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THE OBLIGATORY ROLE OF THE NULL LYMPHOCYTES IN  
IMMUNOGLOBULIN SYNTHESIS BY HUMAN B LYMPHOCYTES.

By

SHARON TAYLOR

Thesis submitted to the School of Graduate Studies in  
partial fulfillment of the requirements for the M.Sc.  
degree in Microbiology and Immunology.

University of Ottawa

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ABSTRACT

It has previously been shown in this laboratory that B cells require the collaboration of Null cells, in addition to the T<sub>H</sub> helper cells and monocytes and a signal from pokeweed mitogen (PWM), in order to synthesize immunoglobulins (Ig) as determined by immunofluorescence. The objective of this investigation was to identify the mechanism whereby the Null cells collaborate with the B cells to enable them to synthesize and secrete Ig, using both immunofluorescence to detect Ig synthesis and the ELISA assay to determine the amounts of Ig secreted by the cells into the culture medium.

It was demonstrated that the Null cells secrete a soluble factor in culture, referred to as immunoglobulin synthesis/secretion facilitating factor or ISFF, which can replace the Null cells in the cultures. Neither the Null cells nor ISFF secreted by the Null cells need be present in the cultures from the beginning of the culture period. In point of fact, the B cells can synthesize and secrete very significant quantities of Ig providing the Null cells or ISFF are added no later than day 4 or day 6, respectively, to the 7 day culture. Thus, the Null cell signal to the B cells, which is provided by ISFF, is transmitted late in the culture period and suggests that the B cell must attain a certain degree of functional maturity before it becomes responsive to the Null cell signal.

It was also demonstrated that the Null cells require  $T_H$  (helper) cells or Interleukin 2 (IL-2), monocytes, and PWM in order to secrete maximum amounts of ISFF.

Furthermore, in order for the B cells to respond to the ISFF signal, monocytes must be present in the culture.

It is concluded that Ig synthesis and secretion by the B cells is the culmination of a complex but orderly sequence of reactions and that the Null cell and the factor it secretes, ISFF, play a prominent role in facilitating Ig synthesis by the B cells.

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LIST OF ABBREVIATIONS

Ab	Antibody
ADCC	Antibody-dependent cell-mediated cytotoxicity
AFC	Antibody forming cell
APC	Antigen presenting cell
ar	autologous rosetting
ARC	Antigen receptor bearing cell
BCDF	B cell differentiation factor
BCGF	B cell growth factor
BSF	B cell stimulating factor
C'	Complement
C'2	Second component of complement
C'3	Third component of complement
C'4	Fourth component of complement
C14	Carbon 14
CD3	Antigen cluster designation
CMI	Cell-mediated immunity
Con-A	Concanavalin A
E	Erythrocytes
EA	Erythrocyte complexed with antibody
EAG	IgG-coated ox erythrocytes
EAM	IgM-coated ox erythrocytes
ELISA	Enzyme linked immunosorbent assay
FCS	Fetal calf serum
FITC	Fluorescein isothiocyanate
FNCS	Filtered null cell sonicate
Fc	Crystallizable fragment of Ig

FcG	Fc of IgG
FcM	Fc of IgM
g	Gravity
HBSS	Hank's Balanced Salt Solution
HLA-Dr	Human class II histocompatibility antigen
HI	Humoral immunity
HLA	Human Leukocyte antigen
hrs	Hours
IF	Immunofluorescence
Ig	Immunoglobulin
IgA	Immunoglobulin A
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IL	Interleukin
ISFF	Immunoglobulin synthesis/secretion facilitating factor
IU	International units
KLH	Keyhole limpet hemocyanin
M $\phi$	Monocyte
MF	Mitogenic Factor
MHC	Major histocompatibility complex
MLR	Mixed leukocyte reaction
MNC	Mononuclear cells
MNC <sup>-1</sup>	MNC depleted once of monocytes
MNC <sup>-2</sup>	MNC depleted twice of monocytes
mw	Molecular weight
NK	Natural killer cell
NSE	Non specific esterase staining

ORBC      Ox whole blood cells  
PB        Peripheral blood  
PBS      Phosphate buffer solution  
PHA      Phytohemagglutinin  
PWM      Pokeweed mitogen  
SG        Specific Gravity  
SRBC     Sheep whole blood cells  
T4        }  
T+4+8-    } T helper cell  
T+G-M+    }  
T+4+8-M+G- }  
T8        }  
T+4-8+    } T suppressor cell  
T+G+M-    }  
T+4-8+M-G+ }  
TCGF     T cell growth factor  
T<sub>M</sub>      T cells with receptor for Fc of IgM  
T<sub>G</sub>      T cells with receptor for Fc of IgG  
U.S.P.    United States Pharmacopeia



## 1. GENERAL INTRODUCTION

Resistance to infectious diseases is provided by what is referred to as the non-specific immune system and the specific immune system. The former includes all the mechanisms which are not induced or activated as a result of antigenic stimulation. These include: (i) the physical barriers of the skin and the mucous membranes, (ii) the secretions (lysozyme, IgA) bathing them, (iii) the phagocytic cells, and (iv) the complement system which can be activated by various pathogens. For example, gram negative bacteria are capable of directly activating C'3 and bypassing C'1, 2 and 4 in the absence of antibodies. The specific immune system constitutes those mechanisms which are activated as a consequence of antigenic stimulation. These are humoral immunity which is mediated by antibodies secreted by antibody forming B cells and cell-mediated immunity which is mediated by sensitized T cells. The antibodies act in conjunction with complement to lyse the pathogenic microorganism. The sensitized cells secrete the lymphokines which are the effector molecules in this form of immunity.

Antibody synthesis and non-specific (polyclonal) immunoglobulin synthesis and secretion appear to be the function of the bone marrow-derived B lymphocytes which have infiltrated the spleen and lymph nodes in the immunologically mature animal. However, the B cells require the participation of other circulating cells in order to

synthesize Ig. It has been demonstrated that the cells which participate in the synthesis of antibodies by the B cells include: (i) antigen-specific T cells which possess surface membrane receptors for the antigenic determinants (ARC)

(ii) monocytes/macrophages which may function in both an accessory manner as well as in a more specific manner to present antigen to the T and/or B cell and

(iii) the regulatory cells which include the antigen-specific T helper cells and the antigen-specific T suppressor cells.

It has also been demonstrated that the cells which participate in the synthesis of non-specific immunoglobulins by the B cells include:

(i) monocytes/macrophages, which appear to function in an accessory fashion and

(ii) the regulatory non-antigen specific T helper and T suppressor cells.

It is accepted that the concentration of circulating Ig (specifically IgA, IgM and IgG) remains constant in humans from the onset of puberty (10-12 years of age) well into old age (80-95 years of age). This is considered to be the result of continuous synthesis and degradation of non-specific immunoglobulins in the presence of continual regulation by the T helper and T suppressor cell. The net helper or suppressor activity expressed by these two cells determines the final concentration of the circulating immunoglobulins. Since the numbers and/or activity of these

T helper and T suppressor cells may vary between individuals, it is obvious that the concentration of the circulating immunoglobulins will also differ between individuals. Both of these T regulatory cells are in a constant state of activation whereas the T antigen-specific helper and suppressor cells become activated only upon antigenic stimulation.

The synthesis of non-specific immunoglobulins by the B cells and the conditions which facilitate immunoglobulin synthesis by these cells have been defined using the in vitro culture system first described in the early 1970s. It was demonstrated that cultures of the circulating human mononuclear cells with a phyto mitogen prepared from the pokeweed plant, referred to as pokeweed mitogen or PWM, results in the synthesis of immunoglobulins by the B cells. PWM was shown to be essential in the culture and the stimulus provided by PWM probably has its in vivo counterpart. It was subsequently demonstrated that the B cells must be cultured with T helper cells, Null cells and monocytes, in addition to PWM, in order to synthesize non-specific immunoglobulins. It would therefore appear that the B cell requires multiple signals from other cells, in addition to that provided by PWM, to stimulate it to synthesize non-specific immunoglobulins. However, it is not known at the present time whether the B lymphocyte must receive signals directly from the T cells, Null cells and monocytes as well as from PWM, or whether the different

cells require signals among themselves with only the signal from one of these cells stimulating the B cells.

This thesis is concerned with the investigation of the role of the Null cells in the synthesis of non-specific immunoglobulins by the B cells. That is, the synthesis of Ig by the B cells in the absence of direct antigenic stimulation.

It should be noted by the reader that the references concerned with the relationships of the cells involved in the synthesis of non-specific immunoglobulins (T cells, B cells, monocytes) do not go much beyond 1980. The interest by the research community in this particular subject appeared to wane at this time and very few relevant papers have been published on this subject since 1980.

The points I have raised in the general introduction have not been referenced as they will be discussed in further detail in the appropriate sections of this thesis.

## 2. RATIONALE, HYPOTHESIS AND OBJECTIVE

As was stated in the preceding chapter, immunoglobulin synthesis by the B lymphocytes requires the obligatory participation of the T<sub>H</sub> helper cells, monocytes, Null cells and PWM. The rationale for this investigation is that Ig synthesis by the B lymphocytes requires the obligatory participation of the T<sub>H</sub> helper cells, monocytes, Null cells and PWM. The hypothesis is that the Null cell secretes a factor which can replace the Null cell in the culture. The objective is to characterize the Null cell replacement factor and to define its mechanism of action.

3. HISTORICAL REVIEW

3.1 Sites of immunoglobulin synthesis

Shoenheimer, in 1942 (1), utilizing radioactively-labelled (C14) amino acids, demonstrated for the first time that the circulating serum proteins are not permanently present in the circulation. He showed that they continually undergo degradation and synthesis and that there is a dynamic balance between degradation (catabolism) and synthesis (anabolism) of the serum proteins. Thus, the circulating proteins are replaced by new proteins at a rate which is defined by their half-life. It was not until the early 1960's that techniques were developed to enable investigators to identify the organ(s) of origin of the serum proteins. These techniques included organ and cell culture, radioimmuno-electrophoresis and immunofluorescence. Since this thesis is concerned only with the regulation of the synthesis of the non-antibody immunoglobulins (Ig) by human cells, the ensuing discussion will concern itself only with the synthesis of non-antigen specific immunoglobulin in the human.

Immunoglobulins are synthesized by lymphoid cells in organs composed of organized lymphoid structures or elsewhere in the body in areas in which lymphoid aggregates develop under certain conditions. These cells are found mainly in the spleen, lymph nodes, bone marrow, appendix, Peyer's patches and the lamina propria in the gastrointestinal tract. Immunoglobulin synthesis has been

observed with cells of all these organs and tissues except for the appendix and Peyer's patches (2-14). Within the spleen and lymph nodes, immunoglobulin containing cells have been observed most characteristically throughout the red pulp in a perifollicular location (spleen), in the subcapsular areas (lymph node) and infrequently in the germinal centers of the follicles (7). Although the appendix and Peyer's patches contain lymphocytes in an organized fashion, failure to detect significant immunoglobulin synthesis by lymphocytes in these organs indicate that those lymphocytes may have functions other than the synthesis of immunoglobulins. It has been estimated that a single immunoglobulin synthesizing cell can secrete 2,000 molecules per second or  $1.7 \times 10^6$  molecules per day. The roughly  $9.5 \times 10^{12}$  immunoglobulin molecules secreted per day are synthesized by approximately  $5.5 \times 10^{10}$  cells (15).

