defective immune response to antigens, but not to mitogens. Other patients with a remarkably identical immunodeficiency associated with HLA-A and -B expression failure have been described and are presented in paper 6.7. The "HLA-null" disease appears to represent a postthymic T cell defect especially in the response to antigens.

In the immune response not only the determinants on the lymphocyte surface are important but also the integrity of the membrane-cytoskeleton interactions. An "experiment of nature", indicating a role of membrane

receptor capping in the development of the immune response, is presented in paper 6.6. This patient had a severe combined immunodeficiency (SCID). Marker tests showed normal numbers and distribution of T and B lymphocytes. Furthermore, the patient had normal immunoglobulin levels, an unusual phenomenon in SCID. These immunoglobulins had no antibody activity against the various viral and bacterial antigens tested. Mitogenic T cell proliferation was absent. The explanation for the discrepancy between the presence of mature T cells and the absence of T cell functions was found in a membrane abnormality. Receptors for mitogens were demonstrated on both T and B lymphocytes, but the surface receptor capping phenomenon following ligand-receptor interaction was disturbed. Lateral mobility of cell surface receptors for ConA was increased, leading to unusually fast accumulation of receptor bound ConA in the surface caps. The STA response of B lymphocytes was normal, indicating a normal B lymphocyte maturation capacity, which was anticipated (paper 6.1), but was unexpected because B lymphocyte also displayed the increased ConA capping phenomenon. An explanation was found in the rigid structure of the STA bacterium carrying the mitogenic protein A molecules, which probably prevents increased capping and thus allows the normal transduction of the proliferation signal. Mitogen induced helper T cell activity of SCID-T cells was absent (paper 6.7), when tested on normal B cells. Patient B lymphocytes responded to T cell helper activity induced by mitogenic activation of normal T lymphocytes. These findings support the theory that plasma membrane cytoskeleton interactions have a role in the expression of specific immunity.

In paper 6.7 a comparison between both types of immunodeficiency diseases associated with membrane defects has been made. In both the immune response defect had

been postulated to be a defect in the response of T cells to antigens, caused by their membrane defect. It was striking that both phenotypically combined immunodeficiencies, both with defective antigenic T cell responses to antigens, had such a completely different histology of the peripheral lymphoid organs. The hypothesis was raised that HLA determinants and/or B2M, which were normally expressed in the capping disorder, play a crucial role in the homing of lymphocytes to peripheral lymphoid organs. Furthermore some indication emerged that in vitro studies on the circulating pool of lymphocytes do not necessarily reflect local situations in the peripheral lymphoid organs.

In summary the studies indicate a network of cell interactions in the human immune response. Lymphocyte membrane structures encoded in the HLA complex play an important but still elusive role. This holds for membrane receptor cytoskeleton interaction as well. B lymphocyte mitogens were valuable tools in the investigation of these immunodeficiency diseases. The classical lymphocyte differentiation model by which immunodeficiency diseases were analyzed is interwoven with a concept of a regulatory unit consisting of subpopulations of T lymphocytes. Histocompatibility antigens encoded by the MHC play a major role in the interactions between T lymphocytes, possibly between T and B lymphocytes and between T lymphocytes and macrophages. Some indications of the role of human MHC products emerged from studies in two types of membrane defects associated with combined immunodeficiency.

The newer models derived from in vivo and in vitro studies on the cell interactions in the immune response, on the role of membrane determinants in these interactions, and especially on the defects in immune responsiveness in immunodeficiencies may eventually lead to immunoprophylaxis and immunotherapy for patients with immunological diseases.

4.2. SAMENVATTING

Hoofdstuk 1

Het doel van de onderzoekingen was een verdieping van het inzicht in het humane immuunsysteem door a) ontwikkeling van nieuwe(re) analysemethoden, en b) toepassing van deze methoden bij het onderzoek van immunodeficiënties. Door een beter inzicht wordt uitbreiding van de mogelijkheden tot beïnvloeding van immuunstoornissen bij kinderen nagestreefd.

Hoofdstuk 2

Drie modellen voor het humane immuunsysteem worden geïntroduceerd:

1) een ontogenetisch model, waarbij twee compartimenten binnen het immuunsysteem worden onderscheiden: de thymus-afhankelijke cellulaire immuunreactiviteit en de thymus-onafhankelijke humorale immuunreactiviteit. De dragers van deze twee vormen van immuunreactiviteit zijn respectievelijk de T-lymfocyten en de B-lymfocyten.
2) een cellulair interactie model, waarbij de T lymfocyt een centrale regelende functie heeft, zowel in de cellualire als in de humorale immuunreactiviteit. Hierbij wordt een functioneel onderscheid gemaakt tussen T helper en T suppressor lymfocyten.
3) een model waarbij de belangrijkste histocompatibiliteits (MHC) determinanten functioneren als gids voor o.m. de T lymfocyten. Antigeen presentatie door macrophagen aan T lymfocyten verloopt aanzienlijk beter wanneer beide cellen elkaar herkennen via de MHC determinant. Voorwaarde is een zekere mate van identiciteit van de determinanten op beide cellen. De "killing" door T lymfocyten van met virus geïnfecteerde cellen verloopt eveneens beter wanneer "killer" en "target" tenminste identiek

zijn voor een deel van de MHC determinanten.
De drie modellen sluiten elkaar niet uit, integendeel, ze zijn met elkaar vervlochten. Er zijn echter nog vele onduidelijkheden, zeker met betrekking tot het humane immuunsysteem.

De analyse van immunodeficiëntie ziekten wordt in eerste instantie volgens het eerste model verricht. Sommige humorale deficiënties blijken echter niet te berusten op een defect in de aanleg van B lymfocyten doch blijken te berusten op een buitensporige suppressor T cel activiteit. De in principe normaal aanwezige B cel reactiviteit kan in vitro aangetoond worden. Onderzoek met behulp van B cel mitogenen kan verdere informatie hierover verschaffen.

Artikel 6.1

Een polyclonaal B cel mitogeen, Staphylococcus aureus Cowan I (STA) activeert humane B lymfocyten van sommige patiënten met humorale immunodeficiënties. Voorwaarde is dat ze circulerende membraan-immunoglobuline-positieve (sIg^+) lymfocyten hebben. Sommige patiënten met een gecombineerde (T+B) immunodeficiëntie blijken sIg^+-B lymfocyten te hebben, die geactiveerd kunnen worden door STA. De kweekomstandigheden van de lymfocyten transformatietest met STA worden besproken. Humaan IgG1, doch niet IgG3, blokkeert de mitogene activiteit van STA. Op STA reagerende lymfocyten komen voor in perifeer blood en in alle perifere lymfoïde organen, doch niet in de thymus. De op STA reagerende lymfocyt blijkt E rosette negatief (non-T), C3 receptor positief, STA receptor positief, Ia-positief en waarschijnlijk sIg-positief. Door lymfocyten van patiënten met verschillende immunodeficiënties te onderzoeken op STA reactiviteit kan de conclusie bevestigd worden, dat STA een vrij ver ontwikkelde B lymfocyt activeert.

Artikel 6.2

De STA reactiviteit van lymfocyten werd vergeleken met de reactiviteit ten opzichte van bekende T cel mitogenen. Na bestraling van T lymfocyten kan de door T cel mitogenen geïnduceerde helper activiteit van T lymfocyten gedemonstreerd worden door de proliferatie en/of IgM secretie van (onbestraalde) B lymfocyten te meten. Bestraling van T lymfocyten heeft een tweeledig effect: a) de transformatie van T lymfocyten wordt geblokkeerd, zodat de transformatie van alleen de B lymfocyten gemeten kan worden, en b) de suppressor activiteit van T lymfocyten wordt uitgeschakeld, zodat de helperactiviteit apart gemeten kan worden. Bij een hemolytische plaque vormende cel (PFC) analyse storen onbestraalde T lymfocyten niet, zodat hiermee de balans tussen helper en suppressor activiteit gemeten kan worden. De T cel fracties in perifeer blod, in de thymus en in perifere lymfoïde organan, hadden alle de door mitogeen geïnduceerde helper activiteit. Normale beenmergcellen en perifeer bloedlymfocyten van SCID patiënten vertoonden geen helperactiviteit. In de onderzochte humorale immunodeficiënties was de helperactiviteit aanwezig. Door middel van mengexperimenten waarbij het percentage van T of B lymfocyten gevarieerd werd, kon worden aangetoond dat de T cel mitogenen interacties tussen twee (of meer) cellen bewerkstelligen terwijl STA slechts één celpopulatie activeert. Op zich wederom een bewijs voor de T cel onafhankelijkheid van de STA reactiviteit. De balans tussen helper activiteit en suppressor activiteit, zoals gemeten met de PFC methode, is alleen na activatie met het T cel mitogeen ConA verschoven in de richting van een suppressief effect.

Artikel 6.3

Suppressor T cellen, zoals deze geanalyseerd werden in een antigeen specifieke PFC methode, behoren tot een bepaald deel van de T lymfocyten, de theophylline sensitive T lymfocyten (T-sens). De isolatie van T-sens lymfocyten kan onder andere geschieden door verwijdering van theophylline resistente T lymfocyten (T-res) gevolgd door verwijdering van C3 receptor positieve lymfocyten. De analyse van membraankenmerken, van de overgebleven cellen (E rosette positief en -negatief voor sIg, C3-R en NSE) bevestigde dit. De T-sens populatie bevat 25-45% Ia-positieve T lymfocyten en heeft een hoge stimulatoire capaciteit in een gemengde lymfocytenkweek (MLR). Berekend op basis van het aantal Ia^+ lymfocyten, is de MLR stimulatie 10 maal zo sterk als die van B lymfocyten.

Artikel 6.4

Na activatie van T lymfocyten neemt het relatieve aantal Ia^+-T lymfocyten snel toe. Mitogen geconditioneerd medium wordt gebruikt om T cel kolonies te laten groeien. Een hoog percentage van de gekweekte cellen draagt dan de Ia determinant. De gekweekte cellen stimuleren in een MLR. De kolonievormende T lymfocyten zijn afkomstig van de T-sens populatie in perifeer bloed.

Artikel 6.5

De rol van HLA determinanten, met name van HLA-A en -B determinanten, in de immuunreactiviteit van de mens, wordt belicht door een "experiment of nature", een gecombineerde immunodeficiëntie geassocieerd met de afwezigheid van HLA-A en -B antigeen op de lymfocyten. De verankering van HLA-A en-B antigenen op de lymfocytenmembraan vindt plaats in combinatie met Beta-2-microglobuline (B2M). De lymfocytenmembraan

van T lymfocyten vertoonde een gestoorde B2M fixatie. Alle tot nu toe beschreven patiënten (artikel 6.7) hadden een af te grenzen type immunodeficiëntie. Hierbij is met name de antigenenherkenning van T lymfocyten gestoord.

Artikel 6.6

De belangrijke rol van niet alleen membraandeterminanten doch ook van de integriteit van de lymfocytenmembraan zelf, wordt gedemonstreerd aan de hand van een ander "experiment of nature". Een stoornis in de laterale mobiliteit van de ConA receptors op de lymfocytenmembraan blijkt geassocieerd te zijn met een gecombineerde immunodeficiëntie. Met betrekking tot membraandeterminanten lijken de T lymfocyten volledig gedifferentiëerd te zijn doch de bijbehorende ontwikkeling van hun functies blijkt afwezig.

Artikel 6.7

Beide types van immunodeficiëntie geassocieerd met membraandefecten werden vergeleken. Er bleek een duidelijk verschillende histologie van de perifere lymfoïde organen te bestaan. Een mogelijke rol van HLA determinanten en/of B2M bij de migratie van lymfocyten naar de perifere lymfoïde organen werden besproken.

De onderzoekingen leiden tot de volgende conclusies: Immunodeficiëntieziekten kunnen niet alleen volgens het ontogenetische model worden geanalyseerd, doch ook aan de hand van cellulaire interactiemodellen. Daarin staan met name T lymfocyten subpopulaties en hun activiteiten centraal, en vormen polyclonale B cel mitogenen een waardevolle aanvulling bij het onderzoek. B2M geassocieerde HLA determinanten blijken een rol te spelen in de cellulaire reacties en moge-

lijk ook bij de migratie van lymfocyten.
Een meer functionele analyse van de immuunreactiviteit bij de mens waarvan de beschreven onderzoekingen deel uitmaken, zal verdere mogelijkheden openen voor immunoprophylaxe en immunotherapie bij patiënten met immunologische stoornissen.

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POLYCLONAL ACTIVATION OF HUMAN LYMPHOCYTES *IN VITRO*

I. Characterization of the Lymphocyte Response to a T Cell-Independent B Cell Mitogen[1]

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The staphylococcal cell wall component protein A (SpA) and formalinized, Cowan I strain Staphylococcal organisms (STA) were compared with the lectins phytohemagglutinin, concanavalin A, and pokeweed mitogen for their ability to trigger proliferation of normal human lymphocytes, lymphocyte subpopulations, and cells from patients with primary immune deficiency diseases. SpA was found to be a potent T cell mitogen, very similar to the other lectins tested. It failed to stimulate purified non-T cells and peripheral blood lymphocytes from patients with different forms of severe combined immunodeficiency disease (SCID). STA, treated to prevent the leakage of soluble SpA during culture, exclusively stimulated non-T cells: the responding cell population was characterized to be E-rosette negative but positive for C3 receptors, surface Ia, a receptor for STA itself, and likely carried surface immunoglobulin. Normal responses to STA were found in patients with the adenosine deaminase-positive form of SCID. In 18 patients with humoral immune deficiency syndromes, the presence of STA responses was correlated with the presence of circulating, surface immunoglobulin-bearing cells. A commercial STA preparation was rendered B cell specific after reformalinization, a procedure that eliminated the shedding of soluble SpA under culture conditions.

Mitogenic activation of lymphocytes is an important tool in both diagnostic and experimental immunology. In contrast to rodents, virtually all mitogens tested in man result in T cell proliferation, whereas purified B lymphocytes are triggered to no more than marginal increases in thymidine uptake or differentiation into immunoglobulin-producing cells. Exceptions have been described and include water-soluble extracts from Nocardia and live Epstein-Barr virus (1, 2). On the other hand, there is a much wider experience with indirect or T cell-dependent B cell activation by pokeweed mitogen (PWM)[4] (reviewed in 3). However, the dependence on T lymphocytes or their products limits the application of PWM: for example, the absence of responses in a given patient may not only indicate a primary B cell deficiency but the absence of appropriate "helper" T cells or the presence of suppressor cells that may be abnormal in function or quantity (4).

Recently, Cowan I strain Staphylococci (STA) and its soluble cell wall product, protein A (SpA), have been described to be mitogenic for both B and T cells (5–10). Since among these reports controversy exists with respect to culture conditions and cell lineage specificity, we have reevaluated the properties and requirements for STA and SpA-induced mitogenesis. In the present study, we used lymphocytes from normals and patients with different forms of immunodeficiency. Our data delineate the cell lineage specificity in the mitogenic properties of STA and SpA: STA exclusively stimulates B lymphocytes that appear to bind STA and carry surface immunoglobulin (sIg) as well as C3 receptors; SpA, in contrast, is a T cell mitogen, equal in potency to phytohemagglutinin (PHA) or concanavalin A (Con A). The experimental requirements for both mitogens are strikingly different, although both seem to involve the protein A moiety. Thus, the T cell triggering site of SpA appears not to be accessible on bacterial cell walls and is distinct from the immunoglobulin binding site of the molecule. In contrast, binding of IgG1 to STA interfered with its mitogenic effect.

MATERIALS AND METHODS

Cell source and preparations. Mononuclear cells from peripheral blood, tonsil, or thymus were obtained by density separation on Ficoll-Hypaque. If indicated, further purification utilized E-rosette depletion on Ficoll gradients as described (11). STA-binding cells were enriched in tonsil cell suspensions by a similar rosette depletion procedure using erythrocyte-IgG-STA complexes. These were prepared by incubating (30 min at 37°C) 1×10^{11} STA particles with 1×10^{9} erythrocyte-IgG complexes. The latter were formed using ox red blood cells and

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Abbreviations used in this paper: ADA, adenosine deaminase; Ay, agammaglobulinemia; CVID, common variable immunodeficiency disease; $C3^+$, complement rosette-forming cells; E^+, E-rosette-forming (T) lymphocytes; PBL, peripheral blood lympho-mononuclear cells; PWM, pokeweed mitogen; SCID, severe combined immunodeficiency disease; sIg, surface immunoglobulin (G, M, D); SpA, Staphylococcal protein A; STA, formalinized *Staphylococcus aureus* (Cowan I); STA^+, STA-binding cells.

a rabbit IgG antibody. Red cell-IgG complexes were formed at low antibody concentrations and did not result in the depletion of $Fc_{(IgG)}$ receptor-bearing tonsillar B lymphocytes. STA was removed from STA-binding cells on a third Ficoll gradient after incubation (90 min at 4°C) in the presence of 50% AB serum. Complement (C3) receptor-bearing cells were isolated from adherence depleted PBL by using C3-coated yeast particles for rosette depletion on Ficoll gradients (12).

Patients. The majority of patients studied have been reported previously (11, 13, 14). *In vivo*, all were incapable of specific antibody formation. Three of the patients with severe combined immunodeficiency disease (SCID) and normal levels of adenosine deaminase (ADA^+) were shown to have the intrathymic form of the disease (15) with normal to elevated numbers of sIg-positive lymphocytes; one patient (case 6) presented with a variant SCID secondary to a cytoskeletal abnormality (14). The two patients with SCID and ADA deficiency (ADA^-) lacked detectable (B or T) lymphocytes (15, 16). Patients with agammaglobulinemia ($A\gamma$) lacked circulating or tissue B lymphocytes and plasma cells (11, 13). All but two patients with common variable immunodeficiency disease (CVID) had up to normal numbers of circulatory B lymphocytes and varying degrees of hypogammaglobulinemia or, as in one case, hypergammaglobulinemia, but lacked serum antibody activity. All patients were receiving γ-globulin or plasma replacement therapy at the time of study.

Tissue culture conditions and reagents. Cultures were maintained for 80 to 90 hr in flat-bottom Microtest-II plates (Falcon Plastics, Oxnard, Calif.) in supplemented RPMI 1640 (GIBCO, Grand Island, N. Y.) and were harvested after a 4-hour pulse with 1 μCi ^{3}H-thymidine and counted in a scintillation counter. All cultures contained 10% fetal calf serum (FCS, GIBCO, batch 14-144). Where indicated, pooled human AB serum was added in varying amounts. In one series of experiments, an aliquot of this AB serum pool was depleted of immunoglobulin by using either insolubilized protein A (Pharmacia, Ltd., Montreal, Quebec) or Sepharose-bound purified sheep antibody to human IgG or an IgM-specific immunosorbent. The latter were a gift from Dr. B. Underdown (University of Toronto, Toronto, Ontario). Human IgG1 and IgG3 myeloma proteins were a gift from Dr. W. Pruzanski (Wellesley Hospital, Toronto, Ontario).

Mitogens. PHA and PWM were obtained from Difco Laboratories (St. Louis, Mo.) and purified SpA from Pharmacia, (Montreal, Quebec). Staphylococci (Cowan strain I) were a gift from Dr. B. Barber (University of Toronto, Toronto, Ontario). One bacterial colony was grown in 10 ml culture broth overnight at 37°C and then propagated in 41 aerated mass cultures as described (17). Staphylococcal organisms were harvested and washed by centifugation, characterized by routine bacteriologic procedures, and killed and hardened by formalinization (130 min at 23°C) in 1.4% formaldehyde and 5 min boiling. Stock suspensions of 1×10^8 killed organisms/ml RPMI 1640 were stored at −20°C. This batch (STA-Toronto) was used throughout this study. It was found not to shed SpA during 3 days of culture at 37°C as tested by binding of radioiodinated IgG. A second batch of STA was purchased (Pansorbin, Batch 830187, Calbiochem-Behring Corp., La Jolla, Calif.) (STA-Cal). Prepared for use in radioimmunoprecipitation assays, this preparation had a high binding capacity for IgG, but there was also significant shedding of SpA if incubated for 3 days under culture conditions. An aliquot was therefore reformalinized for 60 min. This reduced its binding capacity for IgG and virtually eliminated SpA shedding.

Data analysis. Data were obtained as cpm thymidine incorporation and the means for triplicate cultures are given ± 1 S.D. Quench, as estimated by H-numbers, indicated no significant differences between test and control samples in any given experiment and was disregarded. When data from different experiments were pooled, results of each were expressed as a fraction of the maximal responses obtained in each experiment or in the appropriate cell population with the mitogen indicated. Graphic and statistical analysis used Plot 10 based software on a Tektronix microcomputer. All *t*-tests were two-tailed.

RESULTS

Requirements for mitogenic responses to STA and SpA. Cultures of 2×10^5 normal peripheral blood lympho-mononuclear cells (PBL) were incubated with varying concentrations of STA or SpA. When cultures contained 10% pooled AB serum, consistent proliferative responses were only observed with SpA. These responses were roughly the same as controls stimulated with PHA (7 to 10×10^4 cpm). In contrast, no significant responses were observed in cultures stimulated with STA. However, when cultures were supplemented with FCS instead of AB serum, a significant mitogenic effect of STA was found (2 to 4×10^4 cpm *vs* 2 to 5×10^2 cpm in unstimulated controls). These differences are analyzed in Figure 1, where cultures containing 10% FCS received increasing amounts of AB serum. AB serum inhibited the STA responses in a dose-dependent fashion. In seven experiments, SpA and PHA responses were little affected. At high concentrations of AB serum, mean values tended to be somewhat lower for SpA responses; the increased variability precluded delineation of a significant difference to cultures containing FCS alone ($p > 0.1$).

Protein A has a lectin-like binding capacity for several species of proteins including immunoglobulins (17–19). High affinity binding is found for IgG1, IgG2, and IgG4 and little binding to IgG3. The effect of AB serum could be mimicked by purified serum IgG (data not shown). We therefore tested the effect of IgG1 and IgG3 myeloma proteins on STA-induced proliferation. As shown in Figure 2, the addition of IgG1, but not IgG3, resulted in abrogation of the STA response. This suppression was dose dependent ($r = 0.91$); PHA and SpA responses were at best only marginally affected.

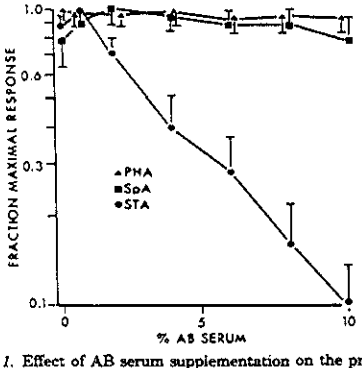

Figure 1. Effect of AB serum supplementation on the proliferative response to PHA, SpA, and STA. Cultures of 2×10^5 normal PBL were maintained in 200 μl RPMI-1640 plus 10% fetal calf serum and the AB serum concentration indicated. Data from four experiments were pooled and expressed as a function of the maximal response to the appropriate mitogen (cpm ^{3}H-thymidine incorporation, means ± 1 S.D.) observed in each experiment.