By 1970 it had been established that Ig synthesis takes place in the cells of the lymphoid organs, primarily the spleen, lymph nodes and bone marrow. However, little was known about the mechanisms which regulate the rate of Ig synthesis nor the nature of the cells and/or factors which facilitate Ig synthesis by the Ig synthesizing cell. It was assumed that Ig synthesis takes place as a result of feedback suppression by the circulating Ig as in a servo-mechanism, in a manner similar to the secretion of the hormones by the endocrine glands, and that only one cell-type was involved, the Ig synthesizing cell.

### 3.2 Cells involved in antibody synthesis

#### 3.2.1 Lymphocytes

In a milestone investigation carried out in 1965 by Claman and his associates (16), it was demonstrated that the primary immune response in the mouse involves the participation and interaction of two lineages of lymphocytes, one derived from the thymus and referred to as the T cells and the other derived from the bone marrow and referred to as the B cells (16). The investigation involved the transfer of lymphoid cells from donor mice to irradiated immunoincompetent recipients of the same inbred strain along with the antigen, SRBC. The transfer of syngeneic thymic (T) lymphocytes or bone marrow (B) cells alone, irrespective of their number, did not confer the capacity to respond with antibody synthesis in the irradiated recipient. However, the injection of a mixture of T and B lymphocytes into the irradiated syngeneic recipient restored the capacity to respond with antibody synthesis within the normal 5 days following immunization. These results were subsequently confirmed by Claman et al (16-19) and by Mitchell and Miller and their associates (20-25). The conclusions reached by these investigators was that the virgin T cells interact initially with the antigen via receptors on the surface of the T cells, and that these T cells are clonally selected and precommitted to interact with the antigenic determinants or epitopes on the antigen. These cells were therefore variously referred

to as antigen receptor-bearing cells, antigen reactive cells, antigen responsive cells or ARC. It was also concluded that B cells are activated by a product of the ARC-antigen interaction and that this activation stage is followed by transformation and proliferation of the virgin uncommitted cells into overt antibody synthesizing and secreting cells (17-25). These cells were therefore referred to as antibody-forming cells or AFC. It was also concluded that the T ARC cells and the B AFC cells are separate lineages of cells and that they do not transform one into the other.

In the outbred rabbit, it has been demonstrated that the primary immune response results from the initial interaction of the antigen with precommitted, unipotent, antigen-receptor bearing cells (ARC) present in the bone marrow followed by the activation, transformation, and proliferation of the virgin (naive) uncommitted pluripotent precursors of the antibody forming cell (AFC), also present in the bone marrow, into overt antibody forming cells (AFC) (26-32). By cell transfer experiments in the rabbit, utilizing allotypic markers, it was shown that these two functionally-distinct cells, ARC and AFC, do not transform into one another (33). In both the mouse and the rabbit, therefore, two lineages of lymphocytes, T and B, interact to facilitate the primary antibody response, and the antibody forming cells are detected primarily, if not only, in the spleen. The progeny of these cells, the memory cells, are also localized in the spleen (32).

### 3.2.2 Antigen presenting cells

#### 3.2.2.1 Phagocytic cells (Macrophages, Langerhans cells and Dendritic cells).

Results of investigations concerned with the immune response in animals, particularly in the rodent and the rabbit between 1965 and 1970, suggested that the rather simple model for Ig synthesis involving only a single cell would have to be revised. It was demonstrated that macrophages are involved in the primary immune response. Sabin, in 1939, injected a soluble antigen, ovalbumin (OA), coupled to a dye (R-salt-azo-benzidine) and observed accumulation of the antigen-dye conjugate principally within the phagocytic cells. He assumed, at this early date, that the phagocytic cells are important in the degradation of antigen and the production of antibodies (cited in 34). In 1968, Sabet et al (35) injected India ink (carbon particles) into a mouse followed by the particulate antigen. All the macrophages phagocytized the carbon particles. The failure of these mice to respond to the antigen was attributed to "blockade" of the phagocytic cells by the carbon particles. Other investigations resulted in the isolation of super-antigen from disrupted macrophages which appeared to be more immunogenic, on an equal weight basis, than the native antigen (36-38). Since the whole sheep red blood cell (SRBC) is too large a structure to stimulate an immunoresponsive cell not much larger than the SRBC, it was reasoned that the

immune response to a particulate antigen like the SRBC must involve the phagocytosis and degradation of the SRBC into small, soluble immunogenic determinants by the macrophage (39-43).

More recent investigations have indicated that the macrophage plays a major role in the immune response, both as an accessory cell and as a regulatory cell (44-46). Unanue and Allen (47) as well as other investigators (48-52) concluded that one of the functions of the macrophage was to process antigen and present it in a biochemically modified form for recognition by T and/or B lymphocytes. These macrophages were referred to as antigen presenting cells or APC. They suggested that the processed antigens, complexed with Ia<sup>h</sup> antigens (molecules coded for in the I region of the major histocompatibility gene complex) on the surface of the macrophage are recognized by antigen receptor bearing T cells (ARC). The B cells either recognize and bind soluble antigen or bind to antigen on the macrophage membrane - thus setting the stage for the T cell-B cell interaction. They speculate, as well, that perhaps the T cell must react with I region antigens on macrophages before the macrophage associated antigen can react with the appropriate T-cell receptor on the T ARC. The APCs have been shown to include the macrophages, the bone marrow derived Langerhans dendritic cells in the skin (within the stratified squamous epithelium) and the intraepidermal (thymus derived?) dendritic mononuclear cells (53-57). However results of an

earlier series of investigations, carried out in vivo, suggested that the T ARC and not the macrophage APC functions physiologically as the antigen-presenting cell to a B cell precursor of the antibody forming B cell (AFC) in vivo. The administration of KLH (keyhole limpet hemocyanin) antigen to rabbits depleted of precommitted antigen-specific ARC cells to KLH failed to evoke an antibody response to the antigen. However, antibody responses to other non cross-reacting antigens were normal (33,58). These rabbits had not been depleted of macrophages, dendritic cells or Langerhans cells. Claman and his associates (16-19) obtained similar results in the mouse. That is, depriving the mouse of T ARC cells but not of the phagocytic APC resulted in loss of the ability to synthesize antibodies. The investigators (33,58) concluded that the cells which function as the antigen presenting cells in vivo are the lymphocyte ARC and not the macrophage APC. However, it may be that macrophages can only process and present the antigen after the antigen has initially reacted with the T ARC. Therefore, it cannot function as the APC in the absence of the T ARC. It is also possible that the antigen is processed by the macrophage but cannot be presented to the B cell in the absence of T ARC-macrophage interaction.

#### 3.2.2.2 B antigen presenting cells

It has also been proposed that the B cell itself can function as the antigen-presenting cell to the T (helper and/or ARC) cells (59-63). However, one may question the

interpretations of the results obtained in these investigations since the B cells used were either (i) from previously immunized rabbits (59) which would be expected to be memory cells with antibody molecules on their surface (63) capable of reacting with the antigen and thereby of presenting it, or (ii) were coated with allogeneic anti-Ig Immunoglobulin (i.e. goat anti-mouse Ig), which is fixed to the cell surface as it interacts with the cell-surface mouse Ig, and can then present the goat Ig (60). These B cells were not naive to start with and were capable of specifically interacting with the "antigen" which they could subsequently present to other cells. In their review of the subject, Swain and Dutton (62) speculate upon the possibility of B cells functioning as presenting cells but they admit that there is no formal, rigorous proof. It would be necessary to demonstrate that randomly isolated B cells can interact with randomly selected antigens and then present these antigens to the appropriate target cells. This has not been done. Furthermore, if B cells could, by themselves, interact with the antigen, they could presumably be activated by the antigen without the participation of the macrophages and T cells. The matter of B antigen-presenting cells (or B APC) is a very controversial one today.

### 3.3 The cells involved in the synthesis of non-specific immunoglobulins by the circulating human B lymphocytes.

#### 3.3.1 The B cell is the immunoglobulin synthesizing cell

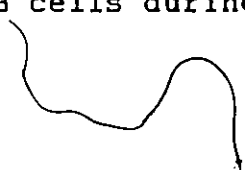
Since antibody synthesis involves the obligatory

participation of macrophages, T lymphocytes and B lymphocytes, the possibility or in fact the probability that similar cell-types are involved in general immunoglobulin synthesis could not be ignored. In order to carry out the appropriate investigations to resolve this question, an in vitro system for immunoglobulin synthesis by human cells was required. Furthermore, since the only accessible cells in the human are the circulating cells, the in vitro system had to function using the circulating cells. Unfortunately, no in vitro system using human circulating cells in culture was available throughout the 1960's. Many attempts were made to culture the circulating cells under various conditions but the cells did not synthesize and secrete immunoglobulins. It was only in 1971, when Cooper, Lawton and Bochman (64) reported that human circulating mononuclear cells cultured in the presence of pokeweed mitogen (PWM) could synthesize immunoglobulins, that an in vitro model system became available for the study of the cellular interactions and contributions in Ig synthesis. This finding by Cooper et al (64) was quickly confirmed by numerous other investigators (65-72). It was also shown that B lymphocytes in man could be identified by the presence of surface membrane-bound immunoglobulins (smIg) in high density (73-79). It was further demonstrated that the pokeweed mitogen induces transformation and differentiation of the B lymphocytes (65,68,69) and that these cells are lacking in individuals presenting with the Bruton or x-linked agammaglobulinemia

syndrome (64,79-82). As a result of these studies, it was generally concluded that it is the B lymphocyte which synthesizes and secretes the immunoglobulins.

3.3.2. The role of the monocytes in Ig synthesis by the B cells.

Monocytes must be present in the cultures of the T<sub>M</sub> cells, B cells and PWM in order for Ig synthesis to take place. Gerrard and Fauci (83), Rosenberg and Lipsky (84), Thiele and Lipsky (85) and Shin and Choi (86) all demonstrated that monocytes had to be present in the cell cultures for Ig synthesis to take place since removal of the monocytes resulted in failure of Ig synthesis. On the other hand, Saxon et al (70) reported that the removal of the phagocytic cells did not adversely affect the capacity of the residual mononuclear cells, or cultures of B cells and T cells, in the presence of PWM to synthesize Ig. These results would appear to conflict with those presented above. However, it is important to stress that Saxon et al (70) examined the phagocyte-depleted cells for the presence of phagocytic cells only at the beginning of the culture and not at the end. Since as many as 2 to 4 percent of the phagocyte depleted cells were identified as phagocytic cells, at the beginning of the culture, it is possible that these cells and the immature cells which are not phagocytic in the freshly isolated state but mature into phagocytic cells in culture (87-89) are sufficient to permit Ig synthesis by the cultured B cells during the 7 day cultures.



Thus, by the early 1980s, it appeared that the cells required in the cultures for the optimal synthesis of Ig are B cells, T<sub>H</sub> cells and monocytes.

### 3.3.3 The role of the T cells in non-specific immunoglobulin synthesis by the B cells.

#### 3.3.3.1 The obligatory participation of the T cells in immunoglobulin synthesis by the B cells.

In the mid 1970's, investigators began to evaluate the role(s) of the circulating non-B lymphocytes and the monocytes in the regulation and facilitation of immunoglobulin synthesis by the B lymphocytes in culture. Saxon et al (70) and Keightley et al (68) demonstrated that the synthesis of immunoglobulins by the B lymphocytes was T cell dependent as purified B lymphocytes cultured with PWM failed to synthesize immunoglobulins. On the other hand, maximum synthesis of immunoglobulins by the B lymphocytes took place when the cells were cultured for 6 to 7 days with an equal number of T lymphocytes in the presence of PWM. The culture medium was fortified with de complemented fetal calf serum (FCS) to a final concentration of 15 to 20 percent (v/v). Penicillin (100 units per ml) and streptomycin (100 ug per ml) were also added to the medium at the initiation of culture. This fortified medium is referred to as culture medium. The method used to assay for immunoglobulin synthesis was immunofluorescence; the degree or extent of immunoglobulin synthesis was considered to be correlated with the percentage of fluorescing cells. The method used to

assay for immunoglobulins secreted into the culture medium was immunoprecipitation of immunoglobulins which had incorporated a radioactive amino acid ( $^{35}\text{S}$ -methionine) into the newly synthesized immunoglobulins during the culture.

#### 3.3.3.2 The regulatory T helper and T suppressor cells

A further improvement of the culture conditions for the optimal synthesis of Ig was introduced by Moretta and his colleagues in the mid 1970s. This group of investigators demonstrated that the T lymphocytes could be separated into two subclasses of cells - one with receptors for the Fc of IgG ( $T_{\alpha}$  or  $T_{\gamma}$  cells) and the other with receptors for the Fc of IgM ( $T_{\mu}$  or  $T_{\mu}$ ). These cells could be physically separated from each other by their ability to rosette with IgG coated ox erythrocytes (EAG) and IgM coated ox erythrocytes (EAM), respectively. They demonstrated that the  $T_{\mu}$  cells facilitate Ig synthesis by the co-cultured B cells in the presence of PWM whereas the  $T_{\alpha}$  cells inhibit Ig synthesis by the co-cultured  $T_{\alpha}$  cells, B cells and PWM (90-93). The  $T_{\mu}$  cells and the  $T_{\alpha}$  cells were therefore referred to as helper and suppressor cells respectively. Thus, by the late 1970's, it was considered that the cells required for optimal Ig synthesis by the B cells in in vitro culture for 7 days at  $37^{\circ}\text{C}$  were B cells,  $T_{\mu}$  cells and monocytes in addition to PWM.

#### 3.3.3.3 The mechanism of helper cell and suppressor cell function.

Numerous investigators have demonstrated that  $T_{\alpha}$  cells

in the rodent (94-98), the rabbit (99) and the human (100,101) can secrete antigen-specific suppressor factors, that is factors capable of down-regulating, aborting or inhibiting specific antibody synthesis. However, Moretta et al (93) were the first to demonstrate the secretion by the  $T_0$  cells of a factor capable of inhibiting the synthesis of non-specific, or polyclonal Ig synthesis, and not just specific antibodies.

Moretta et al (93) demonstrated that the  $T_0$  suppressor cells and the  $T_H$  helper cells secrete soluble factors functionally capable of replacing these cells in the cultures. They demonstrated that the  $T_0$  cells, isolated by rosetting with EAG and incubated for 42 hours with PWM, secreted a suppressor factor capable of inhibiting Ig synthesis by B cells cultured with  $T_H$  cells. Similarly, the  $T_H$  cells, isolated by rosetting with EAM and incubated for 3 days with B cells and PWM, secreted a factor capable of inducing differentiation of B cells and synthesis of Ig in the presence of PWM. Most interestingly, the suppressor factor could not inhibit Ig synthesis by the B cells incubated with the helper factor and PWM but it could inhibit Ig synthesis by the B cells incubated with the  $T_H$  helper cells and PWM. These results suggested that the  $T_0$  cells secrete a suppressor factor which suppresses Ig synthesis by the B cells by acting on the  $T_H$  helper cells to inhibit them from secreting the helper factor. Once the helper factor has been secreted by the  $T_H$  cells, neither the

T<sub>0</sub> cells nor the suppressor factor secreted by the T<sub>0</sub> cells can inhibit B cell differentiation and Ig synthesis (93).

Jelinek and Lipsky (102) confirmed the findings of Moretta et al (93) at least insofar as the secretion of the non-specific helper factor by PWM stimulated T cells is concerned. Cell-free culture supernatants of human circulating T cells cultured with PWM for 48 hours were capable of inducing the differentiation of B cells into immunoglobulin secreting cells in cultures of mononuclear cells depleted of T cells. Similar findings were also reported by Brenner et al (103).

Chiorazzi, Fu and Kundel (104) demonstrated that T cells, activated in the mixed leukocyte culture reaction (MLR) by allogeneic monocytes, also secrete a helper factor which can induce the production of large amounts of polyclonal Ig by human circulating B cells in culture.

#### 3.3.3.4 Phenotypic characterization of the helper and suppressor T cells.

As discussed previously, the helper T cells and the suppressor T cells can be distinguished from each other by the specificity of their surface receptors for the Fc of Ig. The T helper cells bear surface membrane receptors for the Fc of IgM (or u heavy chains) and the T suppressor cells bear surface membrane receptors for the Fc of IgG (or heavy chain). The T helper and suppressor cells were therefore initially referred to as T+G-M+ and T+G-M-, respectively.

In the early 1980's, mouse monoclonal antibodies directed toward two surface constituents on the circulating T cells permitted the delineation of the T cells into helper and suppressor cells on the basis of surface (antigen) markers rather than receptors for FcG or FcM. Reinherz, Schlossman and their associates (105-109) identified a monoclonal antibody directed toward a surface configuration, referred to as the T4 marker, present on 60 to 70 percent of the T cells. Another monoclonal antibody was directed toward a surface configuration, referred to as the T8 marker, present on 30 to 40 percent of cells. They showed that the T4 and the T8 cells behave as helper and suppressor cells, respectively, with respect to Ig synthesis by the cultured B cells (105-109). The helper and suppressor T cells were therefore referred to as T+4+8- and T+4-8+.

A question which had to be resolved was the relationship between the helper and suppressor cells identified on the basis of their Fc receptors, on the one hand, and on the basis of reactivity with the T4 and T8 monoclonal antibodies, on the other. Reinherz et al, (110) showed that the T<sub>m</sub> cells contained both T4+ and T8+ cells and that the T<sub>s</sub> cells were not enriched for either T4+ or T8+ cells. They concluded that there is little correlation between T cell subsets defined by monoclonal antibodies and those defined by Fc receptors.

Richter et al (111) demonstrated that the overt T helper cells constitute less than 50 percent of the T+4+8-

cells and that they are the T+M+G- cells. This finding demonstrated that the T+4+8- cells are functionally heterogeneous and include M+G- and M-G- cells. Only the T+4+8-M+G- cells are helper cells and the T+4+8-M-G- cells are functionless in this regard. Similarly, less than 40 percent of the T+4-8+ cells are overt suppressor cells and these are the T+M-G+ cells. Therefore, the T+4-8+ cells are also functionally heterogeneous and include M-G+ and M-G- cells. Only the T+4-8+M-G+ cells are overt suppressor cells. The function of the T+4-8+M-G- cells, if any, are not known at the present time.

To further complicate matters, Heijnen, Pot and Ballieux (112) and Heijnen and Ballieux (113) have demonstrated the presence of a suppressor T cell induction circuit in man. They presented evidence for the presence of T suppressor inducer, T suppressor precursor and T suppressor effector cells which can all be defined phenotypically with the aid of an additional marker, a receptor for the autologous red blood cell which facilitates autologous rosetting (ar). Thus, the T suppressor inducer cells, the T suppressor precursor cells, and the T suppressor effector cells have been characterized as T+M+G-4+8-ar-, T+M-G-4-8+ar+ and T+M-G+4-8+ar- (112,113). The unexpected finding was that the T suppressor inducer cell was a cell of the M+4+ series, the phenotype of the helper cells. Thus, a T+M+G-4+8-ar- cell induces a T+M-G-4-8+ar+ suppressor precursor cell to differentiate into a T+M-G+4-8+ar- suppressor effector cell.

It has also been demonstrated by Morimoto et al (114) that the T suppressor inducer cell can be identified by the use of a mouse monoclonal antibody referred to as anti-2H4. They showed that the T4<sup>+</sup>2H4<sup>+</sup> subset functions as the inducer of the T8<sup>+</sup> suppressor cell. The suppressor inducer cell can therefore be defined phenotypically as a T<sup>+</sup>M<sup>+</sup>G<sup>-</sup>4<sup>+</sup>8<sup>-</sup> anti-2H4<sup>+</sup> cell.

Futhermore, Morimoto et al (115) demonstrated that the T4<sup>+</sup> helper cell could be identified by the use of a mouse monoclonal antibody referred to as anti-4B4. They showed that the T<sup>+</sup>4<sup>+</sup>B4<sup>+</sup> cells provide the helper signal to the B cells for Ig synthesis in the presence of PWM whereas the T<sup>+</sup>4<sup>+</sup>4B4<sup>-</sup> cells provide little or no help to the B cells. These latter cells are the 2H4<sup>+</sup> suppressor inducer cells.

Calvo et al (116) generated their own monoclonal antibody termed anti-D44 which was shown to react with approximately 70% of T4 cells and 30% of T8 cells. They showed that most of the helper activity for Ig synthesis by the B cells in culture was mediated by the T<sup>+</sup>4<sup>+</sup>D44<sup>+</sup> cells and that the T<sup>+</sup>4<sup>+</sup>D44<sup>-</sup> cells were essentially inactive in this regard. Futhermore, they demonstrated that virtually all the suppressor activity was mediated by the T<sup>+</sup>8<sup>+</sup>D44<sup>-</sup> cells. It may be that the anti-D44 monoclonal antibodies as defined by Calvo et al (116) and the anti-4B4 monoclonal antibodies defined by Morimoto et al (115) are directed towards the same determinant on the T4<sup>+</sup> cell since both these monoclonal antisera identify the T4 helper cells.

3.4 Soluble cell derived factors which mediate the proliferation and differentiation of activated B cells into immunoglobulin synthesizing cells.

It has been known since the late 1970's that the monocytes and the T lymphocytes, especially the T4 helper lymphocytes, secrete factors following activation which maintain T cells in a viable active state and facilitate the activation, proliferation and differentiation of the B cells into the immunoglobulin synthesizing cells. These factors are referred to collectively as the interleukins or IL. The interleukin secreted by the monocyte/macrophage is referred to as IL-1. The T helper cell secretes IL-2, BSF, BCDF, BCGF, IL-5 and IL-6 (to name a few). The IL-1 secreted by the monocyte/macrophage facilitates the secretion of the other interleukins by the T helper cells. It has been shown that IL-1, or a similar factor, activates the dormant B cell, that BCGF stimulates transformation and proliferation of the activated B cell into blast cells and that BCDF then facilitates the subsequent differentiation of these blast cells into immunoglobulin synthesizing B cells. It has also been suggested by some investigators that a different T cell derived factor, BSF, can replace both BCGF and BCDF in the activation, transformation, proliferation and differentiation of the B cells into Ig synthesizing cells. During the past decade, many papers have been published on the subject of IL. However, confusion still reigns and there is no acceptable nomenclature for the IL to date. An

in depth evaluation and discussion of these factors is beyond the scope of this thesis. However, I have broached the subject since Ig synthesis is generated by a B cell and it is essential to recognize that the Ig synthesizing B cell must undergo transformation, proliferation and maturation from the inactive precursor state to the Ig synthesizing state. Soluble factors have been shown to play an accessory role in these processes. The following review articles summarize the current confusing state of affairs concerning the characterization and functions of the interleukins (117-126).