These findings suggested that IgG, by attaching to protein A binding sites on STA, could abrogate its mitogenic activity. This was directly tested in blocking experiments where STA was pretreated with either AB serum or FCS, thoroughly washed, and then used as a stimulator. In addition, AB serum was used after absorption with insolubilized SpA or specific immunoabsorbents for IgM or IgG. Myeloma proteins served as a suppressive (IgG1) or nonsuppressive (IgG3) control. IgG-coated STA failed to elicit proliferative responses: as shown in Figure 3, the suppressive moiety in AB serum could be removed with insolubilized anti-IgG or Sepharose-SpA but not with the anti-IgM immunoabsorbent. In face of the failure of SpA responses to be affected in the same fashion, these data suggested a different mode of action of STA and SpA and the possibility of different responding ("target") lymphocyte populations, only one of which (the STA-responsive) required access to the lectin-like binding site (9). The absence of blocking effects of IgG or AB serum on SpA responses suggested that the lectin-like binding site for a serum protein such as IgG was distinct from the mitogenic region of the SpA molecule and conceivably inaccessible on formalinized STA.

Tissue distribution of mitogenic responses. With cells from 91 different donors, the tissue distribution of mitogenic responses to STA and SpA was compared with those of the T cell mitogens PHA, Con A, and PWM (Table I). As a central lymphoid organ thymus was chosen, containing almost exclusively (>95%) cells of T lineage; tonsil served as a source of more mature cells containing about two-thirds B cells and one-third T lymphocytes; in addition, PBL were used consisting of two-thirds T lymphocytes and one-third non-T cells (20).

PHA, PWM, and Con A had similar response profiles, with the highest values in PBL, followed by tonsil and thymus. STA responses generally were highest in tonsil, somewhat lower in PBL, but consistently absent in thymus. In contrast, SpA elicited vigorous proliferative responses in thymocytes and closely paralleled the other T cell mitogens. In agreement with the suggestion of differences in the mitogenic properties of STA *vs* SpA, these results suggested differences in the tissue distribution of the cell populations responding to STA and SpA, respectively.

Mitogen responses in purified lymphocyte subpopulations. The question of different target cell populations responding to STA or SpA was addressed in cell separation experiments. Observations in purified lymphocyte subpopulations from PBL and tonsil cells were similar and have been pooled in Table II.

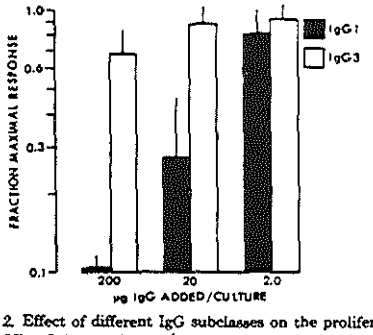

Figure 2. Effect of different IgG subclasses on the proliferative response to STA. Cultures of 2×10^5 normal PBL were maintained in 200 μl RPMI-1640 containing 10% fetal calf serum. Various concentrations of purified myeloma proteins were added at the beginning of culture. Pooled data from three experiments are expressed as a fraction of maximal responses observed in each experiment. Maximal responses were found in control cultures without added IgG.

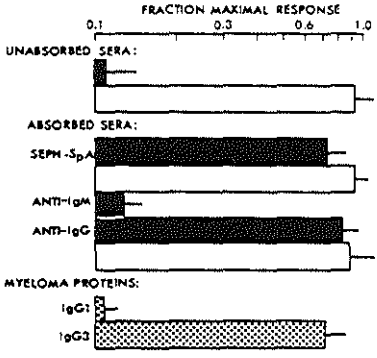

Figure 3. Mitogenicity of STA preparations preincubated (2 hr at 4°C) with different AB serum sources (*solid columns*) or purified IgG myeloma proteins (*stippled columns*). The different AB sera were obtained as described in *Materials and Methods* by using immunoabsorbents with the specificities indicated. Fetal calf serum, treated in parallel, served as control (*open columns*). Following incubation, STA were washed and used for the stimulation of normal PBL in cultures containing 10% fetal calf serum. Responses (means ± 1 S.D.) are expressed as a fraction of maximal responses that were observed in cultures stimulated with untreated STA.

T cells, purified as E-rosette forming cells (>95% E^+) showed vigorous responses to stimulation with PWM, Con A, PHA, and SpA but lacked a response to STA. The converse was true for cultures of E-rosette depleted (E^-) cell populations, which contained 90% or more C3 receptor and surface Ia-bearing B lymphocytes: there was a consistently good response in these populations to stimulation with STA, whereas no response was observed with the T cell mitogens or SpA. More detailed cell dilution experiments and regression analysis will be presented in the accompanying paper (21).

To further define the populations of responding cells, we tested positively selected peripheral B lymphocytes ($C3^+$) and C3-receptor negative cells ($C3^-$). The $C3^+$ preparations contained no E-rosette forming cells, and virtually all cells carried surface Ia and more than 60% had easily detectable surface IgM/IgD. These cells responded exclusively to stimulation with STA (Table II). $C3^-$ populations contained up to >90% E-rosette forming cells, virtually no C3 receptor and surface IgM/IgD bearing cells, and were responsive to PHA, Con A, PWM, and SpA. These results suggested that the STA-responsive cell population was a C3 receptor-positive, Ia-bearing cell, and likely expressed sIg.

By using a sandwich technique, STA was bound to ox erythrocyte-IgG complexes, and this reagent was then used in a rosette-depletion procedure for the depletion of STA binding cells. Approximately 30 to 40% of tonsil cells sedimented together with this complex, and only these cells responded to STA (Table II), indicating that the responsive cell population bears accessible STA receptors. Taken together, these data suggested a clear-cut dichotomy between SpA and STA mitogenicity, with the latter acting exclusively on B lymphocytes and the former selectively stimulating T cells.

Mitogen responses in patients with primary immune deficiency disorders. The results presented characterized STA as

TABLE I

Tissue distribution of mitogen responses[a]

Tissue	(N)	Cell Marker (%)		Mitogen Added (cpm × 10^{-3})					
		E	C3	—	STA	SpA	PHA	Con A	PWM
Thymus	(10)	>95	<3	0.7 ± 0.4	0.8 ± 0.6	48 ± 17	31 ± 14	39 ± 15	30 ± 17
HTC	(29)	33 ± 4	62 ± 9	0.4 ± 0.3	37 ± 11	69 ± 24	74 ± 21	65 ± 21	20 ± 10
PBL	(52)	71 ± 6	23 ± 4	0.3 ± 0.2	26 ± 9	92 ± 26	112 ± 31	89 ± 24	44 ± 17

[a] Mononuclear cells (2×10^5/well) from thymus, tonsil, or peripheral blood were stimulated with the mitogen indicated for 80 to 90 hr in 200 μl RPMI-1640 supplemented with 10% fetal calf serum. Pooled results (mean ± 1 S.D.) are given as cpm ($\times 10^{-3}$). The distribution (mean % ± 1 S.D.) of E and complement (C3) rosette-forming cells was obtained as referred to in *Materials and Methods*. N, number of individual experiments.

TABLE II

Mitogen responses in purified lymphocyte subpopulations[a]

Cell Population	(N)	Cell Marker (%)		Mitogen Added (cpm × 10^{-3})					
		E	C3	—	STA	SpA	PHA	Con A	PWM
E^+	(13)	>95	<3	0.3 ± 0.2	0.9 ± 0.6	68 ± 21	72 ± 18	59 ± 19	38 ± 12
E^-		<3	87 ± 6	0.6 ± 0.4	41 ± 18	2 ± 1	2 ± 1	2 ± 1	2 ± 1
$C3^+$	(4)	<3	>95	0.5 ± 0.4	38 ± 16	3 ± 1	2 ± 1	<2	<2
C3		90 ± 4	<3	0.4 ± 0.2	5 ± 3	72 ± 16	81 ± 23	64 ± 15	49 ± 20
STA^+	(3)	<3	>95	9 ± 6	81 ± 19	8 ± 3	11 ± 4	6 ± 4	13 ± 5
STA		54 ± 18	36 ± 11	2 ± 1	3 ± 1	74 ± 8	72 ± 13	68 ± 8	52 ± 15

[a] Purified lymphocyte subpopulations were obtained as described in *Materials and Methods* and the separated cells (1×10^5/well) were cultured and analyzed as indicated in the legend of Table I. E^+/E^-, E-rosette-forming or non-E-rosette-forming cells obtained by E-rosette depletion of PBL or tonsil; $C3^+/C3^-$, C (C3) receptor positive or negative cells obtained by depletion of PBL; STA^+/STA^-, cells binding or not binding to STA, obtained from tonsil by rosette depletion with erythrocyte-IgG-STA complexes. N, number of individual experiments.

TABLE III

Mitogen responses in severe combined immunodeficiency (SCID)[a]

Case	Diagnosis	Mitogen Added (cpm × 10^{-3})				
		—	STA	SpA	PHA	PWM
1	ADA$^-$-SCID	0.2 ± 0.1	0.4 ± 0.2	0.6 ± 0.4	0.5 ± 0.3	0.4 ± 0.3
2		0.1 ± 0.1	0.2 ± 0.1	0.3 ± 0.1	0.3 ± 0.2	0.2 ± 0.1
3	ADA$^+$-SCID	0.9 ± 0.1	26 ± 1	2.4 ± 0.4	1.8 ± 0.6	1.5 ± 0.3
4		0.6 ± 0.1	24 ± 3	1.4 ± 0.3	1.5 ± 0.4	0.9 ± 0.2
5		0.4 ± 0.1	16 ± 8	1.7 ± 0.6	1.2 ± 0.7	1.5 ± 0.8
6		0.7 ± 0.3	34 ± 5	1.9 ± 1.1	1.4 ± 0.8	2.3 ± 0.9

[a] PBL (2×10^6/well) from patients with severe combined immunodeficiency (SCID) were cultured and analyzed as indicated in the legend of Table I. ADA^+/ADA^- denotes the presence or absence in these patients of adenosine deaminase. Each patient was studied on at least four occasions and results have been pooled.

a B cell mitogen and SpA as a T cell mitogen. This conclusion was tested in patients with immune deficiency (Table III). The two patients with ADA-deficiency (ADA^- SCID) presented with a virtual absence of lymphocytes but elevated numbers of monocytoid cells (16). None of the mitogens were capable of eliciting a proliferative response in PBL. The three patients with the intrathymic form of (ADA^+) SCID lacked T lymphocytes but presented with normal to elevated numbers of sIg^+ B lymphocytes (15). PBL from these patients showed normal responses to STA but no proliferation to any of the other mitogens. This confirmed the cell lineage specificity of STA *vs* SpA and underlined the T cell independence of the STA-induced B cell response.

Following the same protocol, 18 patients with primary antibody deficiency and intact T cell function were studied. In Table IV, these patients are ordered according to the presence or absence of sIg^+ B lymphocytes. This was done to stress the absolute correlation ($r = 1.0$) between the presence of sIg^+ B cells and the presence of STA responses. In contrast, all patients had positive T cell mitogenic responses, including SpA. There was no significant correlation between STA and SpA responses, or SpA response and the presence or absence of sIg^+ B lymphocytes.

Mitogenicity of different STA preparations. One difficulty previously encountered when testing different compounds for B cell mitogenicity was the large variability between different batches. Although all data presented were obtained with one batch of STA (prepared by us), we studied a commercially available STA preparation in parallel. This preparation (STA-Cal) was found to shed soluble SpA under culture conditions and was therefore reformalinized (see *Materials and Methods*). This treated STA preparation and the untreated batch were tested in cultures of purified tonsillar T and B cells or PBL from selected patients (Table V). As shown, no clear-cut dichotomy was found between responses to SpA and untreated STA-Cal in any of the experiments. In contrast, when STA-Cal was treated to prevent SpA leakage, the results with this preparation were similar to those obtained with our own STA preparation.

DISCUSSION

Polyclonal lymphocyte mitogens have been invaluable tools in immunobiology, since they seem able to recall and set into motion parts of the programming of those lymphocyte subpopulations which they activate. Thus, they can provide information about lymphocyte triggering and activation, serve as markers of lymphocyte lineage, and reveal the functional repertoire, such as immunoglobulin production or helper and suppressor cell activities (3, 4). When compared with rodents, human B lymphocytes seem to be less susceptible to direct mitogenic activation or polyclonal initiation of differentiation

TABLE IV

Mitogen responses in antibody deficiency syndromes[a]

Case	Diagnosis	sIg	Mitogen Added (cpm × 10^{-3})				
			—	STA	SpA	PHA	PWM
7	Aγ	−	0.6 ± 0.2	1.4 ± 0.8	170 ± 22	70 ± 23	70 ± 27
8	Aγ	−	0.8 ± 0.5	1.8 ± 0.9	99 ± 12	78 ± 19	37 ± 11
9	Aγ	−	0.5 ± 0.2	0.4 ± 0.2	110 ± 18	41 ± 5	35 ± 7
10	Aγ	−	0.4 ± 0.2	0.3 ± 0.2	116 ± 7	73 ± 1	40 ± 10
11	Aγ	−	0.3 ± 0.1	1.2 ± 0.4	71 ± 10	78 ± 3	61 ± 8
12	Aγ	−	0.4 ± 0.1	0.8 ± 0.2	115 ± 8	51 ± 4	42 ± 4
13	Aγ	−	0.5 ± 0.1	1.3 ± 0.3	175 ± 9	64 ± 6	82 ± 9
14	Aγ	−	0.4 ± 0.1	0.7 ± 0.1	92 ± 16	39 ± 2	35 ± 6
15	Aγ	−	0.7 ± 0.1	1.1 ± 0.3	62 ± 7	44 ± 6	32 ± 5
16	CVID	−	0.8 ± 0.1	1.1 ± 0.3	125 ± 15	115 ± 10	75 ± 14
17	CVID	−	0.5 ± 0.2	0.6 ± 0.1	68 ± 5	48 ± 4	42 ± 10
Mean values:		−	0.6 ± 0.2	1.0 ± 0.5	110 ± 40	67 ± 16	50 ± 18
18	Aγ	+	0.7 ± 0.1	11 ± 2	89 ± 7	31 ± 7	39 ± 6
19	Aγ	+	0.7 ± 0.3	37 ± 9	83 ± 5	51 ± 8	29 ± 4
20	CVID	+	0.9 ± 0.2	9 ± 2	86 ± 7	127 ± 10	21 ± 5
21	CVID	+	0.4 ± 0.2	13 ± 3	90 ± 5	67 ± 5	40 ± 11
22	CVID	+	0.8 ± 0.4	43 ± 13	79 ± 22	84 ± 8	35 ± 6
23	CVID	+	0.5 ± 0.4	19 ± 3	62 ± 6	59 ± 12	34 ± 7
24	CVID	+	0.3 ± 0.1	49 ± 8	108 ± 8	72 ± 9	47 ± 5
Mean values:		+	0.6 ± 0.2	26 ± 4	85 ± 14	70 ± 30	35 ± 8

[a] Proliferative responses of patient PBL (2×10^5/well) to stimulation with the mitogens indicated, (see legend Table I). Patients have been grouped according to the presence or absence of surface immunoglobulin (sIg) positive B lymphocytes. Each patient was studied on at least three occasions and results were pooled. Aγ, agammaglobulinemia; CVID, common variable immunodeficiency disease.

TABLE V

Mitogenicity of different STA-preparations[a]

Cell Source	Diagnosis	Mitogen Added (cpm × 10^{-3})				
		—	STA (Toronto)	Commercial STA: Untreated	Commercial STA: Formulinized	SpA
PBL		0.3 ± 0.1	23 ± 2	49 ± 3	29 ± 4	87 ± 9
E^+	Normal	0.2 ± 0.1	1.1 ± 0.6	26 ± 7	0.9 ± 0.6	79 ± 10
E^-		0.5 ± 0.3	32 ± 2	34 ± 4	35 ± 3	1.3 ± 0.6
	Aγ	0.4 ± 0.2	0.6 ± 0.3	39 ± 5	1.0 ± 0.2	68 ± 7
PBL	CVID	0.6 ± 0.2	9.6 ± 1	57 ± 8	10 ± 1	72 ± 9
	SCID	0.6 ± 0.3	17 ± 2	19 ± 3	18 ± 2	1.4 ± 0.5

[a] Three STA-preparations are compared in their mitogenicity for unseparated normal and patient PBL and purified T and B lymphocytes from tonsil. The three STA preparations are described in *Materials and Methods*. Cultures were treated as indicated in the legend of Table I.

into immunoglobulin-secreting plasma cells.

In the experiments presented, we have demonstrated STA and SpA to be selective lymphocyte mitogens and confirmed their specificity in studies of patients with different immunodeficiency diseases. Our findings indicate that STA is able to activate B lymphocytes, whereas SpA was a potent and selective T cell mitogen. Studies of the culture requirements indicated a role for the protein A moiety not only in T cell triggering but also in the mitogenic effect of STA on B lymphocytes. Thus, the inhibitory effect on only STA responses of normal human serum and IgG1 (but not IgG3) indicated that both the presence of bound IgG and access to the lectin-like IgG binding site on the protein A molecule are irrelevant for T cell activation by SpA. By the same token, access to this lectin-like binding site, and/or the absence of bound IgG are required for the B cell response to STA. It is unclear whether these differences reflect distinct and alternative triggering events in B and T lymphocytes or whether the inhibitory effect of STA-bound IgG is due to post-activation events such as Fc-domain-mediated suppression of induced B cell responses. Although in preliminary studies IgG-coated STA did not interfere with either SpA-induced T cell proliferation or PWM-induced B cell proliferation, IgG-mediated inhibition would have some precedence (22) and cannot be ruled out at present.

The cell lineage specificity of STA and SpA responses did not (only) reflect different response spectra at the level of B and T lymphocytes but was expressed at the molecular level as well: SpA failed to trigger B lymphocytes no matter whether these populations were physically purified or obtained from patients with the appropriate cellular immunodeficiency. If B and T cell stimulation by STA or SpA reflect the presence of distinct mitogenic sites on these agents, then this would allow several conclusions to be drawn concerning the triggering events involved: a) the T cell stimulating domain of the SpA molecule is not accessible on formalinized STA and may be buried in the cell wall; alternatively, the mechanism of T cell triggering by SpA requires the molecule to be soluble, permitting, for example, its uptake and subsequent activation of the cell; b) B cell activation by STA requires the stimulating protein A molecule to be insoluble and conceivably in a certain (rigid) steric conformation; alternatively, STA cell walls may contain a second structure that by providing a "second signal" (23) is fulfilling a requirement for B cell triggering.

The results of cell separation experiments and patient studies indicate that the STA-responsive populations likely express Ia-determinants, C, and STA receptors. The absolute correlation of the presence of sIg^+ cells with the expression of STA responsiveness in patients with antibody deficiency syndromes does not necessarily indicate the presence of sIg on all responding cells, but may rather be indicative of the stages of maturation that have to be completed before the acquisition of STA reactivity. This notion is reinforced by the finding of sIg on only a minority (<40%) of the positively selected STA-binding cells and the failure in preliminary experiments to abrogate STA responses through pretreatment with anti-immunoglobulin re-

agents. Surface immunoglobulin, in particular sIgG, has been implied in B cell triggering and may represent an attractive candidate for the structure recognized by STA. However, our experiences do not support this possibility, including the failure to detect by immunofluorescence and radioimmunochemical procedures, sIgG on SCID B lymphocytes (cases 3 through 6) and on appreciable proportions of normal peripheral blood B cells (24). In addition, the possiblity of B cell stimulation via sIg determinants is still controversial after more than a decade of direct experimental attempts (reviewed in 25). STA-responsive cells bind to STA and can thereby be separated from unresponsive B cells (Table II), suggesting that the binding avidity is considerable. This is difficult to explain as an entirely IgG-mediated event, since even if IgG were expressed on all of these cells (approximately 60% of all C3-bearing B cells), the number of sIgG-molecules must be very small to escape detection after surface radioiodination (24). On the other hand, there may well be other protein A binding surface structures, including histocompatibility-related alloantigens (19). Although difficult to formally disprove, we feel that sIgG is not required for B cell triggering by STA. The physical depletion by STA of only 30 to 40% of B cells and no T lymphocytes suggests rather specific receptor ligand interactions between STA and STA-responsive B cells.

Cell separation experiments utilized both positive and negative selection procedures for different lymphocyte surface properties in order to avoid inadvertant co-purification of smaller cell populations (11). The conclusions derived were tested in studies of patients with a variety of immunodeficiency syndromes. Our findings of normal STA-responsiveness in the group of ADA$^+$-SCID patients confirms the previous reports of intrinsically normal B cell function in such patients (15). The patients with primary antibody deficiency syndromes were heterogenous in several aspects. Multiparameter analysis indicated only one significant correlation to the presence of an STA response, i.e., the presence of sIg-positive lymphocytes. In some of the patients functional, sIg-negative precursors of specific IgM-producing B cells have been demonstrated previously (26). All of these patients (cases 7 through 11) also exhibited spontaneous, T cell-mediated suppressor cell activity, measured in a hemolytic plaque assay by their ability to reversibly interfere with immunoglobulin secretion (11). The failure of these patients to generate a STA response may reflect either the interference of suppressor cells with this response or the inability of the small population of sIg-negative plaque-forming cell precursors to respond to STA. This is currently being investigated.

The preparation of STA was of great importance in determining its B cell specificity. A commercial STA preparation, which showed some shedding of soluble SpA, gave quite different results from our own preparation. When this shedding was eliminated, the preparation was rendered B cell specific. SpA as well as STA have been variously described as B and/or T cell mitogens by using culture conditions with or without human serum supplements (5–10). We suggest that most variations of findings in the different laboratories that used STA may be explained by differences in STA preparations.

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POLYCLONAL ACTIVATION OF HUMAN LYMPHOCYTES *IN VITRO*

II. Reappraisal of T and B Cell-Specific Mitogens[1]

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The capacity of the T cell mitogens phytohemagglutinin (PHA), concanavalin A (Con A), pokeweed mitogen (PWM), and Staphylococcus protein A (SpA) to induce B cell proliferation and differentiation was compared with the B cell mitogen, formalinized *Staphylococcus aureus* (STA). Lymphocyte subpopulations from normal donors and patients with various immunodeficiency diseases were studied. In the presence of the T cell mitogens, irradiated T cells were capable of providing a helper cell activity that enabled co-cultured B lymphocytes to proliferate in response to these mitogens and to differentiate into IgM-secreting (direct) hemolytic plaque-forming cells (PFC). In the PFC response, radioresistant T-helper and radiosensitive T-suppressor cell activities could be demonstrated. T-suppressor cell activity outweighed helper activity only in nonirradiated co-cultures stimulated with Con A. Patients with severe combined immunodeficiency lacked mitogen-induced helper T cells, whereas patients with various forms of humoral immune deficiency were normal in this respect. These findings and the tissue distribution of the helper cell activity in normals indicate that this T-helper activity is acquired early in post-thymic T cell differentiation. The data suggest that experiments with cell lineage-specific lymphocyte mitogens should be considered in the context of more complex cell-cell interactions.

Lymphocyte mitogens are convenient probes in studies of cellular immune function. Regardless of the species studied, most mitogens possess specificity for the lymphocyte lineage stimulated and thus can serve as markers of cell origin. In contrast to rodents, there is a paucity of reliable B cell-specific mitogens in man that are able to trigger proliferative responses in the absence of T cells. Some exceptions have been noted including water-soluble extracts from Nocardia (1), live Epstein-Barr virus (2), and more recently formalinized *Staphylococcus aureus* Cowan I strain (STA)[4] (reviewed in 3). While testing lymphocyte mixtures from normal tissues and immunodeficient patients, we observed an apparent breakdown in the established cell lineage specificity of the T cell mitogens phytohemagglutinin (PHA), concanavalin A (Con A), Staphylococcus protein A (SpA), and pokeweed mitogen (PWM). Cell dilution analysis of various lymphocyte mixtures indicated that a radioresistant T-helper cell activity could be unmasked that permitted co-cultured B lymphocytes to proliferate and differentiate in response to these mitogens. The tissue distribution of this activity indicated that immature (thymic) T lymphocytes were particularly rich helper cell sources. Patients with severe combined immunodeficiency disease (SCID) without T lymphocytes lacked this activity, whereas patients with defective humoral immunity had normal helper activity.

MATERIALS AND METHODS

Cell sources. Ficoll-Hypaque purified mononuclear cells were obtained from thymus, tonsil, bone marrow, thoracic duct, and peripheral blood as described (3). E-rosette-forming (E^+) T cells were separated from nonrosetting cells (E^-) by E-rosette depletion (3). E^+ cell populations contained at least 97% E-rosette-forming cells, no sIg-bearing cells, and less than 1% complement receptor- (C3) bearing cells. The E^- populations contained up to 1% E-rosette-forming cells, greater than 80% C3 receptor-positive cells, 40 to 60% of the cells were sIg (IgM/IgD) positive, and more than 90% of the cells expressed surface Ia determinants as measured by indirect immunofluorescence (4). Peripheral blood mononuclear cells (PBL) were also obtained from patients with both the adenosine deaminase (ADA) positive and negative forms of SCID, agammaglobulinemia (Aγ), and common variable immunodeficiency disease (CVID). All patients have been described in the accompanying paper (3).

Culture conditions. Unless otherwise indicated, cultures of 1×10^6 cells/well (Microtest II plates, Falcon Plastics, Oxnard, Calif.) were maintained for 80 hr in supplemented RPMI 1640 and 10% normal human serum or 10% fetal calf serum (FCS) (3). At the end of culture, cells were harvested after a 4-hr pulse with 1 μCi ^{3}H-thymidine (specific activity 6.7 Ci/mM). Under these conditions, mixed lymphocyte responses in allogeneic cell mixtures were not detectable, and mixtures of purified alloge-

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[4] Abbreviations used in this paper: ADA, adenosine deaminase; Aγ, agammaglobulinemia; CVID, common variable immunodeficiency disease; E^+, E-rosette-forming (T) lymphocytes; E^-, E-rosette-negative cells; PBL, peripheral blood lymphomononuclear cells; PFC, hemolytic plaque-forming cells; PWM, pokeweed mitogen; SCID, severe combined immunodeficiency disease; SpA, Staphylococcal protein A; STA, formalinized *Staphylococcus aureus* (Cowan I); sIg, surface immunoglobulin.

neic or autologous lymphocyte subpopulations showed similar mitogenic responses. In cell dilution experiments, 1 to 100 × 10^3 cells were cultured in the presence or absence of irradiated cells (2000 rads) as indicated.

Mitogens. Mitogens (10 μg/culture) were always added at the beginning of culture. PHA, PWM, and Con A were purchased from Difco Laboratories (Detroit, MI) and SpA from Pharmacia (Montreal, Que.). S. aureus (Cowan I) bacteria were grown and treated as described (3); 1 × 10^6 organisms were added per culture.

Micro-PFC responses. Micro-cultures for the assessment of direct hemolytic plaque-forming cell (PFC) responses towards picrylated sheep erythrocytes (5) were maintained as described above in the presence of 5% FCS. After 5 days of incubation they were washed three times in the culture wells, resuspended in RPMI 1640, and then were transferred to fluid-phase microplaque assays in microtest II plates as described (6).

Data evaluation. Means and standard deviations were calculated from replicate values for thymidine incorporation or PFC responses per culture. Results from larger series of experiments were pooled after expressing responses as fractions of the maximal thymidine incorporation or maximal PFC responses in the individual experiments. For statistical regression analysis as well as curve fitting, we utilized a Tektronix microcomputer and Plot 10 based software (7, 8).

RESULTS

Effects of cell density on B and T cell mitogen responses. Before performing cell mixing experiments, it was necessary to assess the role of cell density in determining the extent of responses to the different mitogens. One to 300 × 10^3 PBL or tonsil cells were stimulated with constant amounts of mitogen (see *Materials and Methods*). Figure 1 shows data from 650 PBL and 860 tonsil cell cultures. These were pooled and submitted to regression analysis and curve fitting. With correlation coefficients of greater than 0.9, results for all T cell mitogens were best fitted with logarithmic functions of the form y = a + b log x. Analyzing the exponential part of cell dose-response curves (Fig. 2), a family of near parallel functions was obtained with slopes between 1.7 and 2.1. In contrast, cell dose effects on STA responses were best fitted with the linear function y = a + b x, and the resulting response curve had a slope of 1. This observation suggested that STA responses likely involved one responding cell population but that more complicated cell interactions were involved in the responses to the T cell mitogens (9, 10). Furthermore, the results suggested that in PBL and tonsil, pool sizes of STA-responding cells may be more than 1 order of magnitude lower than those responding in cultures containing PHA, with intermediate values for the other T cell mitogens.

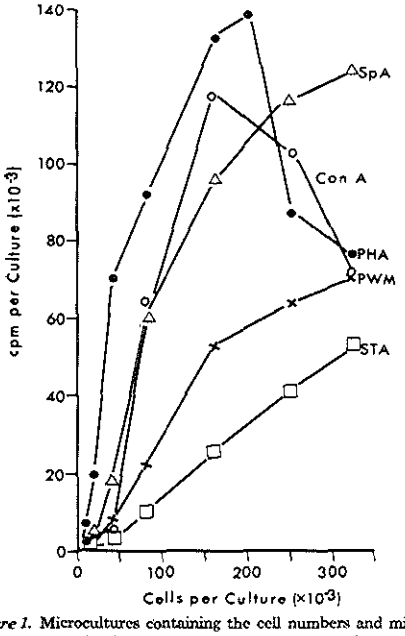

Figure 1. Microcultures containing the cell numbers and mitogens indicated were maintained for 80 hr in supplemented RPMI-1640 as described in *Materials and Methods*; their DNA-synthetic activity was then measured after a 4-hr pulse with 1 μCi H^3-thymidine. The pooled data shown reflect results from 650 PBL and 860 tonsil cell cultures.

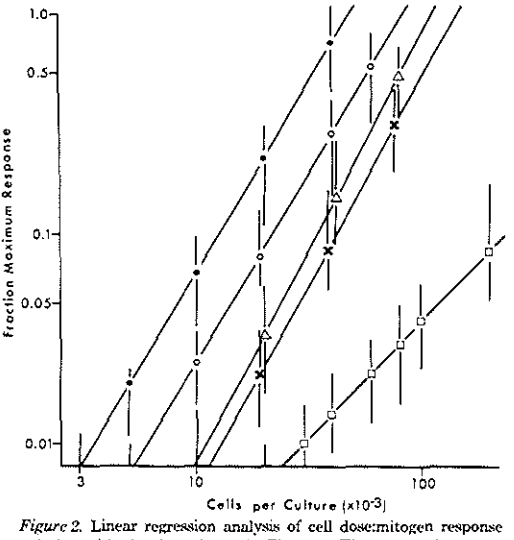

Figure 2. Linear regression analysis of cell dose:mitogen response correlation with the data shown in Figure 1. The pooled data were normalized to express fractions of maximal responses observed with each mitogen in the individual experiments. Curve fitting was done by using Plot 10-based software in a microcomputer. Vertical bars indicate standard errors. Symbols as in Figure 1: △, SpA; ○, Con A; ●, PHA; ×, PWM; □, STA.

Cell co-operation. To analyze the different responses patterns of B and T cell mitogens, we utilized cell mixing experiments and differential irradiation (Table I). Irradiated T cells (E^+, 1 × 10^5/culture) were mixed with nonirradiated, E-rosette-negative cell populations (E^-, 1.5 × 10^5/culture). In addition, PBL from a patient with the ADA^+ form of SCID were used as a cell source rich in B lymphocytes but devoid of T cells. Only STA induced significant responses in E^- and SCID PBL. In the presence of PHA, Con A, PWM, or SpA, comparatively vigorous responses were observed in co-cultures of nonirradiated E^- or SCID PBL with irradiated T cells.

Cellular requirements for B cell proliferation. The cellular requirements for the proliferative responses observed in co-

cultures of E^- and irradiated E^+ lymphocytes were analyzed in cell dilution experiments. Increasing numbers (2 to 100 × 10^3) of irradiated peripheral blood or tonsillar T cells were added to a constant number (1 × 10^5 cells/well) of purified E^- (Fig. 3). In cultures stimulated with STA, the addition of irradiated T cells had little effect, and in all experiments these cultures showed the highest incorporation of thymidine (3 to 5 × 10^4 cpm/culture). Cultures of E^-, stimulated with the T cell mitogens, showed only marginal responses when 2 to 10 × 10^3 irradiated T cells were added. With higher numbers of added T cells, responses increased until a plateau was reached at between 3 × 10^4 to 7.5 × 10^4 irradiated T cells per well. At these plateau values, the proliferative responses observed were a direct function of the number of E^- cells added per well (Fig. 4): at a constant concentration of 10^5 irradiated T cells/culture, the addition of more E^- cells (but not of T cells, Fig. 3) resulted in enhanced proliferation. These data demonstrated that irradiated T cells, incubated in the presence of PHA, Con A, PWM, and SpA, permitted the proliferation of B cells. Furthermore, it was possible to define limiting and saturation conditions for the responding B cells (Fig. 4) as well as for the cooperating T cells (Fig. 3).

TABLE I

Demonstration of T-helper activity[a]

Mitogen Added	Mitogen Responses (cpm × 10^{-3})				
	E^-	SCID	Irradiated E^+ Plus		
			—	E^-	SCID
None	<2	<2	<2	<2	<2
PHA	5 ± 3	<2	3 ± 2	37 ± 8	45 ± 8
PWM	5 ± 1	<2	4 ± 2	29 ± 11	42 ± 6
Con A	2 ± 1	<2	5 ± 2	26 ± 7	34 ± 8
SpA	4 ± 2	<2	4 ± 1	42 ± 12	51 ± 13
STA	36 ± 6	31 ± 7	<2	43 ± 10	38 ± 11

[a] 1.5 × 10^5 purified normal B cells (E^-), 1 × 10^5 irradiated (2000 rad) T cells (E^+) or 2 × 10^5 SCID PBL were cultured alone or in combination in the presence of the mitogen indicated. Proliferative responses (cpm × 10^{-3}, $\bar{x}$ ± 1 S.D.) in 1 of 4 similar experiments are given.

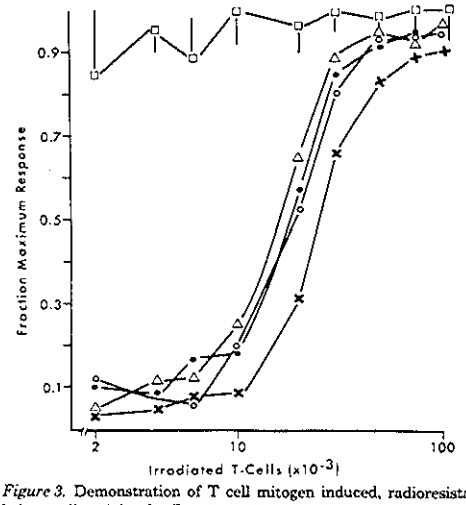

Figure 3. Demonstration of T cell mitogen induced, radioresistant T-helper cell activity for B cell proliferation. Increasing numbers of irradiated T cells were cocultured with 1 × 10^5 nonirradiated B cells in the presence of either SpA (△), Con A (○), PHA (●) or PWM (×). Stimulation with STA (□) served as a control. Proliferative responses in five experiments were corrected for background thymidine incorporation and expressed as a fraction of the maximum response in each experiment (17 to 38 × 10^3 cpm) and pooled.

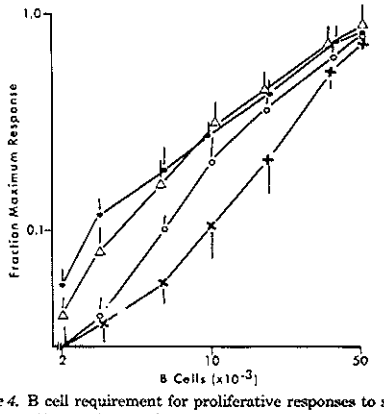

Figure 4. B cell requirement for proliferative responses to stimulation with the T cell mitogens SpA (△), Con A (○), PHA (●) or PWM (×) in cocultures of irradiated T (1 × 10^5/well) and nonirradiated B cells (2 to 50 × 10^{-3}/well). Data from five experiments were normalized and pooled as before (see legend, Figure 3).

TABLE II

Tissue distribution of helper activity[a]

Irradiated Helper Cell Source	Normal B Cells	% Maximum Response		
		SpA	Con A	PHA
None	+	6 ± 1	5 ± 3	4 ± 1
E^- (PBL)	+	8 ± 1	7 ± 2	9 ± 2
E^+ (PBL)	−	11 ± 4	7 ± 2	10 ± 6
	+	88 ± 11	79 ± 9	94 ± 10
Thymus	−	<5	<5	<5
	+	100 ± 7	92 ± 14	81 ± 8
Bone marrow	−	6 ± 2	5 ± 3	6 ± 2
	+	9 ± 2	8 ± 1	8 ± 8
Thoracic duct	−	8 ± 1	9 ± 3	6 ± 1
	+	32 ± 4	27 ± 6	23 ± 4
Tonsil	−	9 ± 2	11 ± 1	12 ± 3
	+	64 ± 3	51 ± 7	67 ± 7
Spleen	−	8 ± 1	7 ± 2	9 ± 3
	+	51 ± 16	n.d.	43 ± 5

[a] 1 × 10^5 irradiated (2000 rad) mononuclear cells per well were tested as sources of mitogen-induced helper activity for B cell proliferation. + denotes the addition of 1 × 10^5 normal tonsillar or peripheral blood B cells. Mean responses (±1 S.D.) are expressed as a fraction of the maximum response observed in two or more experiments. Maximum responses varied between 27 and 43 × 10^3 cpm.

Tissue distribution of T-helper cells. The tissue distribution of the radioresistant T-helper activity is shown in Table II. Data from more than 1000 cultures are summarized. 1 × 10^5 irradiated cells from each tissue served as a source of helper cell activity for mitogenic responses of 1 × 10^5 nonirradiated E^- cells from tonsil. T-helper activity was found in all tissues studied except normal bone marrow. The highest activity was

observed in thymocytes, followed by PBL and tonsil, spleen, and thoracic duct cells.

Helper activity in immunodeficient patients. Four groups of patients with primary immunodeficiency, who share the common feature of impaired specific antibody formation, were studied for the presence of radioresistant helper activity in PBL (Table III). No helper activity was found in patients with SCID. Normal activity was consistently observed in patients with either Aγ or CVID.

Demonstration of polyclonal B cell activation. Mitogen-stimulated microcultures were examined for the generation of direct hemolytic PFC specific for picrylated sheep erythrocytes (Table IV). Tonsillar B cells and irradiated or nonirradiated E^+ were tested in cell mixing experiments similar to those described above. After 5 days incubation, unstimulated cultures contained virtually no spontaneous PFC. STA induced the development of PFC in B cell cultures independent of the presence of T cells. The T cell mitogens failed to induce the generation of PFC in cultures containing E^+ or E^- cells alone, whereas in co-cultures of E^+ and E^- cells the generation of PFC was induced by all mitogens but Con A. Indeed, the addition of both Con A and STA to B-T cell mixtures resulted in the suppression of STA-induced PFC responses. Consistently higher PFC responses were observed in all cultures containing B cells and irradiated T cells. The irradiation of B cells, in contrast, abrogated the PFC response. Furthermore, in cell mixtures of E^- and irradiated E^+ cells, Con A was consistently able to induce the development of PFC. In such co-cultures, Con A was no longer suppressive to STA-induced responses. Thus, cultures stimulated with both Con A and STA generated high numbers of PFC, and albeit somewhat more variable than cultures stimulated with only one mitogen, the STA and Con A responses in these cultures appeared additive. These data suggest that T cell mitogen-induced B cell activation is not restricted to proliferative responses but is accompanied by the differentiation of B lymphocytes into direct PFC.

TABLE III

Helper cell activity in immunodeficient patients[a]

Irradiated Helper Cell Source	(N)	Normal B Cells	% Maximum Response		
			SpA	Con A	PHA
ADA$^+$ SCID	(3)	−	<5	<5	<5
		+	5 ± 2	5 ± 1	<5
ADA$^-$ SCID	(2)	−	<5	<5	<5
		+	<5	<5	<5
Aγ	(5)	−	6 ± 1	7 ± 4	6 ± 4
		+	96 ± 9	66 ± 11	89 ± 7
CVID	(5)	−	6 ± 2	8 ± 3	7 ± 2
		+	82 ± 5	90 ± 6	84 ± 9

[a] See legend, Table 2. ADA, adenosine deaminase; SCID, patients with severe combined immunodeficiency disease; Aγ, patients with congenital agammaglobulinemia; CVID, patients with common variable immunodeficiency disease; N, number of patients tested on at least two occasions. Mean responses (±1 S.D.) are expressed as a fraction of the maximum response observed among all co-cultures in each experiment. Maximum responses varied between 22 to 40 × 10^4 cpm. + denotes the addition of 1 × 10^5 normal B cells.

DISCUSSION

The statistical analysis of a large series of experiments revealed the likelihood of cell-cell interactions in T cell mitogen but not in B cell mitogen-stimulated cultures (9, 10). The theoretical conclusions were confirmed in cell-mixing experiments of B and irradiated T cells. These studies revealed a mitogen-induced, radiation-resistant T-helper activity, which permitted (or induced) B cells to proliferate. The lack of comparable effects in STA-stimulated co-cultures provided an appropriate control for the cell-mixing experiments and not only reinforced the validity of the statistical analyses, but confirmed the T cell independence of STA responses (3).

Radiation-resistant T-helper cells were demonstrated in different lymphoid tissues and certain patients with immunodeficiency. Their absence in bone marrow and SCID indicated that early steps in thymus-dependent T cell differentiation have to be completed before the generation of detectable helper activity (11). The demonstration of normal helper activity in patients with defective humoral immunity is reminiscent of our previous findings in these patients of normal helper activity for specific *in vitro* PFC responses (12), although it is unclear whether the same cell populations are involved.

The B cell proliferative responses were accompanied by the generation of direct PFC responses, which were assayed using heavily TNP-haptenated sheep erythrocytes. Whether the proliferative B cell responses and the generation of PFC are directly linked, coincidental or consecutive events is unclear. Since PFC were not generated if B cells were irradiated, it is likely that these cells undergo cell division once activated in the co-culture system.

The nature of radioresistant T cell help and, in particular, of the activation processes triggered by the T cell mitogens have not been addressed (13–18). Helper activity of irradiated T cells has been described for both mitogen- and antigen-driven B cell

TABLE IV

Demonstration of mitogen-induced hemolytic plaque-forming cells[a]

Cells Cultured	PFC Response/Culture						
	−	PHA	SpA	PWM	Con A	STA	Con A + STA
E^-	<5	11 ± 3	<5	13 ± 3	<5	66 ± 19	51 ± 12
Irradiated E^-	<5	<5	<5	<5	<5	7 ± 5	<5
E^+	<5	8 ± 2	<5	<5	<5	<5	<5
Irradiated E^+	7 ± 3	6 ± 4	6 ± 4	7 ± 2	<5	<5	<5
$E^- + E^+$	<5	38 ± 11	77 ± 19	89 ± 11	<5	59 ± 7	9 ± 6
E^- + Irradiated E^+	8 ± 2	107 ± 18	104 ± 16	115 ± 22	81 ± 10	76 ± 12	136 ± 39

[a] Microcultures of 1 × 10^5 purified B cells (E^-) and/or 1 × 10^5 T lymphocytes (E^+) from peripheral blood or tonsil were incubated with the mitogen indicated. Irradiated cells received 2000 rads before culture. After 5 days of culture the cells were washed in the culture trays and transferred to direct, fluid phase hemolytic plaque assays for anti-TNP sheep erythrocyte plaques. Results from three experiments are pooled and expressed as mean PFC responses generated per microculture (±1 S.D.).

activation in rodents and man (17, 19, 20). In the case of PWM- and Con A-stimulated cultures, this activity has been associated with soluble factors (15, 16). A soluble helper factor released from irradiated T cells has similarly been shown for specific (direct) PFC responses to T cell-dependent antigens (17). The term "radioresistant" in the present context may be somewhat misleading and does not necessarily imply that helper T cells are not triggered by the mitogens to proliferate: the release of soluble T-helper activity, for example, may be completed sufficiently early in culture to be unaffected by irradiation. In the case of specific PFC responses and PWM-induced B cell activation, soluble helper activity has indeed been found to accumulate rapidly in culture supernatants within hours after antigen or mitogen contact (16, 17).

Direct PFC were detected using heavily haptenated sheep erythrocytes as targets. Although T cell mitogens were able to induce the generation of PFC, this reaction was strictly T cell dependent, since purified B cells alone were responsive only to stimulation with STA. This underlines the primary cell lineage specificity of the mitogens studied. The T cell mitogens may be required to trigger the generation of helper activity in T lymphocytes; this activity (e.g., a soluble factor) may then act as a "B cell mitogen" akin to STA. Alternatively, both the T cell mitogen and T-helper activity may be required at the B cell level to initiate the development of PFC. We favor this second alternative, since we observed earlier that the generation of T cell-dependent PFC required two triggering signals at the B cell level: binding of the activating ligand itself plus the presence of a T cell product (17).

Irradiation of the T cell supplement in culture enhanced the number of PFC generated in response to all T cell mitogens. It seems likely that irradiation abrogated the development of mitogen-induced T-suppressor cell activity co-induced with helper activity. This was directly shown for Con A-induced PFC responses. In Con A-stimulated co-cultures of unirradiated cells, radiosensitive suppressor cell activity outweighed co-induced helper function: helper activity was unmasked when suppressor cell development was prevented by irradiation.

Considering the present data together with previous reports (13, 14, 17, 18, 21–27), we may extrapolate several conclusions: a) the absolute height of the PFC response reflects the net helper function induced, i.e., the excess of helper over suppressor activity generated; b) the B cell response generated in untreated B-T cell mixtures may not primarily reflect the functional B cell repertoire present in this mixture, but it may rather depend on the balance between antagonistic regulatory T cell subsets activated by the particular mitogen; and c) nonirradiated cell mixtures would then be characterized by a negative restraint of B cell function.