### 3.5 The role of the Null cell in the synthesis of non-specific immunoglobulins by the B cells.

#### 3.5.1 Evidence in favour of the Null cells as a distinct class of circulating lymphocytes.

Up to the early 1970s, the circulating lymphocytes were considered to consist of only two lineages of cells - T cells and B cells. Approximately 60 to 75 percent of the circulating lymphocytes rosette with SRBC, a universally-accepted property of T cells, and stain positive by immunofluorescence with the monoclonal pan anti-T cell antiserum, OKT3 or CD3. The remaining cells were considered to be B cells since they have surface membrane Ig (smIg) as demonstrated by immunofluorescence using FITC conjugated anti-human Ig. In the late 1960s, it was demonstrated that some circulating lymphocytes possess surface membrane receptors for the Fc of IgG by virtue of which they can

carry out antibody-dependent cell-mediated cytotoxicity (ADCC) (127). These cells were considered to be part of the B cell pool since, in the freshly isolated state, all FcG receptor-bearing cells exhibited smIg. A number of investigators subsequently confirmed the presence of receptors for FcG on some of the circulating lymphocytes (128-131). It was not until the early to mid-1970s that it was demonstrated that the FcG receptor-bearing "B" cells are, indeed, different from the non-FcG receptor-bearing "B" cells since the former cells shed their smIg following incubation at 37°C for 30 minutes and do not regenerate it whereas the latter cells do not shed their smIg following even longer incubation at 37°C (132-135). Although the FcG receptor bearing "B" cells do not regenerate their smIg once it is shed, the non FcG receptor bearing "B" cells regenerate their smIg even after it has been enzymatically degraded by exposure to trypsin or pronase (132-135). On the basis of these findings, the non-T lymphocytes were classified into two separate lineages, the true B cells which display stable smIg and do not exhibit surface membrane receptors for FcG, and the pseudo B or L cells which possess temperature labile smIg, which cannot synthesize smIg, and bear surface membrane receptors for FcG. The L cells were subsequently named Null cells as they lack the critical markers for T cells (receptor for SRBC) and B cells (stable smIg).

Several functional properties also distinguish the Null cells from the T cells and the B cells: (i) only the Null cells participate in the antibody-dependent cell-mediated cytotoxic (ADCC) reaction; the true B cells and the T cells cannot mediate the ADCC cytotoxic reaction (136-141), and (ii) Null cells do not undergo blastogenesis and mitosis when stimulated with the conventional phyto mitogens PHA and CON-A (which stimulate T cells) and PWM (which stimulates T cells and B cells) in culture (142,143).

In recent experiments conducted on cultured isolated B and Null cells, neither the candidate (unpublished results) nor Moretta (personal communication) demonstrated the conversion of B cells to Null cells, and vice versa. B cells do not demonstrate ADCC activity after culture for up to 72 hours. Null cells do not stain with OKB6 monoclonal antibodies, do not generate smIg and do not lose their capacity to mediate ADCC cytotoxicity. Similar findings have previously been reported by Ng et al (143). It must therefore be concluded that the circulating B and Null cells in the immunologically mature individual are distinct from each other and represent separate lineages (Table 1).

3.5.2 Evidence that Null cells are obligatory participants in immunoglobulin synthesis by the B cells.

In all of the investigations concerning immunoglobulin synthesis referred to thus far, immunoglobulin synthesis was successfully attained only when T cells and B cells were cultured in the presence of PWM. A critical assessment of

these studies disclose that the cultures invariably consisted of T and non-T cells, and not T and B cells, since investigators generally assumed that the non-T cells consisted of only B cells. However, in 1985, Richter and Jodouin (144) reported that B cells cultured with T<sub>H</sub> helper cells and PWM, in the absence of the Null cells, failed to synthesize immunoglobulins in vitro. As was anticipated, Null cells cultured with T<sub>H</sub> helper cells and PWM, in the absence of B cells, failed to synthesize immunoglobulins. Monocytes were present in significant numbers (greater than 10 percent) in all of the cultures, and therefore failure of immunoglobulin synthesis in the absence of the Null cells could not be attributed to the absence of monocytes. Furthermore, cultures of reconstituted B and Null cells with T<sub>H</sub> helper cells and PWM resulted in immunoglobulin synthesis by the cultured B cells. These results demonstrated that B cells must be cultured with T<sub>H</sub> helper cells, Null cells, monocytes and PWM in order for immunoglobulin synthesis to take place.

### 3.6 SUMMARY

As a result of the many investigations carried out to identify the immunoglobulin synthesizing cell, an overwhelming consensus has emerged that the B lymphocyte is the only cell which carries out this function. However, it cannot do so in isolation; rather, it requires the collaboration with and participation of T<sub>H</sub> helper cells, monocytes and Null cells, as well as PWM, in culture to

synthesize immunoglobulins. The function of the T cells appears to be a regulatory one with  $T_H$  helper cells and  $T_S$  suppressor cells acting antagonistically, via secreted helper and suppressor factors, to stimulate or inhibit immunoglobulin synthesis by the B cell. The helper factor acts directly on the B cell to stimulate it whereas the suppressor factor acts on the  $T_H$  helper cell to inhibit secretion of the helper factor. The mechanism of action of the monocytes is not clear at the present time. A plausible role for it is the secretion of interleukin-1 (IL-1) which acts on the T cells to maintain them in a viable state and facilitates the secretion of IL-2 or T cell growth factor (TCGF) by the  $T_H$  helper cells. The mechanism of action of the Null cells in the facilitation of immunoglobulin synthesis by the co-cultured B cells is also not known at the present time. The objective of the investigations carried out in this thesis is to shed light on the mechanism of Null cell contribution and participation in immunoglobulin synthesis by the B lymphocytes.

TABLE 1

PROPERTIES WHICH DISTINGUISH BETWEEN THE CIRCULATING HUMAN  
T, B AND NULL LYMPHOCYTES.

Circulating lymphocytes	Property									
	% in circulation	Blastogenic response*	Receptor for SRBC	FcG	C'3b/d	IF** with OKT3	OKB6	Stable SmIg*	Labile SmIg	ADCC activity
T cell	60-75	****	+	-	-	+	-	-	-	-
B cell	8-15	*****	-	-	+ -	-	+	+	-	-
Null cell	12-25	-	-	+	-	-	-	-	+	+

\* Blastogenesis induced by phyto mitogens in vitro (PWM, PHA, Con-A)

\*\* IF = immunofluorescence

\*\*\* Blastogenesis to PHA, Con-A and PWM (3 days)

\*\*\*\* Blastogenesis to PWM only (7 days)

4.0 MATERIALS AND METHODS

4.1 Materials

4.1.1 Reagents

4.1.1.1 Reagents for isolating and culturing of lymphocytes

Carbonyl iron, grade sf - was obtained from Dyestuff and Chemicals, Toronto, Ont. and stored at 18-20°C.

Culture medium - RPMI-1640 fortified with fetal calf serum (FCS)(final concentration 20%), penicillin (100 units/ml), streptomycin (100 ug/ml), garamycin (50 ug/ml) and 1M HEPES (final concentration 2%).

Erythrocytes - Heparinized ox and sheep blood were obtained from Frappier Diagnostic Inc., Laval, Que. and stored at 4°C.

Fetal calf serum - 500 ml bottles were obtained from Whittaker M.A. Bioproducts Inc., Walkersville, MD. Fetal calf serum was decplemented (heated at 56°C for 30 min) and filtered through a 0.22 um Nalgene filter before use and stored at -20°C.

Ficoll 400 - 500 g containers were obtained from Pharmacia Fine Chemicals AB, Uppsala, Sweden and stored at 18-20°C.

Garamycin - (40 mg/ml Gentamycin), 2 ml vials were obtained from Schering Inc., Pointe Claire, Que. and stored at 4°C.

Hanks Balanced Salt Solution (HBSS) - 500 ml bottles were obtained from Mediatech, Herndon, VA and stored at 4°C.

Hematoll-LA Hgb reagent - 15 ml vials were obtained from Fisher Scientific, Orangeburg, N.Y. and stored at 18-20°C.

Hematoll Isotonic Diluent (azide free) - 20 liter cartons were obtained from Fisher Scientific, Orangeburg, N.Y. and stored at 18-20°C.

Heparin (1,000 U.S.P. units/ml) - 30 ml vials were obtained from Organon Canada Ltd., Toronto, Ont. and stored at 4°C.

Hepes buffer solution - 100 ml bottles (1M) were obtained from Whittaker M.A. Bioproducts Inc., Walkersville, MD and stored at 18-20°C.

Hypaque sodium 50% (300 mg/ml diatrizoate sodium) - 30 ml vials were obtained from Winthrop Laboratories, Aurora, Ont. and stored in the dark at 18-20°C.

IgG fraction of rabbit anti-bovine red blood cell antiserum (30 mg/ml total protein) - 5 ml vials of lyophilized antiserum were obtained from Organon Teknika, West Chester, PA. They were rehydrated and stored at -70°C.

Penicillin-streptomycin mixture (5,000 units Potassium Penicillin G/ml, 5,000 mcqs Streptomycin/ml) - 100 ml bottles were obtained from Whittaker M.A. Bioproducts, Walkersville, MD and stored at -20°C.

Pokeweed mitogen - vials containing lyophilized pokeweed mitogen were obtained from Gibco Laboratories, Chagrin Falls, Ohio and stored at 4°C.

RPMI-1640 - 500 ml bottles were obtained from Mediatech, Herdon, VA and stored at 4°C.

#### 4.1.1.2 Reagents for harvesting and staining of lymphocytes

Albumin, human (25% solution) - 100 ml bottles were obtained from Connaught Laboratories Ltd., Willowdale, Ont.

and stored at 4°C.

Ethanol (95%) - 25 liter containers of ethanol were obtained from Commercial Alcohols Ltd., Montreal, Que. and stored at 18-20°C.

Fluorescein conjugated goat anti-human IgA, IgG and IgM immunoglobulins (heavy and light chain specific) - were obtained from Organon Technika Corp., Westchester, PA. and stored at -20°C.

Glycerol - bottles containing 473 ml glycerol were obtained from Fisher Scientific, Orangeburg, N.Y. and stored at 18-20°C.

Methyl green - was obtained from Sigma Chemical Co., St. Louis, Missouri and stored at 4°C.

Monoclonal antibodies (25 µg/ml) - Leu-M3, Anti-Tac, Leu-11a, Leu-7, B-12, B-16 and Anti-HLA-Dr were obtained from Becton Dickinson Immunocytometry Systems, Mountain View, Calif. and stored at 4°C. OKT3, OKT4, OKT8, OKT11 and Goat Anti-Mouse IgG-FITC conjugates were obtained from Ortho Diagnostics, Don Mills, Ont. and stored at 4°C.

Trypan blue - was obtained from Grand Island Biological Co., Grand Island, N.Y. and stored at 18-20°C.

4.1.1.3 Reagents for enzyme linked immunosorbent assay (ELISA).

Albumin, bovine (30% solution) - 50 ml aseptically filled bottles were obtained from Sigma Chemical Co., St. Louis, Missouri and stored at 4°C.

Alkaline phosphatase conjugate - a solution containing

affinity-isolated goat anti-human antibodies to IgM (u chain specific) or IgG ( $\gamma$  chain specific), conjugated with calf intestine alkaline phosphatase, was obtained from Sigma Chemicals Co., St. Louis, Missouri and stored at 4°C. Discs coated with antibodies to human IgM or IgG - were obtained from Dimension Laboratories, Mississauga, Ont. and stored at 4°C.

Human IgG and IgM (unconjugated, chromatically purified) (5 mg/ml) - 5 ml vials were obtained from Organon Technika, Westchester, PA and stored at 4°C.

Magnesium chloride - 1 lb bottles of MgCl<sub>2</sub> crystals were obtained from British Drug Houses, Montreal, Que. and stored at 18-20°C.

p-Nitrophenyl phosphate disodium - 1 g vials were obtained from Sigma Chemicals Co., St. Louis, Missouri and stored in the dark at -20°C.

Sodium azide - 100 g bottles were obtained from Fisher Scientific, Orangeburg, N.Y. and stored at 18-20°C.

Tris (hydroxymethyl) aminomethane - 500 g bottles were obtained from Fisher Scientific, Orangeburg, N.Y. and stored at 18-20°C.

#### 4.1.2 General supplies.

Conical glass centrifuge tubes - 50 ml round-bottomed tubes were obtained from Fisher Scientific, Orangeburg, N.Y.

Cordis glass vials - 5 ml flat-bottomed vials were obtained from Cordis Laboratories Inc., Mississauga, Ont.

Culture tubes, sterile - 5 ml (12x75 mm) falcon 2003 and 15

ml (17x100 mm) falcon 2001 round-bottomed polystyrene tubes were obtained from Becton Dickinson Co., Lincoln Park, N.J. Eppendorf sterile combitips - 1.25 ml, 2.5 ml and 5.0 ml were obtained from Fisher Scientific, Orangeburg, N.Y. Filter cards - were obtained from Johns Scientific, Toronto, Ont.

Gauze sponges - (5.1 x 5.1) were purchased sterile from Kendall Canada, Toronto, Ont.

Microscope slides (75x25 mm) - frosted one end on both sides, were obtained from Serum International Inc., Montreal, Que.

MSI syringe adaptable filters, sterile (0.22  $\mu$ m) - were obtained from MSI, Westborough, MA.

Nalgene 0.22  $\mu$ m filters - were obtained from Nalgene Labware, Rochester, N.Y.

Needles - 18G(1") were obtained from Becton Dickinson Labware, Oxnard, Calif.

Pipettes - 1, 2, 5 and 10 ml pyrex sterile disposable serological pipettes were obtained from Corning Glassworks, Corning, N.Y.

Syringes - 1, 10, and 60 ml plastiplate sterile disposable syringes were obtained from Becton Dickinson Labware, Oxnard, Calif.

#### 4.1.3 Equipment

CO<sub>2</sub> incubator - cultures were maintained in a National incubator, NAPCO, Portland, Oregon. This incubator is fully automated with respect to temperature ( 37.5°C  $\pm$  0.5) and

pCO<sub>2</sub> (5.0% ± 0.1).

Cordis 1250 wash dispenser - manufactured by Hyperion Inc., Miami, Florida.

Coulter counter - was obtained from Coulter Electronics Inc., Hialeah, Florida.

Cytocentrifuge - (cytospin SCA-0030) was obtained from Shandon Southern Instruments, Camberley Surrey, England.

Digital 405 Photometer II - manufactured by Hyperion Inc., Miami, Florida.

Eppendorf repeater 4780 - was obtained from Brinkman Instruments., Westbury, N.Y.

Multipurpose rotator - was obtained from Scientific Industries Inc., Springfield, Massachusetts.

Refrigerated centrifuge CRU 5000 - was obtained from Fisher Scientific, Fairlawn, N.J.

Zeiss fluorescent microscope with a mercury vapor light source (bright field, dark field and epi-illumination) - was obtained from Zeiss, West Germany.

#### 4.2 Methods.

4.2.1 Blood donors - Volunteers consisted of hospital personnel who were free of any major illness and who did not take any form of medication, either for medical or recreational reasons. The volunteers were between 20 and 55 years of age.

4.2.2 Isolation of mononuclear cells (MNC) - The procedure is that essentially described by Boyum (145). Heparinized (50 units heparin/ml drawn blood) venous blood of healthy

volunteers (180 ml), diluted with an equal volume of Hanks Balanced Salt Solution (HBSS), was layered over a Ficoll-Hypaque discontinuous gradient (S.G. 1.077 at 18-20°C.) and centrifuged at 400 g for 30 minutes. The mononuclear cells were isolated from the interface, washed in HBSS and centrifuged at 300 g for 20 minutes. The cells were then washed twice with HBSS at 450 g for 10 minutes and resuspended in HBSS. An automated cell count was taken.

#### 4.2.3 Preparation of sheep and ox red blood cell

suspensions (SRBC and ORBC, respectively) - Heparinized sheep and ox bloods were centrifuged at 450 g for 10

minutes. The cells were washed three times in HBSS at 450 g for 10 minutes at 18-20°C. The packed cells ( $10^{10}$  cells per ml) were then resuspended in HBSS to the desired cell concentration. The SRBC and ORBC suspended at cell concentrations of  $3 \times 10^8$  and  $5 \times 10^8$  cells per ml constitute 3% and 5% suspensions of SRBC and ORBC, respectively.

#### 4.2.4 Preparation of IgG sensitized ox red blood cells (5% EAG)

EAG - Equal volumes of a 5% suspension of ORBC (E) and rabbit IgG anti-ORBC antibodies (A), used in a subagglutinating concentration, were mixed and incubated at 37°C for 60 minutes. The resulting antibody-sensitized ORBC (EA-IgG or EAG) were centrifuged at 450 g for 10 minutes at 18-20°C. The supernatant was aspirated, the cells were resuspended in RPMI-1640, and again centrifuged at 450 g for 10 minutes. This procedure was repeated twice. The EAG indicator cells were resuspended in RPMI-1640 to a

concentration of 5% and kept at 4°C.

4.2.5 Isolation of T and Non-T lymphocytes - The mononuclear cells were incubated with the 3% SRBC suspension (ratio of MNC:SRBC is 1:30) for 15 minutes at 37°C. The cells were centrifuged at 200 g 5 minutes and incubated overnight at 4°C. The cells were gently resuspended, layered over Ficoll-Hypaque and centrifuged at 400 g for 30 minutes. The non-rosetted Non-T cells were collected from the interphase and the T cells from the pellet. Both the T and Non-T cells were washed in HBSS and then in hypotonic ammonium chloride (0.1M) for 2 minutes in order to lyse the SRBC present in either cell suspension. The cells were then washed twice in HBSS, resuspended in HBSS, and an automated cell count was taken.

4.2.6 Isolation of T<sub>H</sub>, B and Null lymphocytes - To isolate T<sub>H</sub> cells, the T cells were incubated with a 5% EAG suspension (ratio of T:EAG is 1:50) for 15 minutes at 37°C. The cells were centrifuged at 200 g for 5 minutes and incubated for 1 hour at 4°C. The cells were gently resuspended, layered over Ficoll-Hypaque (S.G. 1.077) and centrifuged at 400 g for 30 minutes. The non-rosetted T<sub>H</sub> cells were collected from the interphase. To isolate B and Null cells, the Non-T cells were incubated with a 5% EAG suspension and then centrifuged in Ficoll-Hypaque (S.G. 1.077) in exactly the same fashion as described above. The non-rosetted B cells were collected from the interphase and the rosetted Null cells from the pellet. All three cell

preparations (T<sub>M</sub> cells, B cells and Null cells) were washed in HBSS and then in hypotonic ammonium chloride (0.1 M) for 2 minutes in order to lyse any ORBC present. The cells were then washed twice in HBSS, resuspended in HBSS, and an automated cell count was taken.

4.2.7 Preparation of monocyte depleted cells - The mononuclear cells were suspended ( $2 \times 10^6$  cells per ml) in sterile disposable tubes in 5 ml serum-HBSS (serum:HBSS is 1:1) to which were added 10 mg carbonyl iron. The tubes were placed on a multipurpose rotator for 30 minutes at 37°C in an atmosphere of 5% CO<sub>2</sub> in air following which the cells were gently layered over Ficoll-Hypaque (S.G. 1.077) and centrifuged at 400 g for 30 minutes. The non-phagocytic cells were isolated from the interphase and washed twice in HBSS. These cells are referred to as once-depleted MNC (MNC<sup>-1</sup>). These cells were cultured overnight in culture medium and subjected once more to depletion with carbonyl iron. These cells are referred to as twice-depleted MNC (MNC<sup>-2</sup>). The cells were stained for non-specific esterase (NSE) (146) which is a marker for monocytes. It was consistently found that less than 1% of the MNC<sup>-2</sup> could be identified as monocytes, following staining (by NSE) and examination by light microscopy.

4.2.8 Cell culture for Ig synthesis - T<sub>M</sub> cells were incubated with equal numbers of Non-T, Null and/or B cells ( $2 \times 10^5$  cells of each) in 1 ml of culture medium. Pokeweed mitogen (0.05 ml) was added in its optimal mitogenic

concentration. The cultures were maintained in a stationary phase at 37°C in 5% CO<sub>2</sub> in air. After 7 days (unless otherwise stated), the tubes were centrifuged at 450 g for 10 minutes, the supernatants were carefully removed, and the cells were washed with medium RPMI-1640 twice. The cells were stained for intracytoplasmic immunoglobulins (IgG, IgM and IgA) by the conventional direct immunofluorescent assay. The Ig present in the supernatant was determined by the ELISA assay.

#### 4.2.9 Preparation of immunoglobulin (Ig)

synthesis/secretion facilitating factor (ISFF) - T<sub>H</sub> cells (2x10<sup>5</sup>) were incubated with equal numbers of Null cells (2x10<sup>5</sup>) and PWM. After 7 days of culture (unless otherwise stated), the tubes were centrifuged at 450 g for 10 minutes and the supernatants were carefully collected in 15 ml sterile falcon tubes. This supernatant is referred to as 7 day conditioned medium (7DCM). This 7DCM was filtered through 0.22 um Nalgene filters and stored at -20°C. In some experiments 7DCM was prepared from cultures of other cell types and the methodology for their preparation is the same as described above.

#### 4.2.10 Immunofluorescent staining for cytoplasmic

immunoglobulin - Cells to be stained were suspended in 100 ul of a 25% solution of normal human serum albumin and cytocentrifuged on microscope slides in a Shandon cytocentrifuge. The cells were fixed on the slides by incubation in a cold 5% solution of acetic acid in ethanol

for 10 minutes at 4°C. The slides were rinsed well in PBS at room temperature and then incubated with fluorescein (FITC)-conjugated goat antibodies to total human Ig for 30 minutes at room temperature. The slides were rinsed well in PBS and examined by fluorescence microscopy.