Taken together, the data presented suggest that experiments with cell lineage-specific mitogens should be considered in the context of more complex cell-cell interactions.

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IDENTIFICATION OF Ia ON A SUBPOPULATION OF HUMAN T LYMPHOCYTES THAT STIMULATE IN A MIXED LYMPHOCYTE REACTION[1]

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The delineation of discrete subpopulations of human T lymphocytes has permitted preliminary analyses of the complex cellular network regulating the immune response in man. We previously showed that a subset of T lymphocytes, designated as theophylline-sensitive because of their inability to bind sheep red blood cells in the presence of the drug, are responsible for antigen-specific suppression or regulation in an *in vitro* plaque-forming cell assay. We now show that 25 to 45% of these theophylline-sensitive T cells were Ia-positive by immunofluorescence with a rabbit antiserum raised against purified B lymphoblast surface antigenic material. These data suggested that 4 to 7% of peripheral blood T cells carry Ia determinants. The presence of Ia determinants on this T cell subset was confirmed by gel analysis of radioiodinated surface material. Furthermore, in mixed lymphocyte culture, the theophylline-sensitive cells demonstrated HLA-D determinants and were 10-fold more potent stimulators than equal numbers of B lymphocytes. The presence of Ia determinants on these T cells indicates the expression of major histocompatibility complex-related regulatory gene products on a specific human T lymphocyte subpopulation.

By using a variety of allo and heteroantisera, DR or Ia-like determinants have been demonstrated on human B lymphocytes and monocytes (1–3). The heteroantisera recognize a basic structure, common to all allotypes, which is composed of two components, 27,000 daltons and 35,000 daltons; in this respect it is similar to murine Ia (4). The main lymphocyte-activating determinant in the mixed lymphocyte reaction (MLR), encoded by the HLA-D region, appears to be identical or closely linked with Ia (5). In the mouse, Ia is known to play a major role in the regulation of the immune response, and in this species a number of groups have described Ia determinants, expressed predominantly on a T cell subset, responsible for suppression of the immune response (6–8).

We have isolated a subpopulation of human T cells that contain antigen-specific suppressor cells when assayed in a direct plaque-forming cell (PFC) assay (9). These cells, identified by their inability to bind sheep red blood cells (SRBC) in the presence of theophylline, have been labeled theophylline-sensitive T lymphocytes (T-sens)[5] (10). In this report we demonstrate that 25 to 45% of T-sens are Ia-positive and express HLA-D determinants.

MATERIALS AND METHODS

Cell surface markers. The E-rosette assays in which were used untreated or aminoethylisothiouronium bromide- (AET) treated SRBC and incubation with theophylline have been described previously (11). After theophylline treatment, all E-rosette assays were carried out by using AET-treated SRBC (E_{AET}). Surface immunoglobulin- (sIg) positive cells were identified by using direct immunofluorescence, and monocytes were enumerated after α-naphthyl esterase (NSE) staining and latex particle ingestion. C3 receptor-bearing cells were assessed by using a rosette technique with intermediates generated by incubating ox red blood cells (E_{ox}) with rabbit IgM anti-ox antiserum and normal mouse serum as the source of complement (12).

Isolation of lymphocyte subpopulations. The isolation technique is illustrated in Figure 1. Heparinized or defibrinated peripheral blood was subjected to Isopaque-Ficoll gradient centrifugation (9). The mononuclear cells (PBL) were depleted of adherent cells by incubation in plastic Petri dishes for 30 min at 37°C in the presence of 20% FCS. The nonadherent cells (NAC) were incubated with 3 mM theophylline for 45 min at 37°C. SRBC were then added in the presence of 30% FCS, and the mixture was spun for 5 min at 200 × G, maintained at 4°C for 30 min, gently resuspended, and placed on gradients of Isopaque-Ficoll. After 30 min centrifugation at 400 × G, the interface containing T-sens and non-T cells were recovered and treated separately from the pellet containing theophylline-resistant T cells (T-res). The interface cells were incubated with E_{ox}AC3 (1:40 ratio) for 5 min at 37°C, kept at room temperature for 30 min, gently resuspended, and layered onto an Isopaque-Ficoll gradient. After centrifugation at 200 × G for 30 min, the

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[5] Abbreviations used in this paper: AET, aminoethylisothiouronium bromide; MHC, major histocompatibility complex; NAC, nonadherent cells; NSE, α-naphthyl esterase (nonspecific esterase); PBL, peripheral blood mononuclear cells; T-res, theophylline-resistant T-lymphocytes; T-sens, theophylline-sensitive T-lymphocytes; E_{AET}, AET-treated SRBC; sIg, surface immunoglobulin; E_{ox}, ox red blood cells; SDS, sodium dodecylsulfate.

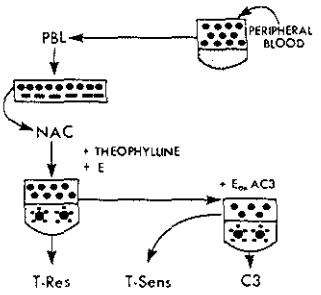

Figure 1. Isolation of theophylline-sensitive peripheral blood T-cells. E, sheep red blood cells; E_{ox}AC3, Ox erythrocyte-IgM-C3 intermediates; C3, C3-positive cell fraction.

interface cells and pellet were recovered. The SRBC and E_{ox} in the cell pellets were lysed in Tris-ammonium chloride (13).

Preparation of the anti-Ia antiserum. Membrane vesicles were prepared from cultured B lymphoblasts by solubilization in Tween 40 (14). The vesicles were solubilized in 0.5% Triton X-100, and the material was applied to a column of Sephadex G-200 equilibrated in 0.01 M Tris-HCl (pH 8.0) and 1% deoxycholate. The position of B cell-specific antigen originally characterized as a molecule of 30 to 35,000 daltons was recognized by precipitation of labeled material with an absorbed anti-B lymphoblast serum (15, 16). Subsequently, material with a m.w. of 20 to 50,000 was recovered, and rabbits were immunized with 50 μg of this material in complete Freund's adjuvant. A second immunization with 10 μg in incomplete adjuvant was carried out, and the animals were bled 7 days later. The serum obtained was absorbed three to five times with cultured T lymphoblasts until the absorbed antiserum no longer precipitated surface labeled material from these lines. This absorbed antiserum was capable of precipitating the B cell antigen, which was resolved into two components of 27,000 and 35,000 daltons from cultured B lymphoblasts, chronic lymphatic leukemia cells, E-rosette-negative acute lymphoblastic leukemia (ALL) cells and acute myelocytic leukemia cells. Precipitation studies with E-positive lymphoblastic leukemia, chronic myelocytic leukemia cells, thymocytes, four different T cell lines, or the K562 line were negative.

Identification of Ia on T lymphocytes

Indirect immunofluorescence. The first incubation was carried out with a 1:60 dilution of the anti-Ia antiserum followed by labeling with a 1:10 dilution of fluoresceinated goat anti-rabbit IgG (Meloy Laboratories). Because of the weak staining of the T cells (compared with B cells or monocytes) and for convenience, all cell preparations were incubated overnight with the anti-Ia reagent. This did not significantly alter the number of positive cells in the different cell preparations but did improve the intensity of staining in T-sens. Control incubations were with a preimmunization serum from the same rabbit.

Lactoperoxidase-catalyzed radioiodination. T-sens were labeled by the lactoperoxidase method, and the labeled antigens were solubilized in 0.5% Triton X-100. The lysates were passed over a DEAE column and precleared with normal rabbit serum and *Staphylococcus aureus* Cowan strain I. Specific immunoprecipitations were carried out with Ig, Ia, and β2-microglobulin antisera and a control normal rabbit serum, followed by incubation with *S. aureus*. The precipitated materials were eluted in 10% sodium dodecylsulfate (SDS), reduced (2 mM 2-mercaptoethanol), and boiled for 2 min. The eluates were analyzed on 10% SDS-acrylamide gels (17).

Mixed lymphocyte reactions (MLR). One-way MLR were performed in microtiter plates by using a constant number of responder cells obtained by Isopaque-Ficoll gradient centrifugation of peripheral blood from normal donors (5×10^4/well) (18). Varying numbers of irradiated stimulator cells in the mixtures were incubated for 7 days. The stimulatory capacity of individual cell suspensions was expressed as a percent of the maximal stimulation in any one experiment. At a concentration of 3×10^5 cells, both T-sens and C3-positive cells were able to stimulate responder cells maximally (range: 47,000 to 158,000 cpm), and these responses were taken as maximal stimulation (100%) in any given experiment.

RESULTS

Table I summarizes the results of four experiments after the cell separation protocol illustrated in Figure 1. The population designated T-sens contained 81 ± 9% E_{AET}, and of these more than 90% were identified as theophylline-sensitive. Less than 5% were positive for C3, sIg, NSE, or latex particle ingestion. Of these cells, 25 to 45% were positively stained with the Ia antiserum, whereas T-res contained <5% Ia-positive cells. The C3-positive fraction contained >90% Ia-positive cells. Absorption of the anti-Ia antiserum with B lymphoblasts removed the reactivity against both the T-sens and the C3-positive fractions. In agreement with previous results for cell recoveries (19), T-sens represented approximately 20% of PBL. These data indicated that Ia-positive T cells could be identified in T-sens, and it was calculated that 4 to 7% of peripheral blood T-cells were Ia-positive.

The presence of Ia on T-sens was confirmed in two additional ways. After incubation of T-sens with anti-Ia, E_{AET} were added, and rosettes formed. Often T lymphocytes were covered with a large "morula" of SRBC and did not permit visualization of Ia on their surface. However, the addition of 1% formaldehyde during the incubation with SRBC permitted the detection of a receptor for SRBC on most of the cells identified as Ia-positive. In the mixed NAC suspension, we were able to detect the SRBC receptor on 10 to 15% of the Ia-positive cells but not in the C3-positive cell preparation.

The presence of Ia determinants on T-sens was demonstrated after lactoperoxidase-catalyzed iodination of T-sens. Figure 2 illustrates the pattern of one such immunoprecipitation with the anti-Ia antiserum and a control serum. The presence of the 27,000- and 35,000-dalton components confirms the presence of the Ia-like determinants on T-sens. Parallel immunoprecipitations with anti-immunoglobulin reagents detected the presence of sIgD/sIgM on only unseparated, NAC, and C3-positive preparations.

It has generally been accepted that B lymphocytes and

TABLE I

Characterization of the T-sens

% Positive Cells		
E_{AET}		Ia
81 ± 9[a]		34 ± 10
C3	sIg	NSE[b]
6 ± 4	1 ± 1	3 ± 2

[a] Results are expressed as the mean ± 1 S.D. of four experiments.
[b] NSE: α-naphthyl esterase stain for monocytes.

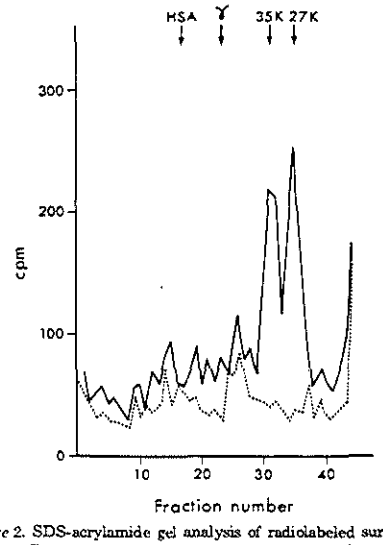

Figure 2. SDS-acrylamide gel analysis of radiolabeled surface material from T-sens. *Solid line,* immunoprecipitate obtained with the rabbit anti-Ia antiserum. Dotted line, immunoprecipitate obtained with the control rabbit serum. *Arrows* indicate the 27,000 and the 35,000 bimolecular complex of Ia, and the markers represent human serum albumin (HSA) and human IgG heavy chain (γ).

monocytes are the most potent stimulator cells in MLR, and T cells have been reported to only stimulate weakly, if at all (20–25). When the isolated subpopulations were used as stimulator cells in the MLR, it was obvious on analysis of cell dilution protocols that not only did T-sens (but not T-res) stimulate in MLR, they appeared to be more potent stimulators than the B cell fraction on a per cell basis (Fig. 3*a*). Further, the number of Ia-positive cells in the B cell fraction was 2- to 3-fold greater than in the T-sens fraction, and when this capacity to stimulate in MLR was expressed as a function of the number of Ia-positive cells, it appeared that T-sens were about 10-fold more potent than the Ia-C3-positive B cells (Fig. 3*b*).

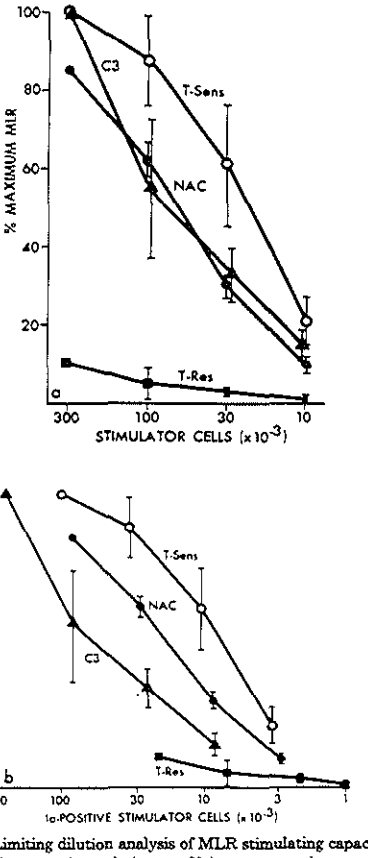

Figure 3. Limiting dilution analysis of MLR stimulating capacity of isolated lymphocyte subpopulations. *a,* Values expressed as a percent of the maximum MLR induced in responder cells by any of the cell populations. Mean (±1 S.D.) of four experiments is shown. *b,* As above except that the MLR stimulatory capacity was expressed on the basis of the number of Ia-positive cells added as stimulator cells.

DISCUSSION

Certain products of the major histocompatibility complex (MHC) in man such as HLA-A, B, and C antigens, can be readily identified by serologic techniques and appear to be expressed equally on all lymphocytes. In contrast, another series of antigenic determinants, designated DR (or Ia-like), appear to be expressed preferentially on B lymphocytes and monocytes (5). Although the presence and functional role of Ia antigens has been demonstrated on certain murine T lymphocyte populations (reviewed in 26), the presence of similar antigens on human T cells is the subject of much discussion and, with one exception (27), appears restricted to activated or proliferating T cells (28).

We demonstrated Ia-like determinants on a subpopulation of human T lymphocytes by immunofluorescence with a rabbit anti-Ia antiserum. This antiserum was raised against purified B lymphoblast surface antigenic material and was rendered specific for the 27,000/35,000-dalton bimolecular complex of Ia by suitable absorption. After a three-step depletion procedure, Ia-positive T cells were identified in the theophylline-sensitive T cell subset, previously shown to mediate antigen-specific suppressor cell activity in an antigen-dependent PFC assay (9, 29). Approximately 20% of all peripheral blood T cells were isolated as T-sens cells. Since 25 to 45% of these cells were shown to be Ia-positive, we estimate that 4 to 7% of peripheral blood T cells carry Ia-like determinants. This figure is very similar to that proposed by Fu *et al.* (27) by using a different antiserum and different separation techniques.

The presence of Ia-like antigens on a significant proportion of theophylline-sensitive T cells was confirmed by surface labeling and immunoprecipitation studies. It is unlikely that the cells responsible for the binding were not T cells (but a B cell, monocyte, or null cell), since a) up to 90% of T-sens cells had E-receptors; b) less than 5% of these cells were non-T cells carrying sIg, C3, stained with the esterase reagent, or were found to be phagocytic; c) the intensity of staining on T cells was less than that observed on B cells; and d) in a double-marker assay, both Ia and SRBC were found on the same cells.

The many contradictory reports on the identity of the cell

responsible for the major allogeneic stimulation in MLR (20–25) prompted us to test the various subpopulations obtained during the depletion protocol. Genes localized in the I region of the H-2 complex of the mouse control several basic immunologic phenomena, including the stimulation of allogeneic cells (26). Stimulation in the human MLR also appears to be controlled by genes of the MHC localized in the D region of chromosome 6. Our studies showed that a minor population of T lymphocytes, the theophylline-sensitive cells, were potent stimulators in the MLR, whereas the majority of the T cells, namely theophylline-resistant lymphocytes, were inactive. The stimulation in MLR by theophylline-sensitive cells could not be explained by a small degree of contamination by B cells or monocytes. In addition, the postulated "back stimulation" of responder cells by mitogenic factors released by irradiated T cells is unlikely, since the theophylline-resistant T cells were incapable of stimulation; both T cell subsets did respond in MLR.

In cell dilution experiments, theophylline-sensitive T cells appeared to be more potent stimulators than either the mixed nonadherent or purified C3-positive cell preparations. The stimulatory capacity of the theophylline-sensitive cells did not reflect (in a quantitative fashion) the expression of surface Ia molecules, since the B cell fraction contained more than twice the number of Ia-positive cells. Thus, when differences in stimulatory capacity were expressed as a function of the number of Ia-positive stimulator cells added, at a level of 50% maximum MLR, the T-sens fraction was about 10-fold more potent than the B cell fraction. Although the results presented are confined to peripheral blood T lymphocytes, similar results were obtained by using tonsillar lymphocytes and an alternative depletion protocol (9).

The strong association between specific HLA-D and Ia-like (DR) antigens makes it difficult to differentiate between the possibility that the genes that code for them are situated on the same locus or are on closely linked loci (30, 31). The MLR findings would suggest that either the Ia/D determinant is more exposed on T lymphocytes or that Ia and D are not identical but that the theophylline-sensitive fraction contains more D-bearing cells. It is also possible that all T-sens are Ia-positive but some cells are below the threshold for detection in our system.

In summary, we have demonstrated the presence of Ia-like determinants on a small subpopulation of human peripheral blood T cells known to contain specific T-suppressor cells and their precursors. This same subpopulation of cells has also been shown to carry MLR-stimulating determinants. Although additional studies are required to document that it is the Ia-positive cells that mediate these activities, we speculate that these "I-region" determinants expressed on theophylline-sensitive cells may be related to MHC-linked regulatory gene products equivalent to murine I-J determinants.

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The Molecular Basis of Immune Cell Function
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GROWTH CHARACTERISTICS AND FUNCTIONAL ANALYSIS OF HUMAN T-CELL COLONIES.

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INTRODUCTION

The maturation of human T-lymphocytes from a pre-thymic precursor stage to that of an immunocompetent T-cell is characterized by a sequence of identifiable stages[1]. Analysis of these stages has been possible using in vitro cultures of human thymic epithelium, epithelium-conditioned medium, thymus extracts and agents capable of modulating intracellular levels of cyclic AMP. In addition, the primary immunodeficiency diseases affecting T-cell differentiation have provided invaluable models for study. Semi-solid culture systems have provided the means for analysing the processes of cell proliferation and differentiation of granulocytes[2,3], erythrocytes[4,5] and megakaryocytes[6] in vitro.

Recently techniques have been adapted for the study of T-lymphocyte colony formation in man[7-9]. We have developed an improved method for human T-cell colony formation in methylcellulose and have attempted to apply this technique to the study of T-cell differentiation. By adapting the system to microculture, we have simplified the system and reduced the requirement for large cell numbers permitting the determination of precursor cell frequencies in the tissues investigated.

METHODS AND RESULTS

Conditions for T-cell colony growth. The conditions for T-cell colony growth are listed in Table 1. Mononuclear cell suspensions were obtained from peripheral blood, bone marrow, tonsil, thymus or thoracic duct lymph. The latter was obtained from an infant with chylothorax. E-rosetting T-lymphocytes were separated from non T-cells by E-rosette depletion[10]. The E-rosetting T-cells were further separated into Fc-IgG receptor positive ($T\gamma^+$) or negative ($T\gamma^-$) suspensions as described[10]. Alternatively theophylline-sensitive and theophylline-resistant T-cell subsets were obtained[10,11].

The culture medium consisted of 20% fetal calf serum (FCS) in alpha medium (K.C. Biologicals, Lenexa, KA), 5×10^{-5}M α-thioglycerol, and 40% PHA-conditioned medium. The latter was prepared by incubating peripheral blood leukocytes with 1% (v/v) PHA for 7 days in 10% FCS in alpha medium and obtaining the cell-free supernate.

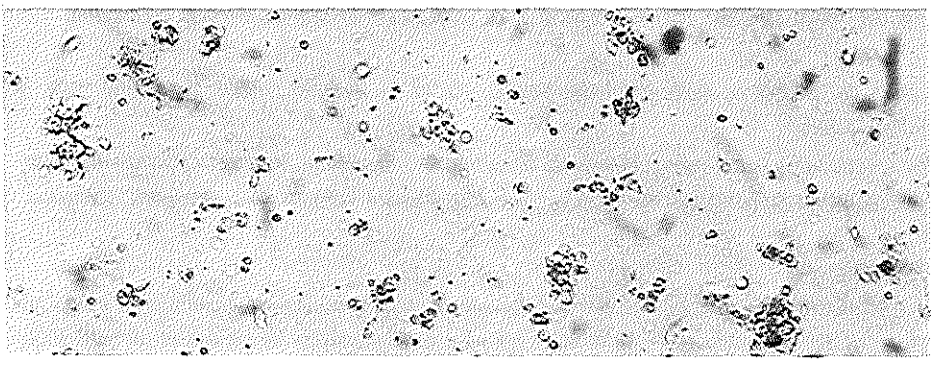

Fig. 1. Photomicrograph of T cell aggregates on Day 2.

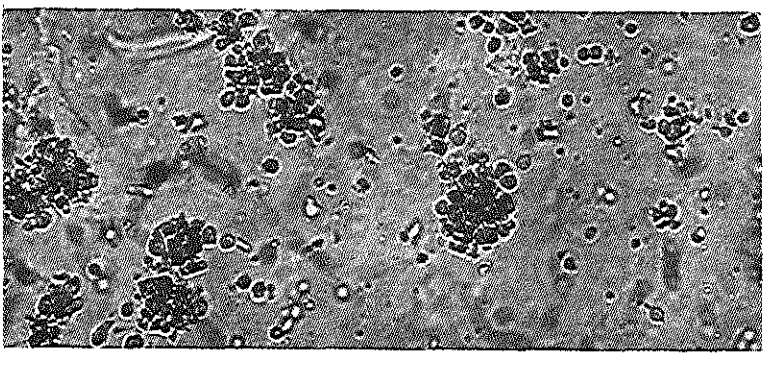

Fig. 2. Photomicrograph of T-cell colonies containing 20-30 cells on Day 3.