4.2.11 ELISA assay - Inert discs coupled with goat anti-human IgM (or IgG) antibodies were incubated with aliquots of the cell culture supernatants (step 1). After a 60 minute incubation period, the discs were washed 5 times in PBS and incubated with the appropriate amount of goat anti-human IgM (or IgG) complexed with alkaline phosphatase enzyme (step 2). After another 60 minute incubation period, the discs were washed 5 times in PBS and transferred to tubes containing the chromogen, p-nitrophenyl phosphate (step 3). Following incubation for a final 60 minutes, aliquots of these supernatants were read in a colorimeter at 405 nm.

4.2.12 Non-specific esterase staining - Monocytes were identified by staining for non-specific esterase as described by Yam et al (146).

4.2.13 Staining cells with monoclonal antibodies - Staining of the various cells with monoclonal antibodies was performed according to the standard procedures for direct and indirect staining as described in detail by Jackson and Warner (147).

## 5. RESULTS

5.1 The optimal conditions for the synthesis of Immunoglobulins (Ig) by cultured human circulating B cells. The roles of the T cell, the monocyte, and pokeweed mitogen (PWM).

### 5.1.1 Rationale and objectives.

Before embarking into experiments to demonstrate the role of the Null cells in Ig synthesis by the circulating B cells, it was essential to demonstrate that the conditions described by other investigators (64-70) as optimal for Ig synthesis by the cultured human circulating cells would also provide for optimal conditions in this laboratory.

The objectives of this series of experiments was to demonstrate that the media and cell composition of the cultures of human circulating cells considered optimal for Ig synthesis by other investigators apply equally well in this laboratory as carried out by the candidate.

### 5.1.2 Experimental protocol.

Normal human volunteers without a history of recent illness and not on medication were bled from the antecubital vein into heparinized tubes as described in Section 4.2.2. The bloods were then diluted 1:1 with HBSS and centrifuged through a Ficoll-Hypaque discontinuous gradient (S.G. 1.077) as described in Section 4.2.2. The mononuclear cells at the interface were washed and suspended in culture medium as described in Section 4.2.2. The mononuclear cells were

depleted of monocytes, using <sup>P</sup>Carbonyl iron, as described in Section 4.2.7. The T cells were isolated by rosetting the MNC with SRBC as described in Section 4.2.5. The T<sub>H</sub> and T<sub>G</sub> cells were isolated by rosetting the T cells with EAG as described in Section 4.2.6. The culture medium used, except where otherwise stated, consisted of RPMI-1640 fortified with penicillin, streptomycin and FCS (final conc.20%) as described in Section 4.1.1.1. Cytoplasmic Ig (cIg) was detected by the immunofluorescent assays (IF) as described in Section 4.2.10 and the IgG and IgM in the culture supernatants were determined by the ELISA assay as described in Section 4.2.11.

#### 5.1.3 Results.

Cultures were set up consisting of equal numbers of T<sub>H</sub> and non-T cells in Medium RPMI-1640 containing penicillin and streptomycin, with or without PWM or FCS, at 37°C in 5% CO<sub>2</sub> in air. The cells were assayed after 2, 5, 7, 10 and 14 days of culture. As can be seen in Table 2, no IF positive cells were detected on day 2, low numbers were detected on day 5 and large numbers on day 7. The percent of IF positive cells decreased significantly by day 10 of culture and a further marked reduction of IF positive cells was observed on day 14 of culture. FCS and PWM were required in order for Ig synthesis to take place; the optimal concentration of the FCS was found to be 20 percent.

It is accepted today, based on previous investigations, that cultures of T<sub>H</sub> helper cells and non-T cells, in a 1:1

ratio ( $2.5 \times 10^5$  cells of each per ml of culture medium) are superior to cultures consisting of unfractionated T cells and non-T cells, or unfractionated MNC, in the facilitation of Ig synthesis by the B cells in vitro (90-93). The different cell combinations obtained from the same individual were therefore cultured for 7 days and assayed for Ig synthesis. As can be seen in Table 3, the cultures consisting of  $T_M$  and non-T cells (1:1) consistently generated the highest percentage of Ig synthesizing cells after 7 days in culture. The non-T cells in the absence of T cells failed to synthesize Ig.

The optimal ratio of  $T_M$  cells to non-T cells in culture which promotes maximum synthesis of Ig by the cultured B cells and/or synthesis of Ig by the highest percentage of B cells in culture was investigated. As can be seen from the data presented in Table 4, the culture of equal numbers of  $T_M$  and non-T cells resulted in the highest percentage of immunofluorescent positive cells. When the number of  $T_M$  cells in the culture was increased by 4 fold, the percentage of IF positive cells was diminished to insignificant numbers (Table 4).

The relationship between the percent of IF positive cells and the IgG and IgM secreted into the culture medium by the B cells was investigated as a function of the time in culture. A good correlation was observed between these two parameters, Ig synthesis and Ig secreted, on days 3, 5 and 7 of culture (Table 5). The amounts of IgM secreted by

the cultured cells, especially on day 7 of culture, was at least 3 fold higher than the amount of IgG secreted. Since in these and other experiments not reported upon here IgM was always secreted in much larger amounts than was IgG and IgG was never found to be secreted in the absence of secretion of IgM, only IgM was subsequently assayed for in the culture supernatants.

T<sub>H</sub> and non-T cells (1:1) were cultured with PWM in culture medium for up to 28 days. The cells were assayed for Ig (IgG, IgM and IgA) synthesis by immunofluorescence at 2 or 3 day intervals. The supernatants were replaced on days 7, 14 and 21 with fresh culture medium and PWM, and the IgM secreted by the cultured cells into the culture supernatants was determined on days 3, 5 and 7 and in the culture supernatants of days 7-14, 14-21 and 21-28. As can be seen in Table 6, the maximum percent of IF positive cells was consistently observed after 7 days of culture. By day 14, the percent of IF positive cells diminished markedly and reached baseline levels by day 21. On the other hand, although IgM was minimally secreted on day 5 of culture and its secretion increased sharply by day 7 of culture, it continued to be secreted in greater amounts between days 7 to 14 and days 14 to 21 of culture in spite of the fact that the cells were no longer IF positive. Large quantities of IgM were secreted between days 21 and 28 of culture (Table 6).

The question which was then addressed was the role of

the monocyte in the synthesis of Ig by cultured cells in vitro. Cultures consisting of monocyte-depleted  $T_M$  and non-T cells in the presence of PWM did not synthesize or secrete Ig (Table 7). Monocytes could partially restore Ig synthesis if they were added to the cultured cells to a final concentration of 5 percent. Ig synthesis was fully restored when the monocytes were added to the cultures to a final concentration of 10 percent of the cultured cells (Table 7).

#### 5.1.4 Discussion.

The cellular composition of the cultures required to facilitate Ig synthesis, and the culture conditions which facilitate maximum Ig synthesis, were established. It was confirmed that maximum synthesis of Ig by the cultured B cells takes place by day 7 of culture and that T cells and monocytes are obligatory participants in the synthesis of Ig by the B cells. Pokeweed mitogen and FCS (final conc. 20 percent) were absolutely required in the cultures of the circulating T and non-T cells (containing monocytes) for Ig synthesis to occur. It was also shown that maximum Ig synthesis takes place when the B cells are cultured with the  $T_M$  helper cells and not with the unfractionated T cells. This may best be explained by the fact that the unfractionated T cells consist of  $T_M$  helper cells (90-93) and  $T_S$  suppressor cells (90-93). The suppressor cells are capable of inhibiting Ig synthesis by the B cells. Normally, the balance of activity is tilted in favour of the  $T_M$  helper

cells. Obviously, removal of the  $T_s$  suppressor cells from the T cells results in the  $T_H$  helper cells stimulating the B cells in an unrestricted manner. Maximum Ig synthesis by the B cells took place when the  $T_H$  and non-T cells (containing the B cells) were cultured in a 1:1 ratio. When lesser or greater numbers of  $T_H$  cells relative to the B cells were present in the culture, the percent of Ig synthesizing B cells decreased.

The secretion of IgM and IgG by the B cells into the culture medium mirrored the synthesis of Ig by the B cells, as determined by immunofluorescence, up to day 7 of culture. The amount of IgM secreted by the cells after 7 days of culture was invariably much greater than the amount of IgG secreted. Furthermore, at no time was IgG secreted in the absence of a greater amount of IgM secreted. It was therefore decided to henceforth only determine the levels of IgM in the culture medium as an index of secreted Ig. IgG was assayed occasionally to verify that it is secreted whenever IgM is secreted, and that it is not secreted when IgM is not secreted.

The finding that IgM is secreted in large amounts between days 21 and 28, when the percent of IF positive cells is negligible (suggesting no active synthesis of Ig), was most unexpected. Nevertheless, it was consistently observed in 6 experiments that the amounts of IgM, and to a lesser extent IgG, secreted into the culture media is very high on days 7 to 14, 14 to 21 and 21 to 28 of culture even

though, very few, if any, of the cultured cells are IF positive. These results indicate that there is no correlation between Ig synthesis (as detected by IF) and secretion (as detected by ELISA) after day 7 of culture. The B cells can secrete large amounts of Ig into the culture medium in spite of immunofluorescent evidence suggesting no detectable intracellular Ig synthesis. It is possible that, after day 7 of culture, the B cells can secrete Ig as rapidly as it is being synthesized and thereby it is not detected by IF. However, during the maturational stage of Ig synthesis (days 0 to 7 of culture), the capacity to synthesize Ig precedes the acquisition of the capacity to secrete Ig. This would explain the good correlation between Ig synthesis as detected by IF and Ig secreted as determined by the ELISA assay up to day 7 of culture. These results make it mandatory to utilize both the immunofluorescent assay and the ELISA technique in order to obtain the maximum information in investigations related to Ig synthesis by B cells in vitro, especially in long-term culture experiments.

In all of the tables, the terms "insignificant", "significant", "marked" and "optimal" refer to the percentage of immunofluorescent cells and the amounts of Ig secreted by the B cells in culture relative to the values of the cells in the control cultures. This approach towards the interpretation of the results is appropriate in this case in view of the individual variations which are displayed by the cells of different individuals.

TABLE 2

IMMUNOGLOBULIN SYNTHESIS BY CULTURES OF T<sub>H</sub> AND NON-T CELLS AS DEFINED BY IMMUNOFLUORESCENCE, IS DEPENDENT UPON THE CONCENTRATION OF FCS AND THE PRESENCE OF PWM IN THE CULTURE MEDIUM AND IS OPTIMAL ON DAY 7.

Cells (T <sub>H</sub> and non-T cells) <sup>a</sup> cultured for the following days in the presence(+) or absence(-) of PWM	The concentration of FCS in the culture medium (percent conc.)	The percentage of IF positive cells		
		I	II	III
0(+) <sup>b</sup>	20	2	3	2
2(+)	20	0	0	0
5(+)	20	3	6	4
7(+)	20	18	28	24
7(-)	20	2	1	0
10(+)	20	12	18	13
14(+)	20	5	7	8
14(-)	20	0	1	0
7(+)	0	3	5	4
7(+)	10	11	13	10
7(+)	40	12	10	13

a  $2 \times 10^5$  T<sub>H</sub> and  $2 \times 10^5$  non-T cells were cultured in 1 ml of medium.

b These cells were cultured for 15 minutes before being assessed.

TABLE 3

CULTURES OF T<sub>M</sub> AND NON-T CELLS ARE SUPERIOR TO CULTURES OF EITHER UNFRACTIONATED T CELLS AND NON-T CELLS OR UNFRACTIONATED MNC IN THE SYNTHESIS OF I<sub>g</sub>.

Cells cultured for 7 days in culture medium (total $4 \times 10^5$ cells in 1 ml)	The percentage of Ig positive cells after 7 days of culture in the presence of PWM		
	I	II	III
MNC	10	7	13
T + non-T (1:1)	11	12	19
T <sub>M</sub> + non-T (1:1)	22	24	30
non-T	0	1	0

TABLE 4

IMMUNOGLOBULIN SYNTHESIS BY THE B LYMPHOCYTES IS A FUNCTION OF THE RATIO OF T<sub>H</sub> TO NON-T CELLS (NON-T CELLS ARE CONSTANT).

Non-T cells ( $2 \times 10^5$ ) cultured with the following numbers of T <sub>H</sub> cells	Ratio of T <sub>H</sub> to non-T cells in culture (non-T cells constant)	The percentage of IF positive cells after 7 days in culture in the presence of PWM		
		I	II	III
Nil	0:1	0	0	1
$1 \times 10^5$	1:2	6	13	13
$2 \times 10^5$	1:1	18	28	22
$4 \times 10^5$	2:1	8	12	10
$8 \times 10^5$	4:1	1	3	4

TABLE 5

THE RELATIONSHIP BETWEEN IMMUNOGLOBULIN (Ig) SYNTHESIS DETECTED BY CYTOPLASMIC IMMUNOFLOURESCENCE AND Ig (IgG AND IgM) SECRETED BY THE CULTURED CELLS DETECTED BY THE ELISA TECHNIQUE.

T <sub>H</sub> and non-T cells cultured in culture medium for the following days	Ig synthesis and Ig secreted by the cells cultured for 3, 5 or 7 days in the presence of PWM								
	I			II			III		
	ISC <sup>a</sup>	IgM <sup>b</sup>	IgG <sup>c</sup>	ISC	IgM	IgG	ISC	IgM	IgG
3	2	<56	<56	2	<56	<56	3	<56	<56
5	8	845	212	5	662	134	12	592	312
7	24	1840	555	22	2600	640	30	4420	475

<sup>a</sup> The percentage of immunoglobulin synthesizing cells (ISC) determined by immunofluorescence.

<sup>b</sup> ng IgM per ml of cell-free culture medium determined by the ELISA technique.

<sup>c</sup> ng IgG per ml of cell-free culture medium determined by the ELISA technique.

TABLE 6

ABSENCE OF CORRELATION BETWEEN THE PERCENTAGE OF IMMUNOGLOBULIN SYNTHESIZING CELLS AND IGM SECRETED INTO THE CULTURE MEDIUM BY THESE CELLS.

T <sub>H</sub> and non-T cells cultured in the presence of PWM	Percentages of Ig synthesizing cells <sup>1</sup> at the following days of culture									IgM (ng per ml) secreted into the culture medium during the following periods of culture (days) <sup>2</sup>					
	3	5	7	10	14	18	21	24	28	0-3	0-5	0-7	7-14	14-21	21-28
Exp. 1	2	4	17	11	4	4	4	5	3	<56	695	2876	5429	4404	2540
Exp. 2	1	5	20	12	7	3	1	<1	<1	<56	844	2540	5578	2083	1916
Exp. 3	1	4	28	ND	10	ND	5	ND	4	125	168	1440	1032	1148	582

<sup>1</sup> Determined by Immunofluorescence.

<sup>2</sup> Determined by the ELISA technique.

TABLE 7

IMMUNOGLOBULIN (Ig) SYNTHESIS AND SECRETION BY THE B CELLS IN CULTURE WITH T<sub>H</sub> HELPER CELLS, NULL CELLS AND PWM IS DEPENDENT UPON THE PRESENCE OF MONOCYTES.

Exp. no.	Cells cultured in the presence of PWM	Ig synthesis and secretion by the cells cultured for			
		7 days		14 days	
		ISC <sup>1</sup>	ngIgM <sup>2</sup>	ISC	ngIgM
1	T <sub>H</sub> + non-T	21	2250	12	1958
	T <sub>H</sub> + non-T + 0.5 non-T <sup>3</sup>	21	2198	15	2934
	T <sub>H</sub> (MD) <sup>4</sup> + non-T(MD) <sup>4</sup>	1	<56	0	<56
	T <sub>H</sub> (MD) + non-T(MD) + M:(2) <sup>5</sup>	6	255	5	492
	T <sub>H</sub> (MD) + non-T(MD) + M:(5)	15	1228	9	1457
	T <sub>H</sub> (MD) + non-T(MD) + M:(10)	21	2051	14	2387
2	T <sub>H</sub> + non-T	20	1609	7	1156
	T <sub>H</sub> + non-T + 0.5 non-T	18	1587	5	1782
	T <sub>H</sub> (MD) + non-T(MD)	0	<56	0	<56
	T <sub>H</sub> (MD) + non-T(MD) + M:(2)	0	<56	1	110
	T <sub>H</sub> (MD) + non-T(MD) + M:(5)	9	501	3	397
	T <sub>H</sub> (MD) + non-T(MD) + M:(10)	17	1571	6	1782
3	T <sub>H</sub> + non-T	25	3073	15	4387
	T <sub>H</sub> + non-T + 0.5 non-T	21	2654	11	3782
	T <sub>H</sub> (MD) + non-T(MD)	4	68	0	<56
	T <sub>H</sub> (MD) + non-T(MD) + M:(2)	9	345	0	119
	T <sub>H</sub> (MD) + non-T(MD) + M:(5)	18	2278	4	2300
	T <sub>H</sub> (MD) + non-T(MD) + M:(10)	20	2986	11	3436
4	T <sub>H</sub> + non-T	16	1723	7	1098
	T <sub>H</sub> + non-T + 0.5 non-T	19	1371	8	1192
	T <sub>H</sub> (MD) + non-T(MD)	0	<56	0	<56
	T <sub>H</sub> (MD) + non-T(MD) + M:(2)	0	<56	0	<56
	T <sub>H</sub> (MD) + non-T(MD) + M:(5)	6	452	5	613
	T <sub>H</sub> (MD) + non-T(MD) + M:(10)	15	1371	8	1523

1 The percentage of immunoglobulin synthesizing cells (ISC) determined by immunofluorescence.

2 ngIgM per ml of cell-free culture medium determined by the ELISA technique.

3 10<sup>5</sup> non-T cells were added to the conventional culture of 2 x 10<sup>5</sup> T<sub>H</sub> cells and 2 x 10<sup>5</sup> non-T cells.

4 Monocyte depleted T<sub>H</sub> and non-T cells. The T<sub>H</sub> and non-T cells were obtained from MNC twice depleted of monocytes before and after incubation of the cells in the culture medium for 24 hours at 37°C.

5 The figures in the brackets represent the percent monocytes in the cultures.

5.2 The obligatory participation of Null lymphocytes in the synthesis and secretion of immunoglobulins (Ig) by the cultured B cells.

5.2.1 Rationale and objectives.

In a previous investigation from this laboratory (144), it was demonstrated that the Null lymphocytes which, along with the B lymphocytes constitute the non-T lymphocytes of the circulating MNC, are required in culture with the  $T_M$  cells, monocytes, B cells and PWM in order for the B cells to synthesize Ig. However, the cells were cultured for only 7 days and it is possible that Ig synthesis was only delayed in the absence of the Null cells. Furthermore, only the immunofluorescent technique was utilized to detect the intracellular synthesis of Ig; the amount of Ig secreted into the culture medium was not assayed. It is possible that Ig could be secreted by the cultured B cells without the cells being IF positive if Ig synthesis is altered in the absence of the Null cells.

The objective of this series of experiments was to demonstrate that Ig fails to be synthesized and secreted by the B cells in the absence of the Null cells in the culture.

5.2.2 Experimental protocol.

Normal volunteers were bled via the antecubital vein into heparinized sterile tubes as described in Section 4.2.2. The bloods were centrifuged through a discontinuous gradient of Ficoll-Hypaque (S.G. 1.077) and the MNC were isolated as described in Section 4.2.2. The  $T_M$  cells, B

cells and Null cells were isolated as described in Section 4.2.6. The cells were cultured for Ig synthesis as described in Section 4.2.8. Ig synthesis was determined using the immunofluorescent assay as described in Section 4.2.10. The amount of IgM and IgG secreted into the culture media was determined by the ELISA technique as described in Section 4.2.11.

### 5.2.3 Results.

Only the cultures consisting of T<sub>M</sub> and non-T cells (which include B and Null cells) and the cultures consisting of T<sub>M</sub> cells, B cells and Null cells (both of which contained monocytes at a concentration greater than 10 percent) synthesized and secreted Ig up to 21 days in culture (Table 8). Cultures consisting of T<sub>M</sub> and B cells or T<sub>M</sub> and Null cells neither synthesized nor secreted Ig for the duration of the 21 day culture period (Table 8).

The effect of decreasing the numbers of either the B cells or the Null cells in the culture on the synthesis and secretion of Ig by the cultured cells was investigated. Decreasing the Null cells 4 fold resulted in only a 40 to 50 percent drop in the amount of Ig secreted by the cells and a decrease of only 15 to 30 percent in the percent of IF positive cells (Table 9). Even when the Null cells were diluted 16 fold, low but still significant quantities of Ig were secreted into the culture medium although no IF positive cells were detected. On the other hand, decreasing the B cells by only 2 fold resulted in a 50 to 80 percent

decrease in the percent of IF positive cells and a 60 to 70 percent decrease in the quantity of secreted Ig. When the B cells were diluted 4 fold, the percentages of IF positive cells were at, or near, baseline and the amounts of Ig secreted by the cells of 2 individuals were at baseline levels (Table 9). These results strongly indicate that it is the B cells and not the Null cells which synthesize and secrete Ig, and that only a relatively small number of Null cells are required in the culture to facilitate Ig synthesis by the B cells.

The question which was then addressed was - how long into the culture can the Null cells or the B cells be added in order for Ig synthesis and secretion to take place by day 7 of culture? It was observed that significant Ig synthesis and secretion took place even if the addition of the Null cells to the cultured  $T_M$  and B cells was delayed until day 4 of culture (Table 10). On the other hand, addition of the B cells to cultured  $T_M$  and Null cells on day 2 of culture resulted in the detection of very low levels of Ig by both the immunofluorescent and ELISA assays (Table 10).

The question was asked whether Ig synthesis and secretion is simply delayed, rather than abrogated, when the B cells are added to the cultured  $T_M$  and Null cells subsequent to the initiation of culture. Cultures were set up consisting of  $T_M$  cells and Null cells (in the presence of PWM). Autologous B cells, cultured by themselves in culture medium at 37°C, were added to the cultures of the  $T_M$  and

Null cells on days 0, 2, 4 or 6 of the 7 day culture. The cultures were terminated on day 7 relative to the  $T_M$  and Null cell cultures, and on days 5 and 7 following the addition of the B cells to the  $T_M$  and Null cell cultures. It was observed that Ig synthesis and secretion took place only if the B cells were added to the cultures of  $T_M$  and Null cells within the first 2 days, irrespective of the length of time the cultures were left after the addition of the B cells (Table 11).