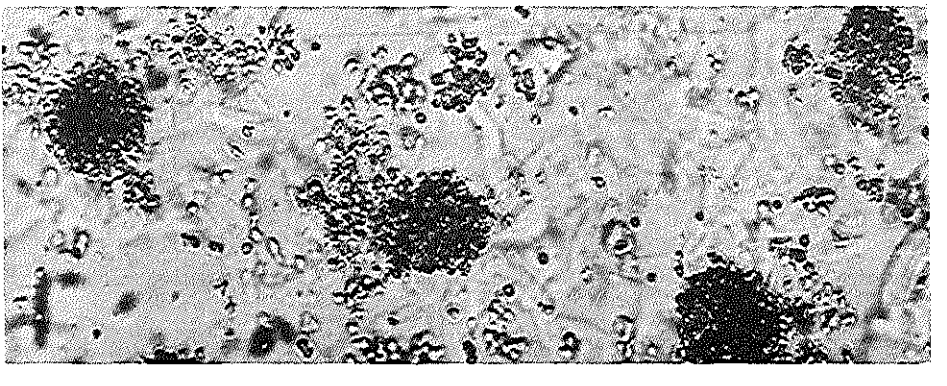

Fig. 3. Photomicrograph of T-cell colonies on Day 7 containing 100-200 lymphocytes.

TABLE 1
MICROCULTURE CONDITIONS FOR T-CELL COLONY GROWTH

CELLS

Post-ficoll mononuclear cells

CULTURE MEDIUM

A. 60% Alpha medium
Methylcellulose
20% FCS
5×10^{-5}M α-thioglycerol

B. 40% PHA-conditioned medium

CULTURE CONDITIONS

Microtest II plates
0.1 ml/well

In the microculture system, cells were suspended in 0.8% methylcellulose prepared in the culture medium and 100 µl added per well to Microtest II plates (Falcon Plastics, Oxnard, CA).

Growth Characteristics. Cell aggregates began to appear within 24 hours of plating peripheral blood cells and colonies containing 20 or more cells were observed by day 3 (Fig. 1-3). The size of the colonies increased exponentially during the first week of culture with maximum size being reached by day 6-7 and containing a mean of approximately 150 cells/colony (Table 2). The colonies could be maintained for 14-18 days after which progressive degeneration ensued.

TABLE 2
ESTIMATION OF COLONY SIZE*

Days in Culture	Cells/colony
1	5 ± 1
2	10 ± 5
3	22 ± 8
4	65 ± 15
5	95 ± 18
6	150 ± 50
7	145 ± 55
10	155 ± 50

* 5×10^4 cells plated per well

In the presence of the conditioned medium there appeared to be a linear relationship between the number of cells added per well and the number of colonies formed. Maximum colony formation was observed with between 3-5 x 10^4 cells/well (Table 3); colony formation was rarely observed if less than 10^4 cells/well were plated.

TABLE 3
NUMBER OF COLONIES FORMED AS A FUNCTION OF THE NUMBER OF CELLS PLATED

Cells/Well	Number of Colonies*
5 x 10^4	390 ± 95
4 x 10^4	341 ± 118
3 x 10^4	301 ± 103
2 x 10^4	69 ± 25
1 x 10^4	27 ± 26
5 x 10^3	0

* Assayed on day 7-10

Role of Irradiated Feeder Cells. In an attempt to optimize colony formation, we determined the effect of the addition of irradiated (2500 rads) feeder cells on plating efficiency. In the presence of 3-5 x 10^4 irradiated feeder cells, plating efficiency was improved by a factor of 10. A variety of cell suspensions were assessed for their efficacy as feeder cells. As shown in Table 4, irradiated cell suspensions from peripheral blood or tonsil were effective as were the T and non-T-cell fractions and lectin-induced lymphoblasts from these tissues. All of the B-cell lines were effective but none of the 4 T-cell

TABLE 4
EFFECT OF DIFFERENT FEEDER CELLS

Source of Cells	Enhancement of Colony Formation
Peripheral blood	+
Tonsil	+
T-cell fraction (E^+)	+
Non-T-cell fraction (E^-)	+
PHA-induced Lymphoblasts	+
ConA-induced Lymphoblasts	+
PWM-induced Lymphoblasts	+
B-cell Lines	+
T-cell Lines	-

lines tested could substitute as feeder cells. The mechanism by which the feeder cells improved plating efficiency remains unanswered and studies evaluating the role of cell-cell contact or soluble factors in supporting colony formation are underway.

Tissue Distribution of T colony Precursor Cells (Table 5). Although the number of colonies varied from individual to individual, T-cell colonies were routinely generated from all normal peripheral blood and tonsil cell suspensions. In general, bone marrow suspensions demonstrated little capacity for colony formation and thymocytes and thoracic duct cells were negative. In an attempt to identify the T-cell subset containing the T-colony precursor cell (TCPC), both peripheral blood and tonsillar lymphocytes were further separated by rosette depletion. The E-rosetting T-cells from both tissues were always shown to contain TCPC whereas the E-rosette negative or non-T-cell fractions were uniformly negative. In tonsil, only the theophylline-resistant and $T\gamma^-$ T-cell subsets contained TCPC and the theophylline-sensitive and $T\gamma^+$ subsets were negative. In limited experiments with peripheral blood, both the theophylline-sensitive and theophylline-resistant T-cell subsets contained TCPC. The discordance between peripheral blood and tonsil was surprising and remains unexplained at the present time.

TABLE 5
TISSUE DISTRIBUTION OF T-COLONY PRECURSOR CELLS

Cell Source	Colonies
Peripheral blood	+++
Tonsil	++
Bone marrow	±
Thymocytes	-
Thoracic duct	-

T-Cell Subset	Peripheral Blood	Tonsil
E^+	++	+++
E^-	-	-
Theophylline-sensitive	++	-
Theophylline-resistant	++	++
$T\gamma^+$		-
$T\gamma^-$		+++

T-cell Colony Formation in Patients with Immunodeficiency. The inability of thymocytes or thoracic duct lymphocytes to give rise to T-cell colonies suggested that not all cells which are responsive to PHA, as measured by de novo DNA synthesis, can give rise to colonies. In addition, since thymocytes and thoracic duct T-cells are less mature than peripheral blood or tonsillar T-cells[1,12], the T-colony precursor cell may be a mature T-lymphocyte. This is in agreement with the failure to detect TCPC in bone marrow, a source of precursor T-cells[13].

Indeed our studies of patients with immunodeficiency, either before or after reconstitution, support the following conclusions a) TCPC are mature T-cells, b) not all PHA-responsive T-lymphocytes can form colonies, c) TCPC are PHA responsive (as measured by DNA-synthesis) and d) the presence of TCPC may be used as evidence for significant functional T-cell differentiation. As shown in Table 6 we were unable to demonstrate TCPC in any of the following patients: Patient A - a patient with normal numbers of T and B cells with combined immune deficiency. There was a complete failure of lectin-induced proliferation. The underlying abnormality appeared to be secondary to an impairment of plasma membrane-cytoskeleton interactions as evidenced by a capping abnormality (Gelfand EW et al, in preparation). Patient B - a patient with severe combined immune deficiency who following reconstitution with a thymic epithelial cell transplant[1,14] showed normal numbers of E-rosettes and a near-normal PHA response. Despite this laboratory evidence for reconstitution and clinical well-being, his T-cells did not form colonies. Patient C - is a patient with severe combined immune deficiency associated with a deficiency of the enzyme, adenosine deaminase. Following red cell transfusion therapy we observed a dramatic increase in numbers of E-rosetting T-cells, the development of a significant PHA response and clinical well-being. Despite these findings, TCPC were not demonstrated.

TABLE 6
T COLONY FORMATION IN IMMUNODEFICIENCY

	% E-Rosettes	PHA (cpm)	No. of Cols/10^4 cells plated
Control	58	48,000	350
A	48	600	0
B	57	16,000	0
C pre-RBC	<1	0	0
post-RBC	25	31,000	0

Functional Capacity of T-colony Cells. Surface marker analysis of the colony cells identified them as being of T-cell lineage. Although some of the original T-cells plated carried receptors for Fc-IgG, Fc-IgM, C3 and approximately 50% of the E-rosetting cells were theophylline-sensitive[11], none of the colony cells could be shown to carry these receptors (Table 7) and all the E-rosettes were theophylline-resistant. In contrast to peripheral blood, but similar to thymocytes, E-rosette formation by the colony cells was stable at 37°C[15]; unlike thymocytes the cells were negative for terminal deoxynucleotidyl transferase and the human thymus leukemia antigen, HThy-L[16].

TABLE 7
CELL SURFACE MARKER STUDIES

	% Rosettes					
	E		C3	Fc-IgM	Fc-IgG	sIg
	4°C	37°C				
Normal PBL	40-65	≤1	15-25	65-80	20-25	5-10
Colony cells	>90	>90	<1	<1	<1	<1

The T-colony cells were capable of proliferating in response to the addition of phytohemagglutin, concanavalin A and to allogeneic cells. The cells were incapable of mediating antibody dependent cytotoxicity against antibody coated M4 melanoma cells or chicken erythrocytes[17]. In addition spontaneous cytotoxicity for the K562 cell line was also absent; however, spontaneous cytotoxicity for the M4 cells was consistently demonstrated.

Using a xenogeneic (rabbit) antiserum raised against human Ia-like determinants, we have shown that approximately 40% of the theophylline-sensitive T-cell subset are Ia-positive in indirect immunofluorescence[18]. Moreover these T-cells appear to be potent stimulators of the mixed lymphocyte response (MLR). In preliminary studies, 40-80% of T-colony cells appeared to be Ia-positive by both cytotoxicity and immunofluorescence using this rabbit anti-Ia antiserum. As shown in Table 8 the T colony cells were potent stimulators in MLR as well. The degree of stimulation between autologous combinations (AA_{TX} or BB_{TX}) may reflect some carry-over of cell bound PHA (or another conditioned medium component) resulting in stimulation of autologous cells or truly represents a form of autologous MLR.

TABLE 8

MLR STIMULATORY CAPACITY OF T-COLONY CELLS

		cpm x 10^{-3} Responders	
		A	B
Stimulators	Ø	.4	.3
	A_{TX}	19.2	48.9
	B_{TX}	51.7	19.4
	A_X	.3	30.8
	B_X	33.7	1.2

X - denotes irradiation and T, colony cells

SUMMARY

The microculture system provides a reliable, simple and sensitive method for studying human T-cell colony formation. Unlike other methods[7-9], no preculture period is required and we were able to eliminate the requirement for a two layer (of agar) culture system. T-colony precursor cells appear to arise from distinct T-cell subsets that are present in peripheral blood and tonsil but not thymus or thoracic duct. Through limiting dilution analysis, we have estimated the frequency of T-colony precursor cells in peripheral blood to be in the order of 1 per 100 mononuclear cells; thus the frequency of TCPC is surprisingly low. The methylcellulose technique now provides the means for the further analysis and isolation of the T-colony precursor cell and determining its function and distribution in various disorders of T-cell immunity. The appearance of TCPC following attempted immune reconstitution may signal true functional differentiation of the T-cell compartment. The demonstration of Ia-like determinants on the T-colony cells offers the means for studying the biochemical nature and potential functional role for human T-cell Ia.

ACKNOWLEDGMENT

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Failure of Lymphocyte-Membrane HLA-A and -B Expression in Two Siblings with Combined Immunodeficiency

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A diagnosis of partial combined immunodeficiency was made in two Turkish siblings with a history of multiple pyogenic infections and persistent candidiasis. They demonstrated severe hypo-γ-globulinemia, with B-lymphocytes, but deficient plasma cell differentiation. T-Lymphocytes were decreased in number and did not respond to antigens, but did proliferate in cultures with lectins and allogeneic cells. HLA-A and -B determinants were not detected on blood lymphocytes, but they were expressed by cultured lymphoblasts, cultured fibroblasts, and were present in serum. MLR-Stimulatory capacity was intermediate and only two of six anti-HLA-DRw7 antisera demonstrated B-cell reactivity. β-2-Microglobulin (B2M) was not detected on the surface of T-lymphocytes, but was found in cross-sectioned T-cell membranes. B-lymphocytes carried B2M normally. The absence of HLA-A and -B determinants on lymphocytes of patients with similar immunodeficiency syndromes suggests a role for HLA determinants in lymphocyte differentiation.

INTRODUCTION

Primary combined immunodeficiencies in man are a heterogeneous group of disorders in which the immune response can be blocked at various stages of differentiation of T- and B-lymphocytes (1, 2). Recent investigations support the view that defective T-cell maturation leads to deficient B-cell response to antigenic stimulation (3). Animal experiments have revealed the important role of gene products of the major histocompatibility complex (MHC) in interactions between T-cells, B-cells, and macrophages (4). A role of HLA determinants in human T-cell differentiation in the thymus has been suggested recently (5). Therefore, abnormal expression of MHC products could be expected to be related to the development of immunodeficiencies. The clinical and laboratory data of two siblings with partial combined immunodeficiency and no detectable HLA antigens on their lymphocytes are reported.[2] A similar patient has been described recently (8).

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[2] Short descriptions of these patients have been presented previously by the authors (6, 7).

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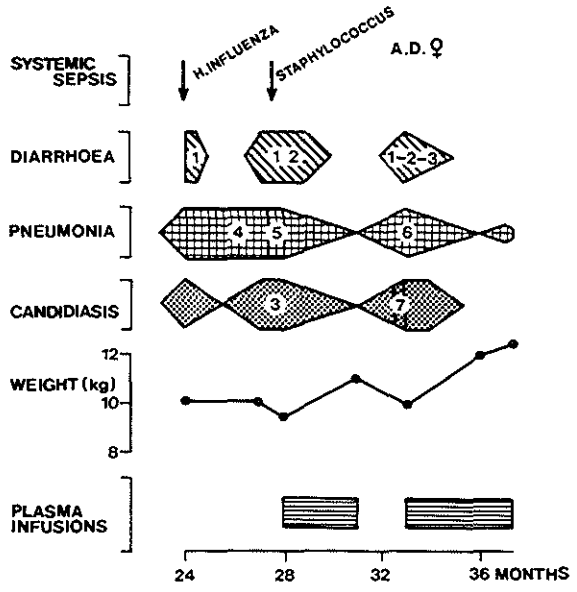


FIG. 1. Schematic representation of the clinical course of patient A.D. The arrows indicate episodes of systemic sepsis. The numbers represent the microorganisms cultured during each episode of infection: (1) *E. coli:* $>10^6$ microorganisms/ml duodenal fluid, (2) *Gardia lamblia*, (3) *Candida albicans*, (4) *S. aureus*, later on: *Klebsiella*, (5) *Pneumocystis carinii* (probably), (6) *H. influenza*, *Pseudomonas aeruginosa*, (7) Herpes simplex hominis, type II.

MATERIALS AND METHODS

Family History

The family of the two children reported here originated from an isolated village in Turkey. There were four children: the eldest, a female, died in Turkey at 4 months; the second child, a male, remains in good health; the two other children (patient A.D. and patient K.D.) will be described.

Patient A.D. This female child was admitted at 12 months of age to the Sophia Children's Hospital, Rotterdam. Gram negative septicemia (*Enterococcus*), intractable diarrhea (*Escherichia coli, Candida albicans, Giardia lamblia*), and extensive mucocutaneous candidiasis were diagnosed. At age 2 years she had several bouts of bronchopneumonia (*Staphylococcus aureus, Klebsiella, Haemophilus influenza*) and interstitial pneumonitis (Fig. 1). She had a further two episodes of septicemia (*H. influenza, S. aureus*). The symptoms were relieved only when antibiotic treatment was supported by plasma therapy. Necrotizing lesions on the nates associated with herpes simplex hominis type II occurred once. There was no evidence of systemic spread of viruses. Patient A.D. died at age 3 from a severe attack of bronchiolitis and bronchopneumonia. At necropsy normal-sized tonsils and lymph nodes were found but the thymus weighed 5 g (normal 20–30 g).

Patient K.D. The youngest child, a boy, was admitted at 5 months of age, because of relapsing pneumonia (*S. aureus, H. influenza, Klebsiella*). He also

developed recurrent diarrhea (*C. albicans*, *G. lamblia*), mucocutaneous candidiasis, and systemic sepsis (*Klebsiella*). These symptoms subsided on antibiotic therapy after ordinary plasma therapy was started. However a pneumonitis persisted. Open-lung biopsy revealed no *Pneumocystis carinii*. Patient K.D. died at age 3 due to rapidly developing bronchiolitis.

Both patients had severe growth retardation after the age of 4 months, but growth rates improved after plasma therapy was initiated. Both had slightly blue sclerae, thick black hair, and normal nails. Lymph nodes were palpable, and tonsils were present. Radiological examination showed normal thymic shadows, a normal-sized spleen, and no skeletal abnormalities.

Serological Studies

These investigations were performed on sera obtained before plasma therapy.

Immunoglobulins. Quantitation of immunoglobulins G, G 1–4, M, A, and D in serum and of IgA and secretory piece in saliva was performed by single radial immunodiffusion. Normal ranges were derived from age-related reference groups, investigated by comparable (9) or the same (10) antisera. IgA in serum was also measured by radioimmunoassay (11). IgE levels in serum were compared with a reference serum in a radioimmunoassay (12).

Complement. Various complement components were investigated in either quantitative single radial immunodiffusion (C1q, C4, C3, C5, and Factor B) or functional assays (Factor B, C2).

Lymphocytotoxic antibodies. The presence of serum antibodies against lymphocytes was assayed by indirect immunofluorescence (13), agglutination (14), and microcytotoxicity methods (15), using a panel of donor lymphocytes.

Granulocyte Functions

The function of granulocytes was measured by the uptake of immune complexes (16), oxidative metabolism (16), and intracellular killing of bacteria (17).

Immunofluorescence Studies

Cytoplasmic fluorescence on bone marrow cells and membrane fluorescence on peripheral blood lymphocytes (PBL) were performed using methods and antisera described by Vossen (18). Normal ranges were derived from age-related reference groups (18). In order to detect all sIg-positive B-lymphocytes in PBL a goat-anti-Fab-antiserum with specificity for Fab fragments of all Ig classes was labeled with fluorescein (FITC) (19).

HTLA-Human thymus-lymphocyte antigen. In order to detect human thymus-lymphocyte antigen (HTLA) on PBL and on tissue cells, respectively, the antisera described by Asma *et al.* (19) and by Brutel de la Riviere *et al.* (20) were used. The first antiserum was directly labeled with rhodamine (TRITC). When used in a double-staining method, the cells were first incubated with anti-Fab-FITC and then incubated with anti-HTLA-TRITC. In normal PBL 1% of the cells show double staining, and 2% do not stain with either antiserum. Control values were derived from age-related reference groups (19). The second HTLA-antiserum was applied to tissues in an indirect fluorescence assay. The first step, a 1:20 dilution

of the HTLA-antiserum was followed by a 1:75 dilution of swine-anti-rabbit IgG-FITC (21). Tissue fluorescence was carried out by methods previously described (21).

β-2-Microglobulin (B2M). Two anti-β-2-microglobulin antisera were used. One, kindly provided by Dr. Radl, REPGO-TNO, Ryswyk, The Netherlands, was prepared by immunizing a rabbit with B2M isolated from human urine. This antiserum had the following specificities in membrane fluorescence assays: normal PBL—100% of the cells positive; acute lymphoblastic leukemia cells—100% positive; erythroid cells—negative; epithelial cells of a bladder tumor—negative; mouse spleen lymphocytes—negative. This antiserum was labeled with TRITC and used in 1:100 dilution in the membrane fluorescence as well as tissue fluorescence studies.

When used in a double-staining technique, the cells were first incubated with anti-B2M-TRITC and then with anti-Fab-FITC. The number of double-marked cells was counted and expressed as a percentage of the B2M-positive cells. When the degree of staining by the fluorescent anti-B2M serum was compared, the same numbers of patient's cells and normal PBL, incubated with the same amount of antiserum, were used in parallel. This anti-B2M serum was also radiolabeled with ^{125}I to measure the difference in B2M expression on patient's lymphocytes and control PBL. The second anti-B2M serum, obtained from Dakopatts, Denmark (Batch 10-472), was applied in an indirect fluorescence technique on tissues in a dilution of 1:100, followed by a 1:120 dilution of swine anti-rabbit IgG-FITC.

Cross-section of blood lymphocytes. In order to be able to visualize the inner side of membranes of peripheral blood T-lymphocytes, PBL were immersed in gelatin, snap-frozen in liquid nitrogen, and cut into 4-μm cryostat sections (22). This cross-section of PBL was compared with PBL in suspension, using both anti-B2M antisera in parallel.

Cell Membrane Markers Detected by Rosette Techniques

Other cell membrane markers were investigated using sheep red blood cells (E rosettes), human D^+ red blood cells sensitized with anti-D serum (EA rosettes) (23), and sensitized sheep red blood cells coated with mouse complement (EAC rosettes) (24).

Cell-Mediated Immunity

Skin tests. In vivo cell-mediated immunity was investigated by a dinitrochlorobenzene (DNCB) patch test using various amounts of DNCB (1-, 3-, 10-, and 30-μg DNCB) 3 weeks after sensitization, and by intracutaneous tests with PPD, *Candida* antigen, mumps antigen, *Trichophyton*, and streptokinase–streptodornase (SK-SD).

Lymphocyte transformation tests. In vitro proliferation of lymphocytes in response to stimulation with lectins, anti-lymphocyte serum (ALS), allogeneic cells (MLR), and Candida antigen was assayed as described by Schweitzer *et al.* (25) and DuBois *et al.* (26). Cultures in triplicate were performed in microtiter plates. The lectins used were: phytohemagglutinin (PHA), Pokeweed mitogen (PWM), and concanavalin A (Con A). Cells were pulsed 16 hr before harvesting with 0.4 μCi [^3H]thymidine (Radiochemical Center, Amersham). Results were expressed

in counts per minute (cpm) thymidine incorporation per 4×10^4 lymphocytes. In the MLR, stimulator cells were either treated with mitomycin or irradiated (2500 rad). Control values were derived from the response of PBL of two healthy donors cultured in parallel. The *in vitro* response of patient K.D.'s PBL to stimulation with diphtheria toxin and tetanus toxin was investigated 3 weeks after a booster injection with these antigens.

Antibody-dependent cytotoxicity (ADC). The killing capacity of patients' PBL was assayed using sensitized target cells derived from a melanoma cell line (24).

Cell-mediated lympholysis (CML). The *in vitro* development of cytolytic capacity was investigated in a cell-mediated lympholysis assay (CML) (27). PBL of donor X were first stimulated by irradiated PBL of donor Y in a mixed lymphocyte culture. In parallel, PBL of donor Y were transformed to lymphoblasts by culture in the presence of PHA. The cytotoxic capacity of stimulated X cells was then assayed using ^{51}Cr-labeled Y-lymphoblasts as targets (27). This CML assay was used in two ways: first, to measure the cytotoxic capacity of stimulated K.D. lymphocytes against nonsensitized unrelated donor lymphoblasts, and second, to investigate whether unrelated donor lymphocytes were able to lyse patient K.D.'s lymphoblasts. Thus, because HLA-A and -B determinants on the target cells have been shown to play a role in the lytic phase of the CML (27), this second mode of the CML was used to detect HLA antigens on patients' lymphoblasts. The lysis of target cells was measured and calculated according to the formula:

$$\frac{\text{cpm release in experiment} - \text{cpm spontaneous } ^{51}\text{Cr release}}{\text{cpm maximum release} - \text{cpm spontaneous release}} \times 100.$$

The cpm represented the release of ^{51}Cr from 10^4 target cells (27).

PWM induced B-cell maturation. In vitro differentiation capacity of patients' PBL was investigated in a PWM induced T-cell dependent B-cell maturation assay (28). After 10 days culture with PWM the cultured cells were spun in a cytocentrifuge and fixed and stained with fluorescinated anti-immunoglobulin sera. The numbers of positive staining cells were enumerated by counting 10^3 blasts cells. Control values were derived from experiments on control PBL, run in parallel.

Immunogenetic Studies

Techniques used to detect HLA determinants. HLA-A, -B, and -C determinants were investigated by the standard typing technique with the NIH lymphocytotoxicity method using 120 different HLA antisera (15), and by an agglutination assay in EDTA blood performed with 30 different HLA antisera (14). The standard cytotoxicity method was also applied to E-rosette depleted B-cell enriched PBL and to PBL which had first been incubated for 24 hr in tissue culture medium supplemented with 20% pooled human serum. The HLA phenotypes of the parents and the plasma donors were determined by the standard HLA typing technique. Because every patient had one plasma donor only, the following assays were performed using antisera against HLA determinants, predicted from the phenotypes of the parents or donors.

HLA determinants on platelets. HLA determinants on platelets were investi-

gated by a platelet complement fixation test (29) and a platelet inhibition assay. In the last assay platelets of both patients were used to adsorb HLA antisera, after which the adsorbed antisera were investigated in the standard lymphocytotoxicity test against a pool of donor lymphocytes (30).

HLA determinants on lymphoblasts. Patients' lymphocytes were transformed to lymphoblasts by 3-day culture in the presence of PHA, after which the lymphoblasts were labeled with ^{51}Cr. These target cells were incubated with HLA antisera and then with normal PBL effector cells, after which the ^{51}Cr release from the target cells was measured (31).

HLA antigens in serum. The presence of HLA antigens in serum was investigated by using radiolabeled HLA antisera in an adsorption–inhibition assay with a panel of typed donor lymphocytes (32).

HLA-determinants on fibroblasts. Fibroblasts of both patients were cultured for 6 weeks and assayed by a selected panel of HLA antisera, used in an indirect immunofluorescence assay (13), a cytotoxicity method using fluorochromatic labeled fibroblasts (33), and a ^{51}Cr release assay (34).

B-Cell allotypic determinants. Enriched B-cell suspensions were tested with 45 antisera against B-cell allotypic determinants (DR-determinants) in an indirect fluorescence method (13).

Additional Assays

Thymus-dependent serum factor (SF) was assayed by methods described by Astaldi *et al.* (35). Karyograms were made according to routine techniques, including the Reverse, Atebrine, and Giemsa banding assays.

RESULTS

Immunological Investigations

Immunoglobulins, Antibodies, and Complement Components

Severe hypo-γ-globulinemia was diagnosed in both patients (Table 1). All immunoglobulin classes and subclasses were below the normal level for age except for a normal level of IgM in the serum of patient A.D. In saliva no IgA could be found whereas a normal amount of secretory piece was present. No titers of isohemagglutinins, nor antibodies against a variety of bacteria, parasites, fungi, viruses, or various tissues were found. No antibodies against allogeneic lymphocytes were detected. Complement components (C2, C4, C3, Factor B, C5) showed normal levels with exception of slightly lowered levels of C1q (60% of normal).

Cellular Immunity

Hematology. In each patient leukocyte counts were about half the normal age-related value. Absolute lymphocyte counts varied between 1.0 and 3.0×10^9 per liter (age-matched controls: $5.0–7.0 \times 10^9$ per liter). Neutrophils were abnormal in size and morphology, suggesting disturbed myeloid cell maturation (36). Functional assays of granulocytes revealed normal values. Absolute monocyte counts were slightly decreased, whereas numbers of eosinophils were consistently increased.

Plasma cells and B-lymphocytes. Bone marrow biopsies showed only few plasma cells. These were shown to contain IgM mainly (Table 2). Only patient

TABLE 1
IMMUNOGLOBULINS (mg/100 ml)

Ig-(Sub)class[a]	Patient A.D. ♀	Normal range	Patient K.D. ♂	Normal range
G	<60	350–1140	<40	140–930
G1	50	350–1180	—[b]	100–850
G2	<15	75–210	—	60–200
G3	12	15–145	6	5–160
G4	<2.5	<2.5–90	—	<2.5–90
A	0.11	13–100	0.13	4–80
Sc-A	<0.5	>30	—	>20
Sc-piece	6.4	4–20	—	2–25
M	80	40–230	4	20–120
D	<0.7	0–3	<0.7	0–2.5
E (IU/ml)	<1	10–500	<1	10–500

[a] Sc-A and Sc-piece, secretory IgA and secretory piece.
[b] Not determined.

A.D. had normal numbers of IgM-containing cells. The IgM cells were plasma cells, morphologically and by fluorescent staining. In PBL suspensions of both children, B-lymphocytes, bearing Ig of various classes were detected (Table 2). With an anti-Fab-antiserum 40% of PBL were shown to be B-lymphocytes (see also Table 9). The relative high percentages of B-lymphocytes were shown to reflect normal absolute numbers when corrected for the lymphopenia. Slightly elevated percentages of Fc-bearing lymphocytes were detected by EA-rosette assay: 31 ± 4% (controls 26 ± 3%), whereas complement receptor-bearing lym-

TABLE 2
CYTOPLASMIC IMMUNOGLOBULINS IN BONE MARROW CELLS

IgCC[a]	Patient A.D. ♀	Normal range	Patient K.D. ♂	Normal range
G	10	35–268	0	33–151
A	0	49–348	0	10–163
M	226	34–203	7	16–272
D	3	0–20	0	0–27

SURFACE IMMUNOGLOBULINS ON BLOOD LYMPHOCYTES

IgBC[b]	Patient A.D. ♀	Normal range	Patient K.D. ♂	Normal range
G	6	1–10	7	0–25
A	0	0–2	1	0–4
M	22	7–22	18	13–31
D	33	8–31	22	15–41

[a] IgCC, immunoglobulin-containing lymphocytes and plasma cells; numbers are expressed per 10^5 lymphocytes and plasma cells.
[b] IgBC, immunoglobulin-bearing cells; expressed in numbers of Ig-positive cells per 100 lymphocytes.

phocytes were slightly decreased: 16 ± 10% (controls 23 ± 4%). T-Lymphocyte percentages were 50–60% (control 70–90%) when investigated by E-rosette and anti-HTLA fluorescence assays (see also Table 9). This implied that absolute T-lymphocyte numbers were about one-third of the normal values for age.

Histopathology of the Immune System

Thymus. Thymic biopsy of patient K.D. showed decreased numbers of Hassall's corpuscles and of cortical lymphocytes. The architecture of cortex and medulla was essentially normal. By fluorescence the thymic cortex contained few HTLA-positive cells (Table 3). Postmortem histology of A.D.'s thymus showed atrophy with Hassall's corpuscles. The cortex was depleted of lymphocytes with few HTLA-positive cells. Thus, both children had a lymphocyte-depleted but essentially normal thymus. The atrophy of A.D.'s thymus appeared to be caused by agonal stress.

Lymph nodes. Lymph nodes of both patients, excised shortly after severe infection, had normal weight and architecture. There were few follicles and no germinal centers. Sporadic plasma cells, scattered through the medulla and through rare follicles, contained only IgM and IgD. The paracortical areas were depleted of lymphocytes, but showed some clusters of HTLA-positive cells.

Spleen. Splenic tissue, obtained at necropsy of patient A.D., showed underdeveloped periarteriolar lymphocyte sheaths containing HTLA-positive cells. Clusters of lymphocytes, staining positively for κ- and λ-chains, appeared to be organized follicles. These scarce follicles, without germinal-center reaction, contained some plasma cells, positive for IgM and IgD only.

Intestinal wall. There were few Peyers' patches in both patients. The intestinal wall contained scarce follicles with no center reactions. Plasma cells, containing IgM and IgD only, were occasionally found in these follicles and throughout the intestinal wall.

TABLE 3
IMMUNOHISTOPATHOLOGY

	Immunoglobulin-containing lymphocytes and plasma cells[a]					
	Thymus	Spleen A.D. ♀	Lymph nodes A.D. ♀	Lymph nodes K.D. ♂	Intestinal wall	Lung A.D. ♀
IgG	–	(+)	–	–	–	(+)
IgA	–	–	–	–	–	–
IgM	–	+	+	(+)	+	+
IgD	–	+	+		+	+
κ and λ	–	+	+	(+)	+	+
	Membrane pattern of immunofluorescence					
HTLA	+	+	+	+	+	+
B2M	+	+	+	+	+	

[a] Numbers of immunoglobulin-containing cells and plasma cells were classified as follows: –, negative, no positive staining cells found; (+), dubious, only a few cells found; +, positive, but lower numbers of cells than normal; ++, positive, normal numbers of cells. Membrane pattern of immunofluorescence: lymphoid cells with a ring of fluorescence around the cells.

TABLE 4
LYMPHOCYTE TRANSFORMATION TESTS[a]

	Patient A.D. ♀	Patient K.D. ♂	Control
PHA	27.4 (2.7)	16.7 (1.8)	24.3 (3.6)
PWM	10.6 (1.1)	6.4 (1.1)	7.1 (1.1)
Con A	7.9 (1.5)	7.8 (1.6)	8.0 (1.2)
ALS	18.3 (1.2)	16.9 (2.0)	17.8 (1.9)
Ø[b]	0.2 (0.1)	0.2 (0.1)	0.2 (0.1)
MLR-R[c]	11.2 (1.5)	9.9 (1.4)	12.4 (1.4)
Candida	0.4 (0.3)	0.2 (0.2)	3.5 (2.6)
Diphtheria		0.3	5.6
Tetanus		0.2	7.2

[a] Mean and (standard deviation) of at least four separate experiments.
[b] Ø, No mitogen added.
[c] MLR-R, MLR of patients' lymphocytes in response to allogeneic lymphocytes.

Lung. Although patient A.D. had severe recurrent lung infections, she had only a few plasma cells, most containing IgM and few IgG.

Cell-Mediated Immunity in Vivo

Skin tests with DNCB were dubious in both children when challenged with 30 μg DNCB only. Negative intracutaneous skin tests were obtained with a variety of antigens including *Candida* antigen.

Lymphocyte Functions in Vitro

Lymphocyte transformation tests. Lymphocyte proliferation was normal when stimulated by lectins, ALS, and allogeneic cells (Table 4). Although both patients had extensive mucocutaneous candidiasis, their lymphocytes did not proliferate in culture with *Candida* antigen. A booster injection of diphtheria and tetanus was not followed by an *in vitro* lymphocyte response to these antigens.

Cytolytic capacity in vitro. Patients' PBL showed normal cytolytic activity against sensitized target cells (ADC). In the CML assay K.D.'s PBL were capable of developing cytotoxicity against unrelated donor lymphoblasts (Table 5) ($P + X_R \rightarrow X$).

T-Cell dependent B-cell differentiation in vitro. PWM induced B-cell maturation was almost absent, although a few lymphoblasts were shown to contain IgM (Table 6). Low numbers of Ig-containing cells did not exceed 15% of the number of control PBL.

Immunogenetic Studies

HLA-A, -B, and -C Determinants

Lymphocytes. On lymphocytes of both patients no HLA-A, -B, and -C determinants could be detected with all HLA antisera available using the standard typing technique and an agglutination assay (Table 7). The standard typing technique was also applied to patients' PBL, after 24 hr culture in medium, to exclude covering of HLA determinants by (auto)antibodies, and to enriched B-lymphocyte suspensions, with negative results.

TABLE 5
CELL-MEDIATED LYMPHOLYSIS[a]

Effector[b]	Target	Ratio		
		1/12.5	1/6.25	1/3.12
$P + X_R$	P	1.1	1.3	
	X	11.1	2.6	
$X + R_R$	P	11.0	6.1	4.1
	X	3.1	1.9	0.6
$X + Y_R$	Y	17.4	12.1	
	X	4.0	2.3	

[a] HLA phenotypes = donor X: A3, W19, B7, W17; donor Y: A2, 11, BW40, 15.
[b] P, patient K.D., X and Y, unrelated donors. P_R, X_R, Y_R: Irradiated stimulator cells in the MLR phase of the CML assay.

Further assays were performed selectively for HLA determinants predicted from the phenotypes of the parents and plasma donors (Table 8).

Platelets. Platelets of both patients were shown to be negative for HLA determinants, in a platelet complement fixation test. However, by the more sensitive platelet adsorbtion inhibition assay some HLA antigens were shown to be present but the amount of HLA antigens detected in this way appeared low.

Cultured lymphoblasts. When patients' lymphocytes were first transformed to lymphoblasts and then investigated by HLA antisera in a lymphocyte-dependent cytotoxicity assay, the lymphoblasts were shown to express some HLA determinants. This assay was not reliable, because the possibility of false positive reactions due to extra antibodies in the reagents used could not be excluded. However, support for the expression of HLA determinants on transformed patients' lymphoblasts came from the CML studies: MLR-Stimulated unrelated donor lymphocytes were shown to develop cytotoxicity against lymphoblasts of patient K.D. (Table 5) ($X + P_R \rightarrow P$).

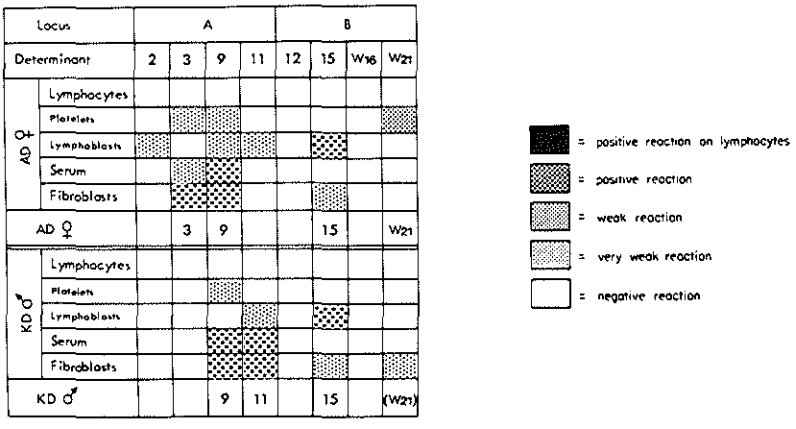
Serum. In contrast to the cell membrane studies, the patients' sera contained HLA antigens. Only HLA-A locus derived antigens were detected. Because the patients were on plasma therapy at that time, the HLA-A3 and A9 antigens in patient A.D.'s serum could theoretically have been derived from her plasma donor. However, donor antigens were not detected in K.D.'s serum (e.g., A2 in K.D.). Furthermore, when plasma transfusions were interrupted for 3 weeks the

TABLE 6
PWM INDUCED B-CELL MATURATION[a]

	Patient A.D.	Patient K.D.	Control	
	+PWM	+PWM	+PWM	−PWM
Ig[b]	15	15	125	<5
IgM		5	25	
IgA	2	<5	45	

[a] Values are expressed in numbers of Ig-containing cells per 10^3 blast cells.
[b] Ig, immunoglobulins, all classes.

TABLE 7
ATTEMPTS TO DETECT HLA ANTIGENS[a]



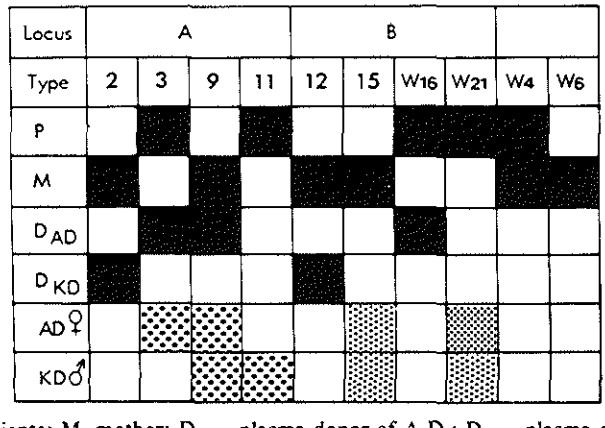
[a] *Lymphocytes:* Standard HLA typing on lymphocytes, on enriched B-cell suspensions, and on PBL after 24 hr incubation in culture medium and typing by an EDTA agglutination assay. *Platelets:* Platelet complement fixation and platelet inhibition assays. *Lymphoblasts:* PHA-induced lymphoblasts investigated by an antibody-dependent cytotoxicity method. *Serum:* HLA antigens in serum, detected by radioimmunoassay. *Fibroblasts:* Indirect immunofluorescence, and fluorochromatic-labeled or ^{51}Cr-labeled target cell release in a cytotoxicity assay.

HLA-A9 and A-11 persisted in K.D.'s serum. These determinants could not have been derived from the donor.

Fibroblasts. Three methods were used to investigate HLA antigens on cultured fibroblasts. Only the HLA-A antigens were detected. For the HLA-B antigens some weak reactions were demonstrated.

These studies made it possible to draw tentative HLA-A and -B genotypes

TABLE 8
HLA-A AND -B DETERMINANTS ON LYMPHOCYTES OF THE PARENTS AND THE PLASMA DONORS[a]



[a] P, father of patients; M, mother; $D_{A.D.}$, plasma donor of A.D.; $D_{K.D.}$, plasma donor of K.D. See Table 7 for explanation of symbols.

(Tables 7 and 8). It was concluded from these data that the children inherited the same haplotype from the mother (A9-B15) but different haplotypes from the father. The HLA-B antigens from the father were not reliably determined. The occurrence of BW21 in both patients could be explained by assuming either the existence of a paternal recombination or technical error.

HLA-D and HLA-DR (Allotypic B-cell) Determinants

D-Determinants. In one-way MLR with unrelated donor lymphocytes, the stimulating capacities of patients' lymphocytes were positive but moderate (40–60% of normal levels).

DR-Determinants. Two of six anti-DRw7 sera were positive with enriched B-cell suspensions of the patients. The other anti-DRw7 sera showed weak or negative reactions. The majority of the other anti-B-cell sera was negative. Removal of the monocytes by iron depletion did not alter these results.

B-2-Microglobulin

Peripheral blood cells. By immunofluorescence B2M was detected on only 40% of patient K.D.'s PBL and these cells were identified as B-cells (Fab-positive) by a double-staining technique (Table 9). The density of the fluorescent staining for B2M on B-cells was reduced. Likewise patients' monocytes, identified by phase contrast microscope, showed reduced fluorescence for B2M. The reaction of radiolabeled anti-B2M serum with mononuclear cells was half of normal. Thrombocytes did not show B2M staining. Cultured fibroblasts were positive. Of PBL, 50–60% were not stained by the anti-B2M sera. Because 95% of B2M-staining cells in PBL were shown to be B-lymphocytes (Fab-positive) it was concluded that T-lymphocytes did not have B2M on their membranes.

TABLE 9

LYMPHOCYTE (SUB)POPULATIONS AND OTHER CELLS WITH SURFACE β-2-MICROGLOBULIN[a]

	Antiserum	Patient K.D. ♂	Control Mean (ranges)
Peripheral blood			
Mononuclear cells	B2M	36–46[b]	100
Lymphocytes	Fab	41	17 (10–26)
	HTLA	54	81 (68–89)
Fab-positive lymphocytes	HTLA	0[c]	0[c]
B2M-positive lymphocytes	Fab	95[d]	17 (10–26)[d]
		Density of B2M[e]	
B2M-positive lymphocytes	B2M	+	++
Monocytes	B2M	+	++
Thrombocytes	B2M	−	++
Cultured fibroblasts	B2M	+	++

[a] Values expressed as numbers of positive cells/100 cells in isolated cell suspensions.
[b] Results of two investigations.
[c] Percentage of Fab-positive lymphocytes, which also stained with α-HTLA.
[d] Percentage of B2M positive cells, also staining with α-Fab.
[e] ++, Normal density per cell: +, lowered density per cell; −, no reaction.

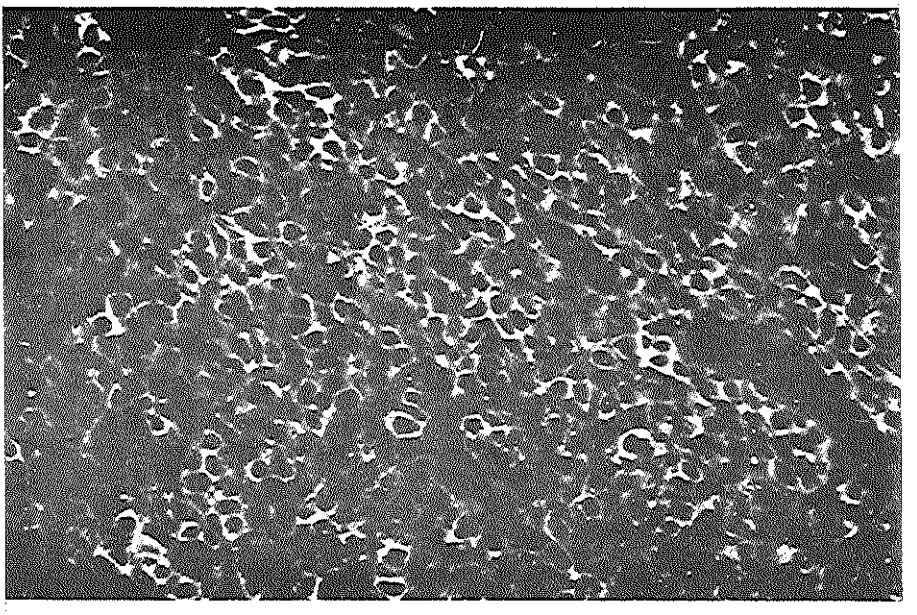


FIG. 2. Paracortical area of an inguinal lymph node of patient K.D. stained with a fluoresceinated anti-β-2-microglobulin serum. ×160.

Immunohistopathology of B2M. With the use of two different anti-B2M antisera, a clearly positive honeycomb pattern of fluorescence was seen in the thymus, spleen, intestinal wall, and lymph nodes (Fig. 2 and Table 3). Compared with tissues from other patients, the intensity of the fluorescent staining per cell appeared to be normal. Lymphoid cells were positive for B2M, as were epithelial cells and to a lesser degree endothelial cells. T-cells in the thymus, spleen, and lymph nodes were shown to carry B2M.

B2M on T-lymphocytes. There was a clear discrepancy between B2M staining on T-lymphocytes whether in tissues or in cell suspensions; the former were positive, while the latter were negative. When patient K.D.'s PBL suspensions were immersed in gelatin and the cells were further processed as tissue, all the mononuclear cells stained positive for B2M. Viable cell suspensions of K.D.'s PBL investigated in parallel showed only 40% of the cells positive (Table 9). Furthermore, the lower density of B2M on PBL membranes was not seen when PBL were processed as tissue specimen. It was concluded that T-lymphocytes either failed to express B2M on their membranes or lost B2M as soon as it appeared at the cell surface. At the same time T-lymphocytes contained B2M in or at the inner side of the membrane, only visible when cells were cross-sectioned.

Additional Data

Thymus-dependent serum factor (SF) was hardly detectable (1.5–2.7 pmol cAMP per 10^7 thymocytes; normal values 35 ± 10 pmol) (34). The erythrocytes of both patients had high levels of the enzymes adenosine deaminase and purine

nucleoside phosphorylase. Both children had normal karyograms. Chimerism in K.D.'s peripheral blood was excluded by investigation of 10^3 blasts. Glyoxalase was detected (phenotype 2-1) as well as phosphoglucomutase-3. Patient K.D.'s serum inhibited three anti-Chido and two anti-Rogers sera, which implied that he possessed these blood group antigens.

DISCUSSION

Two Turkish siblings with a history of multiple pyogenic infections and persistent candidiasis had a severe hypo-γ-globulinemia. One patient (A.D.) produced IgM in sufficient amounts, the other (K.D.) had a low level. This was reflected in the numbers of IgM plasma cells found in bone marrow biopsies. Despite the presence of B-lymphocytes carrying immunoglobulins of all classes, both patients showed a failure of B-cell differentiation to IgG- and IgA- containing cells. This might indicate a disturbance in the switch from IgM to IgG and IgA production (2). This particular B-cell differentiation phase is considered to be induced by antigenic stimulation and to be mediated by T-cells (3). There was no defect in the early stages of T-cell differentiation (stem cell → T-precursor → thymus-lymphocyte) as can be concluded from positive *in vitro* proliferative responses to lectins and the relatively normal percentages of E-rosette-forming cells and $HTLA^+$ cells. The grossly normal thymus histology also excluded severe T-cell defects. The absence of thymus-derived serum factor might indicate deficient maturation of T-cells from the intrathymic stage on. The low absolute numbers of T-lymphocytes also indicated insufficient maturation. Furthermore, defective T-cell function was shown by the lack of lymphocyte proliferation after antigenic stimulation. Lack of T-cell dependent B-cell maturation, induced by PWM stimulation, does not distinguish between a T-cell function defect or an intrinsic B-cell defect.

The final diagnosis was partial combined immunodeficiency, characterized by severe hypo-γ-globulinemia with B-cells present and partial T-cell deficiency, mainly expressed at the level of post-thymic functions. Immunogenetic studies revealed a lack of HLA-A and -B determinants on lymphocytes. A structural genetic defect in the HLA region on chromosome 6 was unlikely for the following reasons: Gross defects in chromosomes 6 in the area of the HLA loci were excluded by normal karyograms and by the presence of various gene products coded in that area (complement components: C2, C4, Factor B; enzymes: phosphoglucomutase-3, glyoxalase; and blood groups: Chido, Rogers); selective defects within the HLA region appeared to be excluded by the presence of HLA-A and -B determinants on platelets, on lymphoblasts, and especially in serum and on cultured fibroblasts. The studies on lymphoblasts were hampered by technical difficulties. The lymphocyte-dependent cytotoxicity assay was unreliable due to the possibility of additional specificities in the HLA antisera used. The CML assay, especially the lytic phase in this assay, is supposed to be mediated by HLA-A and -B determinants on the target lymphoblasts but does not exclude a role for other target cell determinants. However, a lymphoblast cell line derived from patient K.D.'s blood lymphocytes also expressed the predicted HLA-A and -B determinants (37). The studies on the D- and DR-determinants were inconclusive. The intermediate stimulatory capacity did not prove the presence of the

D-locus determinants, because theoretically this might have been mediated by determinants coded by minor D-loci within or outside the HLA region. Because only two of six well-characterized HLA-DRw7 antisera showed clearly positive reactions, it could not be concluded that patients had the HLA-DRw7 determinant. In conclusion, the immunogenetic studies showed a lack of HLA-A and -B determinants on lymphocytes, which appeared not to be caused by a structural gene defect.

Lack of HLA-A and -B determinants on lymphocytes has been described in two other cases. One was a patient with Hodgkin's disease, in which the "loss" of HLA determinants was transient (38). The other, an Algerian infant, was strikingly similar to the Turkish siblings (8). He had partial combined immunodeficiency, characterized by severe antibody deficiency, with B-lymphocytes, normal mitogenic response of lymphocytes, and a low absolute T-cell number. There was an absence of HLA-A and -B antigens on lymphocytes but they were present in serum. The parents were consanguineous. HLA typing data of the family showed one healthy sibling with the same genotype as the patient, which excluded linkage of either the immunodeficiency or the lack of HLA determinants with a particular HLA genotype.

Although there was no known parental consanguinity in the Turkish family, the coincidence of two siblings of different sex in one family from an isolated Turkish village, argues for an autosomal recessive genetic trait.

The association of lack of HLA determinants with similar lymphocyte differentiation defects in patients from two separate families suggests a causal relationship between HLA determinants expression and normal immune function. In view of the existing knowledge (4, 5) it is tempting to speculate that an autosomal genetic trait leads to an HLA determinants expression defect, which in its turn leads to a lymphocyte differentiation disorder.

The studies of B2M may indicate how the genetic defect is exposed by the absence of HLA-A and -B determinants. B2M is required for the expression of HLA-A and -B determinants on the surface of lymphocytes, as has been shown by hybridization experiments with Daudi cells (39). The finding of B2M without detectable HLA determinants on patients' B-lymphocytes argues against a defect in HLA antigen expression by mere absence of B2M. However, the fact remains that T-cells did not express B2M on their surface, although they appeared to contain B2M in the membrane. This might be explained by postulating either an immunologically undetectable structural defect in B2M or a membrane defect, in both cases leading to defective "anchorage" of B2M on the T-cell membrane. This anchorage defect would then cause rapid loss of B2M from the membrane and defective fixation of HLA determinants, which is normally accomplished in cooperation with B2M (40). The presence of B2M in patients' sera suggested that B2M reached the surface but actually did not imbed in the lymphocyte membrane. Although B-cells appeared to express reduced amounts of B2M on their surface, it is difficult to explain why there was a difference between B- and T-cells other than by assuming that there is a difference in B2M anchorage and/or B2M-mediated HLA determinant fixation on B- and T-cells. Also cell membrane turnover might be different. That cell turnover disorders can lead to loss of HLA determinants, has been shown in fibroblast cultures from patients with progeria (41). Normal

expression of other cell membrane markers such as Ig on B-lymphocytes, E-receptors on T-lymphocytes, and the Fc and complement receptors suggests that any membrane defect, if present, is selective.

In conclusion, the described association of a partial combined immunodeficiency, absence of HLA-A and -B determinants on lymphocytes, and a loss of B2M from T-lymphocytes, indicates an interrelationship between lymphocyte differentiation and HLA determinants and B2M on lymphocyte membranes.

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ABNORMAL LYMPHOCYTE CAPPING IN A PATIENT WITH SEVERE COMBINED IMMUNODEFICIENCY DISEASE

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Abstract Despite the presence of normal numbers and distribution of T and B lymphocytes and normal levels of serum immunoglobulins, a five-month-old infant failed to show any evidence of T-cell or B-cell immunity. In trying to identify a specific membrane abnormality as a potential cause of the immunologic dysfunction, we examined the lateral mobility of the cell-surface receptor for concanavalin A. In contrast to normal cells, in which the receptor is distributed uniformly over the cell surface, the patient's lymphocytes showed an unusually high accumulation of concanavalin A receptors in surface caps. This capping abnormality appeared in both T and B lymphocytes and was exaggerated by colchicine, an inhibitor of microtubule assembly. These findings support the theory that plasma-membrane–cytoskeleton interactions have a role in the expression of specific immunity; the findings also identify new areas that should be considered in trying to understand the primary immunodeficiency diseases. (N Engl J Med 301:1245-1249, 1979)

SEVERE combined immune deficiency (SCID) is a heterogeneous and uniformly fatal disorder characterized by the failure of specific humoral and cellular immunity to develop.[1-4] With rare exception,[5,6] it presents with a marked impairment in the expansion of cells of T lineage; normal numbers of peripheral blood lymphocytes and, indeed, elevated numbers of B lymphocytes often prevail, despite the uniform presence of profound hypogammaglobulinemia. Thus, the combined deficiency often reflects the failure of T-precursor cells to mature; they show arrests at various stages of development, including a primary failure of function of thymic epithelial cells.[3,4]

We report on an infant with an unusual, inherited form of SCID in the presence of normal numbers of T and B cells. These cells had surface receptors that were indistinguishable from normal; after interaction with a number of ligands, however, there was no T-cell proliferation. In trying to find a specific membrane abnormality that resulted in failure to transmit or translate triggering signals from the surface membrane to the nucleus, we examined the lateral mobility of a surface ligand, concanavalin A (con A),[7] in the membranes of the patient's lymphocytes. Under normal conditions, con A binds to uniformly distributed receptors and maintains a uniform surface distribution during short periods of incubation at 37°C.[8] The patient's lymphocytes showed an unusually high accumulation of con A receptors in surface caps during brief incubation with fluorescein-con A. This capping abnormality was exaggerated by exposure of the cells to colchicine, which inhibits microtubule assembly. Our findings support the theory that the lymphocyte cytoskeleton has a role in the expression of normal immune responsiveness, and reveal new clues to the pathogenesis of primary, inherited disorders of the immune system.

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CASE REPORT

A five-month-old boy, the secondborn of a consanguineous marriage, was referred to the Hospital for Sick Children with suspected immunodeficiency, because a brother had died at the age of three years from parainfluenza Type 3 pneumonitis after a lifelong history of infections. Laboratory evaluation of the brother had revealed an absence of lymphocyte-proliferative response to lectins and allogeneic cells; he had normal immunoglobulin levels but weak, if any, antibody responses.

On admission the patient was well developed, well nourished, and at the 60th percentile for height and 75th percentile for weight. There were no abnormal findings other than a diffuse, scaly, macular rash and a generalized paucity of lymphoid tissues. Results of routine laboratory tests were entirely normal. Levels of adenosine deaminase and nucleoside phosphorylase in the red cells (kindly assayed by Dr. E. Giblett, Seattle, WA) were normal.

When the patient was seven months old, after the studies described here were completed and the diagnosis of combined immunodeficiency was established, he received a bone-marrow trans-

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plant from his father who was HLA-D compatible. Five days after the transplantation, fever, diarrhea, and a morbilliform skin rash developed in the infant, indicating acute graft-versus-host disease. He died four weeks later with marked hyperbilirubinemia, skin lesions resembling toxic epidermal necrolysis, and total marrow aplasia.

Methods

Cell Suspensions

Buffy-coat leukocytes were obtained from heparinized blood and suspended in modified Hanks' medium. The leukocyte suspensions contained approximately 70 to 80 per cent polymorphonuclear cells and 20 to 30 per cent lymphocytes and monocytes. Mononuclear-cell suspensions from peripheral blood or bone marrow were obtained after Isopaque-Ficoll gradient centrifugation.[1] These cell suspensions contained 70 to 85 per cent lymphocytes, 15 to 20 per cent monocytes, and less than 5 per cent granulocytes.

Immunologic Evaluation

Serum immunoglobulins, isohemagglutinins, tetanus, diphtheria, and polio (Types 1, 2, and 3) antibody titers were obtained with standard techniques.

Delayed cutaneous reactivity was assessed with *Candida albicans*, streptokinase-streptodornase, dermatophyton, diphtheria-tetanus, and phytohemagglutinin. Lymphocyte proliferation in response to phytohemagglutinin, con A, pokeweed mitogen, and allogeneic cells was determined as previously described.[2] Cultures containing formalinized *Staphylococcus aureus*, Cowan strain I, were grown in 10 per cent fetal calf serum (Schuurman RKB, Gelfand EW, Dosch H-M: unpublished data).

Confluent monolayer cultures of human thymic epithelium, supernates of these cultures (human-thymus conditioned medium), thymosin fraction V (kindly provided by Dr. C. Joseph, Nutley, NJ), and theophylline were used in studies of the induction of T-cell differentiation.[4]

Identification of lymphocytes bearing receptors for sheep erythrocytes, the third component of complement, the Fc fragment of IgG, or surface immunoglobulin was performed as previously described.[1,5,10] The number of rosette-forming or immunoglobulin-bearing cells was expressed as a percentage of total mononuclear cells. The presence of HLA-like and Ia-like determinants was assessed by complement-dependent cytotoxicity with monospecific and oligospecific alloantiserums (kindly performed by Mrs. J. Falk, Toronto, Ontario). Ia and β2-microglobulin antigens were also detected by means of indirect immunofluorescence with xenoantiserums.

Studies of Cap Formation by Fluorescence Microscopy

Buffy-coat cells ($\approx 10^6$ leukocytes per milliliter) were labeled with rhodamine goat antihuman IgM (Cappel Laboratories, Cochranville, PA) for two minutes at 37°C, and then labeled with fluorescein-con A (30 μg per milliliter) for 10 minutes at 37°C.[7] The cells were then washed and fixed with 2 per cent paraformaldehyde for 10 minutes. The nuclei were labeled with 2 μg per milliliter of Hoechst 33258 as previously described.[11] The Hoechst fluorochrome was employed for the observation of nuclear morphology and identification of lymphocytes in mixed populations of cells (Fig. 1c); the anti-IgM reagent enabled us to categorize lymphocytes as B or T cells (Fig. 1b). The surface distribution of fluorescein con A was categorized as uniform when fluorescein was distributed over the whole surface in either a diffuse pattern or, less frequently, in surface patches. Capped cells showed concentration of fluorescein exclusively at the pole of the cell. The typical distributions are shown in Figure 1a. Small monocytes were identified by their tendency to internalize the con A. The sequence in which the three labels (Hoechst, fluorescein con A, and anti-IgM) were examined ensured that T and B cells were observed at random. Preliminary experiments showed no differences in con A capping of lymphocytes between normal adult cells and cells from two infants matched for age with the patient.

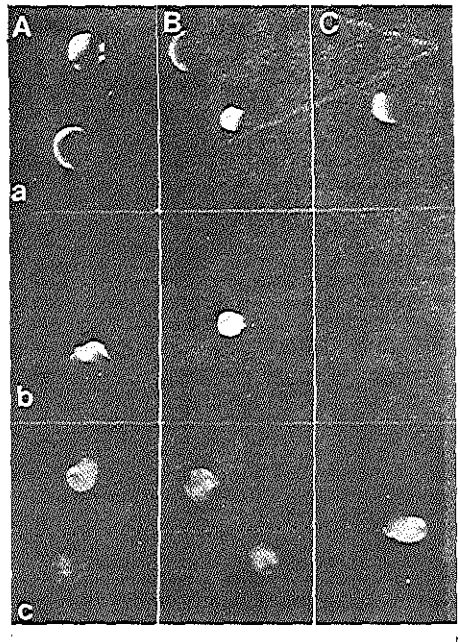

Figure 1. Distribution of Con A on Human Lymphocytes (× 650).

Frames a, b, and c show the distribution of fluorescence due to (a) fluorescein-con A, (b) rhodamine antihuman IgM, and (c) Hoechst 33258 on typical buffy-coat leukocytes not treated with drugs. The cells are all lymphocytes, as judged from nuclear morphology (c). The control cells (A) consist of a T cell (IgM-negative) with a patchy surface distribution of con A and a B cell with a uniform surface distribution of con A and a separate patchy distribution of anti-IgM. The patient's cells (B and C) consist of a capped B cell with co-capping of anti-IgM and a uniformly labeled T cell (B) and a capped T cell (C).

Results

Immunologic Studies

Serum immunoglobulin quantitation revealed a mean IgG of 400 mg per 100 ml, IgA of 85 mg per 100 ml, IgM of 70 mg per 100 ml, and IgE of <10 ng per milliliter. No abnormal proteins were detected by serum immunoelectrophoresis. Five to 12 per cent of peripheral blood mononuclear cells were stained with the fluorescein anti-IgM or anti-IgD reagents, and plasma cells were detected in the bone marrow. The patient was in blood group O, but isohemagglutinins were not detected. Despite repeated immunization with diphtheria-tetanus-pertussis-trivalent poliomyelitis vaccine (Connaught Laboratories, Toronto, Ontario), no antibodies were detected and the Schick test remained positive.

There was no evidence of delayed cutaneous reactivity to any of the antigens tested. Absolute lympho-

cyte counts never fell below 3000 per cubic millimeter and appeared morphologically normal under the light microscope. Surprisingly, E-rosette formation, including the expression of theophylline sensitivity in approximately 40 to 45 per cent of the patient's E-rosetting cells, was normal (Table 1). Peripheral blood mononuclear cells also normally expressed receptors for the third component of complement (C3) (mean, 27 per cent) and for Fc-IgG (mean, 30 per cent) as well as $\beta 2$ microglobulin and appropriate HLA and Ia-like determinants.

As shown in Table 2, the patient's T cells did not respond to phytohemagglutinin, con A, pokeweed mitogen, allogeneic cells, or a number of specific antigens including *C. albicans*, streptokinase-streptodornase and tetanus toxoid. In constrast, there was a statistically significant response to formalinized *Staphylococcus aureus*, a T-cell independent B-cell mitogen

Table 1. E-Rosette Formation in a Patient with Combined Immunodeficiency.

Subject	% Rosettes*		
	E†	E AET†	E theoph‡
Patient	48±10	68±4	29±7
Control	58±6	77±5	30±6

*Mean ±1 SD of 12 experiments.

†E (AET) denotes rosettes formed with sheep erythrocytes treated with aminoethylisothiouronium bromide.

‡E (theoph) denotes rosettes formed in the presence of 3 mM theophylline.

(Schuurman RKB, Gelfand EW, Dosch H-M: unpublished data), and this was confirmed in the patient in cell-separation studies. The failure to respond in culture to phytohemagglutinin and con A was confirmed with blast-count analysis and studies of [^{14}C]leucine incorporation. In mixed lymphocyte cultures the patient's cells stimulated normally, and there was no evidence of suppressor cells or suppressive substances in the patient's serum. Lymphocyte proliferation in both parents was normal.

Because lymphocyte numbers and proportions of E-rosetting T cells were both normal, the combined immunodeficiency did not appear to reflect the abnormality of T-cell differentiation that has been described in most patients with SCID.[4] The normal induction of E-rosette formation with the patient's cells after incubation with thymic epithelial cells, thymic epithelium conditioned medium, thymosin, and theophylline demonstrated the presence of all identifiable precursor T cells at all stages.[4]

Fluorescence Microscopy (Fig. 1)

Since the translational mobility of membrane proteins and the phenomenon of capping have been implicated in the triggering of cell activation, the patient's leukocytes were examined for an abnormality in the mobility of con A bound to surface glycoprotein receptors. A striking difference between normal cells and the patient's cells in the surface distribution of con A was immediately apparent (Table

Table 2. Lymphocyte Proliferation in a Patient with Combined Immunodeficiency.*

Mitogen/Antigen	Patient	Control
	cpm × 10^{-3}	
None	0.4±0.1	0.5±0.2
Phytohemagglutinin	0.6±0.3	68.4±15.6
Concanavalin A	0.6±0.3	42.7±9.3
Pokeweed	0.3±0.1	18.4±9.4
Formalinized *S. aureus*	16.3±8.1	18.1±8.2
Autologous cells	0.5±0.4	0.4±0.1
Allogeneic cells	1.5±0.6	41.9±0.9

*Values represent [^{3}H]thymidine incorporation expressed as the mean ±1 SD of at least 6 experiments.

3). A mean of 30 per cent of the patient's cells showed the spontaneous accumulation of con A into a surface cap, whereas only 9 per cent of the normal cells did so. In addition, more than 60 per cent of the patient's cells showed con A capping after exposure (30 minutes at 37°C) to 10 μM colchicine, an alkaloid that inhibits microtubule assembly.[3] In contrast, only 26 per cent of the control cells capped after incubation with this drug. Lymphocytes from the patient's mother were essentially normal. In none of these studies could we define an abnormality in the distribution of con A-receptor complexes on the membranes of either the patient's polymorphonuclear leukocytes or monocytes.

The abnormal formation of con A caps and the exaggerated response to colchicine observed in the patient's lymphocytes applied to both purified T and B cells (Table 3). Although, in general, B cells from the patient or controls were more readily capped by con A than were T cells and were more susceptible to enhancement of capping by colchicine, both T and B cells from the patient showed a rate of spontaneous con A capping that was three times the normal rate (Table 3). After colchicine, the number of B and T cells that capped in the patient was twice the number of normal B and T cells that capped (Table 3).

Table 3. Distribution of Con A-Receptor Complexes in a Patient with Combined Immunodeficiency.

Treatment	Cells	Percentage of Con A Capping*		
		Total Lymphocytes	T Lymphocytes	B Lymphocytes
Buffer	Patient	29.7±1.5	20.5±0.7	47.6±3.5
	Normal	8.7±1.2	8.5±0.9	13.5±4.8
	Maternal	9	6	23
Colchicine (10^{-3} M)	Patient	63.0±9.6	42.5±6.3	78.5±2.1
	Normal	26.3±3.0	27.5±6.4	41.0±6.7
	Maternal	28	25	58

*Results are expressed as the mean ±1 SD of 3 separate experiments. Results for maternal cells are from a single study, and further experiments are required to determine the statistical significance of the slightly elevated B-cell capping response.

Lymphocytes were identified by Hoechst fluorescence. The distribution of con A on each of at least 200 lymphocytes was then classified as randomly distributed or capped. From this we derived the percentage of total lymphocyte caps. Finally, each cell was examined for the presence or absence of fluorescence with the anti-IgM antiserum. Counting was continued until 100 IgM-negative (T) lymphocytes or IgM-positive (B) lymphocytes had been examined, and these counts gave the percentage of T lymphocytes and of B lymphocytes capped.

Discussion

We have described a patient who, despite having normal numbers of cells with markers characteristic of T and B lymphocytes, lacked the functions normally attributed to these cells. The family history and laboratory assessment of lymphocyte function favored the diagnosis of severe combined immunodeficiency. The patient's brother had died after a lifelong history of infections, an absence of in vitro or in vivo evidence of cell-mediated immunity, and the presence of normal levels of immunoglobulins but feeble antibody responses. Although many patients with SCID have normal or elevated numbers of B lymphocytes[3,12-14] and even normal levels of serum immunoglobulins,[15] the presence of normal numbers of T cells is unusual. Thus, these patients represent one variant of SCID that in many ways differs from the usual form, which is associated with a marked reduction in T cells and profound hypogammaglobulinemia.

The presence of all stages of precursor T-cell development in bone marrow correlated with the normal lymphocyte count as well as with normal proportions of T cells. This relation implied that the T-cell compartment was able to proliferate and expand normally in response to T-cell-inductive signals and that the defect was a more specific one unrelated to an abnormality of T-cell differentiation itself. Both T and B lymphocytes had a variety of normal receptors and surface determinants, including receptors for both phytohemagglutinin and con A. Furthermore, at least one early lymphocyte response to both lectin binding and exposure to the calcium ionophore A23187 — the uptake of calcium ions by the cells — was indistinguishable from the response in controls, indicating normal ligand-receptor interaction and activation of the cells (studies carried out in collaboration with Dr. M. H. Freedman, Toronto, Ontario).[16]

The specific redistribution of membrane-receptor complexes after interaction with exogenous agents such as antibody or lectin may represent a primary event in signal transduction across membranes.[17,18] Since Edelman et al. described con A-cap formation induced in the splenic lymphocytes of mice,[19] interest has focused on the potential role of capping in cell-cell interactions, cell activation, and cell function.[20-22] Analysis of the patient's lymphocytes after brief exposure to con A at 37°C revealed a unique abnormality: his lymphocytes showed a marked increase in both spontaneous and colchicine-induced surface con A-cap formation. This capping abnormality was expressed on both B and T cells even though T-lymphocyte function appeared more profoundly impaired in the family. T cells may have provided sufficient helper activity to permit some B cells to differentiate into immunoglobulin-secreting plasma cells, but not in response to challenge by specific antigens.

The basis of the capping abnormality in these lymphocytes remains undefined. Since colchicine exaggerated the abnormality, the most obvious explanation is a defect in the assembly of cytoplasmic microtubules, analogous to the defect in leukocytes from patients with the Chediak-Higashi syndrome.[23] However, preliminary studies with the electron microscope suggested that the patient's lymphocytes contained a normal density of both centriole-associated and peripheral microtubules. Another possibility is a direct alteration of membrane fluidity that encourages con A-receptor movement in SCID lymphocytes. Studies of the composition of phospholipid, fatty acid, and cholesterol in erythrocytes, as well as fluorescence-depolarization studies with diphenylhexatriene, showed no differences between normal red cells and those from the patient (Berlin RD, Fera JP: unpublished data). However, specific changes in the membranes of both B and T lymphocytes that do not extend to other blood cells cannot be ruled out. Since concentrations of microfilaments generally underlie the caps, an additional explanation is an intrinsic defect in the structure or regulation of lymphocyte microfilaments that encourages cap formation.

These observations provide direct support for the hypothesis that interactions between plasma membrane and cytoskeleton have a role in regulating the expression of specific immunity. The profound effect of these interactions on lymphocyte function was emphasized by the development of acute and fatal graft-versus-host disease after attempted reconstitution with bone marrow from the HLA-D-compatible father. Further analysis of the functional interaction between membrane and cytoskeleton components may not only clarify the molecular basis of this disorder, but may also provide insight into the possible therapeutic approaches to this and other diseases.

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LYMPHOCYTE-MEMBRANE ABNORMALITIES ASSOCIATED WITH PRIMARY IMMUNODEFICIENCY DISEASE.

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INTRODUCTION

Primary combined immunodeficiency disease is a heterogeneous group of disorders affecting both T- and B-lymphocyte differentiation[1]. Two types of lymphocyte membrane defects have been described in association with T- and B-lymphocyte dysfunction:

a) Lack of expression of Beta-2-microglobulin (B2M) and B2M-associated-HLA determinants on lymphocytes[2,3];

b) Abnormality of lymphocyte-surface-receptor capping[4].

These disorders will be discussed in view of the role of the lymphocyte plasma membrane in normal T- and B-lymphocyte functions and properties.

Specific antibody secretion is the endresult of a series of interactions between antigen, mononuclear phagocytes, T-lymphocytes and B-lymphocytes. These interactions are studied in vitro following antigenic or mitogenic activation of peripheral blood mononuclear cells. The endpoint of the interactions is best measured by the induced maturation of B-lymphocytes. This is almost exclusively a T-dependent phenomenon which means that T-lymphocytes serve as obligatory regulators of the induction of B-lymphocyte maturation. However some exceptions do exist: in vitro activation of human B-lymphocytes by EB virus[5], Nocardia antigen[6] and formalized Staph. Aureus Cowan A strain (STA)[7,8] are all T-independent. The soluble mitogens PHA, spA (protein A, derived from

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Staph. Aureus) and PWM activate T-lymphocytes first. B-lymphocyte maturation is the consequence of the generation of T-helper cell activity. This helper cell function is radio-resistant in the assays discussed here[8], which allows detection of the helper function at the B-cell level.

Although mitogens are widely used to study human T- and B-lymphocyte function the understanding of the molecular events induced by mitogens at the level of the plasma membrane is still fragmentary[9]. Lymphocyte activation is initiated following the ligand binding to membrane glycoproteins[10]. Ligands also induce the movement of lymphocyte surface macromolecules to form patches or caps composed of receptor-ligand complexes. A relationship between the capping phenomenon and lymphocyte activation has been demonstrated although the exact mechanism is still elusive[10,11]. It has been suggested that the HLA determinants, a major source of cell surface glycoproteins, are receptors involved in the binding of ligands[12].

The HLA-A, -B and -C determinants are expressed on the membranes of almost all human cell types in association with and probably anchored by B2M. Thus far, they have been shown to bind antigen to lymphocytes[13] and to be essential for T-cell mediated killing of virus infected cells[14].

A role for HLA-A and -B determinants in the cooperation between T- and B-lymphocytes has also been suggested by studies on the generation of antigen specific suppressor T-cells[15]. When mitogenic assays are used no MHC restriction can be demonstrated: allogeneic T-B-cell mixtures give results comparable to those obtained from autologous or HLA-identical combinations.

The Ia-like HLA-D/DR determinants are largely found on B-lymphocytes, mononuclear phagocytes, a small percentage of resting T-lymphocytes[16], and on activated T-lymphoblasts[17]. These determinants are expressed on the membrane independently of and not associated with B2M. Their role in human immune responses has thus far been demonstrated in macrophage-T-cell interactions in a secondary immune response against certain antigens (HSV, PPD)[18,19].

The delineation of lymphocyte membrane abnormalities in association with primary immunodeficiency has revealed the potential roles of B2M associated HLA determinants and of surface receptor capping in human T- and B-lymphocyte differentiation and responsiveness.

PATIENTS AND IMMUNOLOGICAL STUDIES

Lack of expression of HLA determinants associated with combined immunodeficiency

The B2M-associated HLA defect was studied in two unrelated families, one of Turkish origin, the other Algerian. Consanguinity was likely in the Turkish family and certain in the Algerian. The affected children were of both sexes; first symptoms presented after the age of 3-4 months; all children had severe and persistent diarrhea, mucocutaneous candidiasis, interstitial pneumonia and bacterial infections of various types. No systemic viral infections could be substantiated. In fact, proven viral infections (e.g. Herpes Simplex in the Turkish patients) evolved and cleared in a normal way.

Predominance of the defect in lymphocytes. The lack of expression of B2M associated HLA determinants is summarized in Table 1. The defect was mainly restricted to resting lymphocytes. Platelets were also deficient in surface HLA. Other cells in the patients (e.g. fibroblasts) did express HLA antigens thus explaining the presence of HLA antigens in the serum.

TABLE 1

B2M AND HLA DETERMINANTS IN THE HLA DEFICIENT PATIENTS.

Lymphocytes:	B2M	HLA-A and -B	HLA-D/DR
T-lymphocytes	-	-	n.d.
B-lymphocytes	(+)or-	-	+
Lymphoblasts:			
PHA induced	n.d.	(+)	n.d.
Cell line	(+)	(+)	n.d.
Serum	+	+	n.d.

Legends: + positive, normal amount; (+) positive, low amount
- negative; n.d. not determined.

Lymphoblasts express small amounts of HLA-A and -B determinants

In a standard cytotoxic assay against PHA-induced patient lymphoblasts as well as in CML studies (in which the targets were patient lymphoblasts), small amounts of HLA-A and -B determinants were detected. This was further substantiated by the expression of HLA-A and -B determinants on EBV-transformed B-lymphoblastoid cell lines grown from PBL of these patients. From these investigations the HLA genotypes of the patients could be delineated (Table 2). Both Algerian patients had healthy siblings with the same HLA genotypes, but normal phenotypic expression on lymphocytes. The presence of HLA determinants in serum, on lymphoblasts and on fibroblasts excluded a genetic defect in the MHC locus on chromosome 6.

TABLE 2
HLA GENOTYPES

	A	B
Turkish 1	3, 9	15, W21
Turkish 2	9,11	15,(W21)
Algerian 1	2, 9	W35, W35
Algerian 2	11,W30	5, W21

Non B2M associated HLA determinants. HLA-D/DR was detected by MLR typing and indirect immunofluorescence using allotypic anti-B-cell sera. These determinants were present on both B-lymphocytes and mononuclear phagocytes. Thus HLA determinants associated with B2M were absent and the determinants not associated with B2M were present, suggesting a possible role for B2M in the defect.

Beta 2 Microblobulin. B2M was not present on viable T-lymphocytes. Tissue sections of T-cell areas in various lymphoid organs showed the presence of B2M which, however, was found between the cells rather than really fixed in the cell membranes. It was concluded that the deficient fixation of B2M in T-cell membranes was either due to a structural defect of B2M or to an intrinsic lymphocyte membrane abnormality.

On the B-lymphocytes of the Turkish patients B2M was expressed in a far lower amount than normal - without the parallel detection of HLA-A or -B. The Algerian patients did not show any B2M on the surface of B-lymphocytes. After EBV-induced transformation of patient PBL the lymphoblastoid cells, especially after several passages, did express B2M together with the HLA-A and-B determinants[2,20].

Relationship between HLA determinants and the immune system. The B2M associated HLA membrane defect did affect the development of T-lymphocytes in the complete form of the disease observed in the Algerian family. The thymuses were small, hypoplastic and with few thymocytes in one patient, and virtually absent in the other. The serum thymic factor activity was abnormally low and the number of T-cell precursors able to undergo "in vitro differentation" was also decreased. Numbers and functional activity of T-lymphocytes were extremely reduced in peripheral blood and no antibody production was demonstrable. The spleen and lymph nodes contained few lymphocytes. No plasma cell was found (Table 3).
In the Turkish patients the development of T- and B-lymphocytes was less affected. Thymus histology was essentially normal, but the thymic cortex contained few lymphocytes. Serum thymic factor was extremely low and target cells for serum factor were virtually absent. Absolute numbers of circulating T-lymphocytes were 30% of normal. T-lymphocyte homing in peripheral lymphoid organs was also disturbed in that T-dependent areas in spleen and lymph nodes were sparse populated. However circulating T-lymphocytes had normal responses to mitogenic activation and it was concluded that the lack of surface HLA glycoproteins did not interfere with normal ligand receptor interactions and subsequent lymphocyte activation. This was in striking contrast to the response to several antigens (Candida, Diphtheria, Tetanus toxoid, Poliovirus): even after booster immunization in vivo with DTP and despite persistent candidiasis proliferative responses were undetectable. This implied that B2M-associated HLA determinants may play a role in the T-cell response to antigens.

TABLE 3 IMMUNOLOGICAL DATA

	B2M-HLA defect	Capping abnormality
T-lymphocytes		
E-rosettes	very low-low	normal
Mitogenic response	very weak-normal	negative
Antigenic response	absent	absent
Thymus architecture	hypoplastic-normal	normal
T-cells in lymphoid organs:-thymus	low numbers	normal
-lymph nodes	virtually absent	normal
Helper function*	absent	absent
B-lymphocytes		
sIg^+ B-cells	low-normal	normal
STA response	weak	normal
T dependent responses*	absent	normal
Germinal centres	absent	absent
Plasma cells	absent-some IgM	normal
Serum immunoglobulins	IgM only	normal, all classes
Specific antibodies	absent	absent

Legends: * tested by PWM induced B cell maturation or T-B cell cooperation studies (see text).

HLA determinants and cytotoxic reactions. The lack of HLA determinants did not lead to deficient killing by the lymphocytes. In vivo the Turkish patients showed a normal response to Herpes Simplex virus and had no signs of systemic viral spreading. In vitro, antibody dependent (ADCC) and non dependent (CML) cytotoxic functions were normal. Thus in cytotoxic interactions the HLA determinants apparently are not necessary at the effector cell level.

Capping abnormality associated with severe combined immunodeficiency.

A male infant from a consanguineous marriage was referred because his brother had died as a result of combined immunodeficiency. The patient was in good health at 5 months of age. Immunological investigations showed normal numbers of E-rosettes and sIg^+ B-lymphocytes. In serum normal immunoglobulin levels were not accompanied by specific antibodies against various bacteria or viruses[4].

Proliferative capacity of lymphocytes and capping of mitogen receptors. Responses to various T-cell mitogens (PHA, PWM, ConA, ALS, spA) were repeatedly absent (Table 3). Because studies with calcium-ionophore A23187 showed no difference between patient and control lymphocytes, the early events in lymphocyte triggering by ligand-receptor interaction were interpreted as normal. This was however not followed by measurable DNA or RNA synthesis. Thus the transduction of the proliferation signal was deficient.

When lymphocytes were incubated with fluoresceinated ConA, increased capping of the ligand-receptor complex was demonstrated on T-lymphocytes as well as on B-lymphocytes. The response of lymphocytes to the polyclonal B-cell mitogen STA was also normal. This might indicate that if a mitogen is fixed to a carrier -- in this case the protein A moiety to the cell wall of the Staph. Aureus bacterium - increased capping is prevented and normal activation follows.

The maturation capacity of patient's B-cells was also demonstrated in the following experiments. Allogeneic irradiated T-cells were mixed with patient's cells and incubated with various mitogens. Neither the irradiated T-cells nor patient's cells alone showed any response. The mixtures however were clearly responsive (Fig. 1). The level of response was comparable to the response of the control allogeneic T-B-cell mixtures. Taken together with the positive response of patient's lymphocytes to stimulation with STA, these investigations showed a normal maturation capacity of the patient's B-lymphocytes.

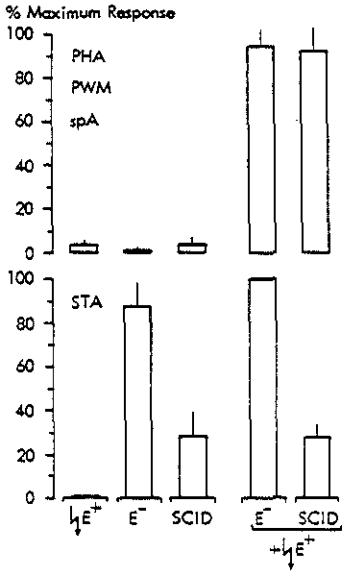

Fig.1. T-dependent and T-independent maturation of B-lymphocytes.

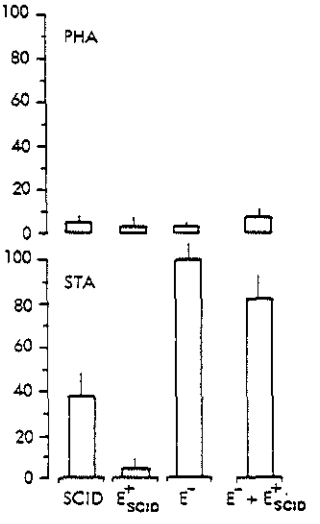

Fig.2. Helper T-cell activity.

Legends: SCID = Severe combined immunodeficiency; E^+ and E^- = T- and B-lymphocytes obtained by E rosette depletion techniques (Ref. 7,8); both E^+ and E^- are allogeneic to patients lymphocytes and each other. 2 x 10^5 cells in 0.2 ml RPMI 1640 - 10% FCS were incubated for 3 days; ↯ = irradiated (2500 rads). Responses were measured by ^{3}H-thymidine incorporation (Ref. 7,8).

Lymphocyte differentiation. The patient had normal levels of serum immunoglobulins and plasma cells were present in various lymphoid organs, although these organs were underdeveloped. B2M associated HLA determinants were expressed normally on the lymphocyte membranes as well as HLA-D/DR, Ia-like antigen, B2M, sIg and receptors for SRBC, C3 and ConA. These findings imply that the capping disorder did not interfere with the normal development of either T-lymphocytes or B-lymphocytes. Furthermore the presence of plasma cells synthesizing immunoglobulins in vivo suggests that the patient's T-cells must have been able to provide some non-specific helper cell activity.

The following experiments were performed to investigate the helper function (the experiments are reciprocal of those described above). Patient T-lymphocytes obtained by E-rosette-depletion, were mixed with allogeneic B-lymphocytes and incubated with various mitogens. None of the cell populations alone showed a proliferative response but the mixtures were also negative (Fig. 2). All the mitogens (PHA, spA, PWM gave the same (negative) results. The figure also shows the results of stimulation by STA. The E^+-fraction of SCID cells did not react whereas the unseparated population did. Allogeneic E^--lymphocytes had a high response to STA which was not changed by adding E^+-SCID cells. Taken together with the results using unseparated SCID lymphocytes, these experiments indicate the absence of helper effects in vitro when T-dependent mitogens were used. The patient did not have any measurable response to specific antigens either in vivo or in vitro. Thus, although T- and B-lymphocytes were developed in this SCID and although T-lymphocytes likely could bind antigen, the capping abnormality appeared to interfere with the antigen-induced immune responsiveness, because a) T-cell proliferation is a necessary event or b) antigen presentation to B-lymphocytes is hampered as a result of the increased capping.

DISCUSSION

Two types of lymphocyte membrane defects were both associated with combined immunodeficiency disease. Both membrane disorders suggested an anchorage defect, the HLA deficiency by a defect in B2M fixation and the capping abnormality by a defect in receptor-capping modulation. In both instances specific antibody production failed. T-cell differentiation was essentially unimpaired up to the level of postthymic T-cells in the sense that circulating E-rosetting lymphocytes were present. Likewise B-cell maturation was intact since sIg^+ B-cells were present and the response to STA, a T-cell independent polyclonal B-cell mitogen, was positive. Despite the presence of T-cells and B-cells both types of immunodeficiency were characterized by a lack of antigenic responsiveness.

B-lymphocyte differentiation appeared less impaired especially in the Turkish patients since normal absolute numbers of sIg^+ B-cells were detected in peripheral blood and bone marrow, whereas the Algerian patients had low numbers of circulating B-cells. However numbers of plasma cells and levels of serum immunoglobulins were abnormally low and PWM failed to induce plasma cell maturation. Thus T-dependent B-cell maturation was significantly compromised although T-cells were present and able to proliferate. The recent demonstration of a weak but positive response of patient PBL to STA activation indicated that B-cells had normal proliferative capabilities. Taken together, these data argue for a role of HLA determinants in T-B-cell cooperation.

In vivo the very low numbers of plasma cells found were mainly IgM containing cells and possibly reflect some degree of T-independent maturation. The IgM secreted in vivo lacked specificity for the various antigens tested. The presence of follicles in lymph nodes appeared to be an important factor in IgM plasmacell maturation, in that one of the patients, who had low IgM serum levels and low IgM plasma cell numbers, virtually lacked follicles, whereas the other had some follicles, normal numbers of IgM plasma cells and a normal serum IgM level. The complete absence of germinal centre activity in these follicles might reflect an essential role of germinal centres in T-dependent B-cell maturation.

In summary the findings indicate the potential for an important role of HLA-A and -B determinants in antigen recognition by T-lymphocytes. Lack of HLA determinants appeared to interfere with the response to antigens and mitogens at the level of T-B-cell cooperation. In addition the differentiation of T-lymphocytes was impaired already at the intrathymic stage but not completely blocked. The Turkish patients particularly showed some degree of T-cell maturation to the post-thymic stage. T-cell homing in peripheral lymphoid organs was severly impeded and T-dependent B-cell maturation to plasma cells was virtually absent. HLA determinants therefore appear to be important in T-cell differentiation, T-cell homing and T-B-cell collaboration especially in the response to antigens.

In both, the antigenic response was shown to be defective at the level of the T-lymphocyte.
In the HLA deficient patients T-lymphocytes had either a normal or a very reduced proliferative response to mitogens, possibly depending on the numbers of T-cells in PBL. In the capping abnormality the reaction to antigens was absent because of a lack of induction of proliferation, shown by negative responses to mitogens, whereas the receptor-ligand interaction, as such, was undisturbed. Likewise the negative antigenic response in the Algerian HLA deficient patients could be explained by the low mitogenic responses. However the Turkish patients had normal mitogenic responses but failed to respond to antigens.

HLA-A and B-glycoproteins on the lymphocyte membrane have been shown to act as receptors for Semliki Forest virus[13]. Thus the lack of response to antigens either in vivo or in vitro might be explained by the absence of B2M associated HLA determinants. At the same time it was evident that these glycoproteins were not the receptors for mitogens.

In the capping disorder the lymphocyte surface was intact. Normal surface determinants and receptors, especially the receptors for mitogens were present. Clear proof for antigen receptor binding was not obtained, since lymphocytes were unable to proliferate in response to antigens. The lack of proliferation was shown to be associated with abnormally increased capping of ligandreceptor complexes on the lymphocyte membranes. Proliferation (of B cells) was demonstrated by the response to STA. It was suggested that this bacterium, being in essence a rigid large carrier of mitogenic protein A molecules (spA), prevented increased capping which allowed normal induction of proliferation, whereas soluble spA was unable to activate lymphocytes. The increased capping also affected normal T-B-cell cooperation when T-dependent mitogens were used. In these assays helper effects were absent, whereas it was demonstrated that the patient's B-lymphocytes could be activated by allogeneic T-lymphocytes.

In these assays only part of the in vivo situation could be simulated. The finding of plasma cells and immunoglobulins in vivo, suggests that some helper function was present in vivo, perhaps non-specific in origin without the elucidation of antigen-specific responses.

An alternative view of both membrane abnormalities may explain the differences between the in vivo and in vitro studies. The sparse population of T-cells in central and especially peripheral lymphoid organs of the HLA deficient patients was striking. In contrast all T-dependent areas in the lymphoid tissues of the patient with the capping abnormality were normally populated. At the same time it was concluded that in both disorders, T-cell differentiation was essentially unimpaired up to the post-thymic stage[1]. This argues for a crucial role of HLA determinants in homing of T-lymphocytes and pre-T-cells. Some experimental evidence for this argument is available. Syngeneic cells have a better homing performance than H2disparate (congenic) cells in reconstitution experiments (21, 22). The difference in homing capabilities of the HLA deficient lymphocytes and the HLA bearing lymphocytes in the capping abnormality might thus explain the presence of plasma cells and serum immunoglobulins in vivo in the patient with the capping abnormality because T-cells were capable of migrating to the environment where B-cell maturation normally is induced.

In summary: These studies indicate that both a normal cytoskeleton and the presence of HLA determinants may be necessary for normal immune responsiveness. The HLA defect with either a lack of antigen-receptor interaction or severely disturbed homing of T-cells and the capping disorder with defective antigen-receptor complex fixation, but essentially normal homing, are both examples of the crucial role of the T-lymphocyte membrane in cell differentiation and in antigen-specific immunity in vitro and in vivo.

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ADDENDUM

Huius farinae sunt et isti, qui libris edendis famam immortalem aucupantur. Hi cum omnes mihi plurimum debent, tum praecipue ii, qui meras nugas chartis illinunt. Nam qui erudite ad paucorum doctorum iudicium scribunt, quique nec Persium nec Laelium iudicem recusant, mihi quidem miserandi magis, quam beati videntur, ut qui sese perpetuo torqueant: addunt, mutant, adimunt, reponunt, repetunt, recudunt, ostendunt, nonum in annum premunt, nec umquam satisfaciunt acfutile praemium, nempe laudem eamque perpaucorum, tanti emunt, tot vigiliis, somnique, rerum omnium dulcissimi, tanta iactura, tot sudoribus, tot crucibus. Adde nunc valetudinis dispendium, formae perniciem, lippitudinem aut etiam caecitatem, paupertatem, invidiam, voluptatum abstinentiam, senectutem praeproperam, mortem praematuram, et si qua sunt alia eiusmodi. Tantis malis spiens ille redimemdum existimat, ut ab uno aut altero lippo probetur.

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