It was speculated that the failure to observe significant Ig synthesis and secretion may be due to degenerative changes occurring in the B cells cultured in medium prior to their addition to the  $T_M$  and Null cell cultures. The B cells were therefore cultured in culture medium in the presence of  $T_M$  cells and PWM and washed prior to their addition to the cultures consisting of  $T_M$  cells and Null cells. Significant Ig synthesis and secretion was now observed even when the B cells were added to the cultures of  $T_M$  and Null cells 6 days following the initiation of the culture and assayed 7 days later (Table 12). These results indicate that the B cells were maintained in a viable state susceptible to activation into Ig synthesis by the co-cultured  $T_M$  cells and Null cells.

#### 5.2.4 Discussion.

It had previously been demonstrated that the B cells must be cultured with the  $T_M$  cells, monocytes, PWM and Null cells in order to synthesize Ig. The objective of this

series of experiments was to assess the relationship between the Null cells and the B cells in the culture leading to Ig synthesis by the B cells, and to measure Ig secreted into the cultures by the ELISA technique.

The results of the experiments carried out in this part of the investigation demonstrated that neither Ig synthesis nor secretion took place in the absence of the Null cells, even when the culture period was extended to 21 days. In the absence of other findings, it could be speculated that it is the Null cells, and not the B cells, which synthesize and secrete Ig. However, all of the evidence presented strongly indicates that it is the B cells which synthesize and secrete Ig and that the Null cells have a helper or support function which can be expressed even if they are present in less than optimal numbers. Decreasing the number of B cells in the cultures by only 2 fold resulted in a drastic decrease in Ig synthesis and secretion. Furthermore, the B cells could synthesize and secrete Ig in culture even when the Null cells were added to the cultures (consisting of  $T_H$  cells, B cells, monocytes and PWM) as late as day 4 of the 7 day culture. Thus, the Null cell signal is not required by the B cell until late in the culture and only 3 days before maximum synthesis and large-scale secretion of Ig takes place. On the other hand, the addition of viable B cells to cultures of  $T_H$  cells, Null cells, monocytes and PWM required an additional 7 days before the B cells could synthesize and secrete Ig. All of these findings support the conclusion

that it is the B cells which synthesize and secrete Ig and that the role of the Null cells is strictly a supportive one.

TABLE 8

B CELLS CULTURED IN THE PRESENCE OF T<sub>H</sub> HELPER CELLS,  
MONOCYTES AND PWM, BUT IN THE ABSENCE OF NULL CELLS  
DO NOT SYNTHESIZE AND SECRETE Ig<sub>α</sub> IRRESPECTIVE OF THE  
LENGTH OF TIME OF THE CULTURE PERIOD.

Exp. no.	Cells cultured in the presence of PWM and monocytes <sup>1</sup>	Percent ISC <sup>2</sup> on day			IgM (ng per ml) secreted into the culture supernatants during the following periods of culture (days) <sup>3</sup>		
		7	14	21	0-7	7-14	14-21
1	T <sub>H</sub>	<1	<1	<1	<56	<56	<56
	Non-T <sup>4</sup>	<1	<1	<1	<56	<56	<56
	B	<1	<1	<1	<56	<56	<56
	Null	<1	<1	<1	<56	<56	<56
	T <sub>H</sub> + Non-T	19	12	7	1525	1908	2740
	T <sub>H</sub> + B	1	<1	<1	<56	<56	67
	T <sub>H</sub> + Null	2	<1	<1	117	73	60
T <sub>H</sub> + B + Null	21	10	4	2228	3763	4020	
2	T <sub>H</sub>	2	<1	<1	<56	<56	<56
	Non-T	4	<1	<1	198	<56	<56
	B	1	<1	<1	212	96	93
	Null	<1	<1	<1	116	<56	<56
	T <sub>H</sub> + Non-T	23	15	7	2265	1650	1920
	T <sub>H</sub> + B	3	<1	<1	<56	<56	<56
	T <sub>H</sub> + Null	2	<1	<1	130	<56	<56
T <sub>H</sub> + B + Null	18	10	5	2653	2456	2163	

- 1 The percentage of monocytes in all the cultures was invariably greater than 10 percent.
- 2 The percentage of immunoglobulin synthesizing cells (ISC) was determined by immunofluorescence (IF).
- 3 Determined by the ELISA technique.
- 4 Non-T cells = B cells + Null cells.



TABLE 9

IMMUNOGLOBULIN SYNTHESIS AND SECRETION AS A FUNCTION OF THE CONCENTRATION OF B AND NULL CELLS IN THE SEVEN DAY CULTURE.

T <sub>H</sub> cells(2 x 10 <sup>5</sup> ) cultured with PWM and			Percentage of cells in culture		Percentage of Ig synthesizing cells <sup>1</sup>			IgM secreted into the cell-free culture supernatants(ng per ml) <sup>2</sup>		
Non-T	B	Null	B cells	Null cells	Exp. 1	Exp. 2	Exp. 3	Exp. 1	Exp. 2	Exp. 3
2 x 10 <sup>5</sup>	-	-	25	25	19	17	14	2613	2645	2670
-	2 x 10 <sup>5</sup>	-	50	0	7	3	5	508	190	310
-	-	2 x 10 <sup>5</sup>	0	50	2	2	2	103	50	68
-	2 x 10 <sup>5</sup>	2 x 10 <sup>5</sup>	33	33	23	16	12	2200	2792	2714
-	2 x 10 <sup>5</sup>	1 x 10 <sup>5</sup>	40	20	15	14	9	1860	2263	1900
-	2 x 10 <sup>5</sup>	0.5 x 10 <sup>5</sup>	44	11	16	11	10	1650	1072	1434
-	2 x 10 <sup>5</sup>	0.25 x 10 <sup>5</sup>	47	6	12	2	4	810	224	475
-	2 x 10 <sup>5</sup>	0.13 x 10 <sup>5</sup>	48	3	2	4	1	318	210	310
-	1 x 10 <sup>5</sup>	2 x 10 <sup>5</sup>	20	40	10	7	3	765	1140	916
-	0.5 x 10 <sup>5</sup>	2 x 10 <sup>5</sup>	11	44	3	6	1	110	475	66
-	0.25 x 10 <sup>5</sup>	2 x 10 <sup>5</sup>	6	47	3	6	<1	113	250	146
-	0.13 x 10 <sup>5</sup>	2 x 10 <sup>5</sup>	3	49	2	4	<1	56	206	173

1 The percent of Ig synthesizing cells was determined by immunofluorescence.

2 Determined by the ELISA technique.

TABLE 10

THE B CELLS SYNTHESIZE AND SECRETE IMMUNOGLOBULINS  
PROVIDING THE NULL CELLS ARE ADDED BY DAY 6 OF  
THE 7 DAY CULTURE.

Cells cultured for 7 days in the presence of PWM	Cells added to the cultures on the following day of the 7 day culture		Percentage of Ig synthesizing cells <sup>1</sup>		IgM secreted into the cell-free culture supernatant (ng per ml) <sup>2</sup>	
	Null	B	Exp 1	Exp 2	Exp 1	Exp 2
T <sub>H</sub> + B	-	-	<1	1	<56	<56
T <sub>H</sub> + B	0	-	23	19	2793	2594
T <sub>H</sub> + B	2	-	21	15	2665	2834
T <sub>H</sub> + B	4	-	18	6	2105	1266
T <sub>H</sub> + B	6	-	8	4	972	854
-----						
T <sub>H</sub> + Null	-	-	<1	1	81	<56
T <sub>H</sub> + Null	-	0	23	19	2793	2594
T <sub>H</sub> + Null	-	2	5	7	266	508
T <sub>H</sub> + Null	-	4	5	4	324	191
T <sub>H</sub> + Null	-	6	0	1	59	<56

1 Determined by Immunofluorescence.

2 Determined by the ELISA technique.

TABLE 11

B CELLS LOSE THE CAPACITY TO SYNTHESIZE IMMUNOGLOBULINS  
AFTER CULTURE AT 37°C FOR 4 DAYS IN THE ABSENCE OF THE T<sub>H</sub>  
CELLS, NULL CELLS AND PWM.

Cells cultured with PWM <sup>1</sup> on day 0 (primary cultures)	B cells <sup>2</sup> added on the following day after the initiation of the primary cultures	Cultures terminated on the following days after the initiation of the primary cultures	Ig synthesis and secretion by the B cells.			
			Percentage of Ig synthesizing cells <sup>3</sup>		IgM secreted into the cell- free culture medium (ng per ml) <sup>4</sup>	
			Exp. 1	Exp. 2	Exp. 1	Exp. 2
T <sub>H</sub> + Null	-	7	0	<1	<56	<56
T <sub>H</sub> + Null	0	7	24	18	2492	2298
T <sub>H</sub> + Null	2	7	5	8	1092	672
T <sub>H</sub> + Null	2	9	6	6	969	693
T <sub>H</sub> + Null	4	7	<1	2	<56	<56
T <sub>H</sub> + Null	4	9	4	2	<56	<56
T <sub>H</sub> + Null	4	11	<1	3	<56	<56
T <sub>H</sub> + Null	6	7	0	0	<56	<56
T <sub>H</sub> + Null	6	11	<1	0	<56	<56
T <sub>H</sub> + Null	6	13	<1	<1	<56	<56

1  $2 \times 10^5$  T<sub>H</sub> cells were cultured with  $2 \times 10^5$  Null cells and PWM  
in 1 ml of culture medium.

2  $2 \times 10^5$  B cells in 0.1 ml of culture medium were added to the cultures  
of T<sub>H</sub> cells, Null cells and PWM. The B cells were kept at 37°C until  
they were added to the primary cultures.

3 Determined by immunofluorescence.

4 Determined by the ELISA technique.

TABLE 12

IMMUNOGLOBULIN SYNTHESIS BY B CELLS PRECULTURED WITH T<sub>H</sub> CELLS AND PWM PRIOR TO THEIR ADDITION TO ONGOING CULTURES OF T<sub>H</sub> CELLS, NULL CELLS AND PWM.

Cells cultured with PWM <sup>1</sup> on day 0 (primary cultures)	Precultured B cells <sup>2</sup> added on the following day after the initiation of the primary cultures	Cultures terminated on the following days after the initiation of the primary cultures	Ig synthesis and secretion by the B cells			
			percentage of Ig synthesizing cells <sup>3</sup>		IgM secreted into the cell-free culture medium (ng per ml) <sup>4</sup>	
			Exp. 1	Exp. 2	Exp. 1	Exp. 2
T <sub>H</sub> + Null	-	7	<1	<1	<56	<56
T <sub>H</sub> + Null	0	7	21	20	2270	2554
T <sub>H</sub> + Null	2	7	11	15	1707	1327
T <sub>H</sub> + Null	2	9	15	19	1920	1153
T <sub>H</sub> + Null	4	7	2	<1	130	<56
T <sub>H</sub> + Null	4	9	12	11	795	867
T <sub>H</sub> + Null	4	11	13	9	693	764
T <sub>H</sub> + Null	6	7	<1	<1	<56	<56
T <sub>H</sub> + Null	6	11	9	5	713	795
T <sub>H</sub> + Null	6	13	7	9	815	926

- 1 2 x 10<sup>5</sup> T<sub>H</sub> cells were cultured with 2 x 10<sup>5</sup> Null cells and PWM in 1 ml of culture medium.
- 2 2 x 10<sup>6</sup> B cells were cultured with 2 x 10<sup>6</sup> T<sub>H</sub> cells and PWM in 1 ml of culture medium at 37°C. On the appropriate day, 0.1 ml of this cell suspension, containing 2 x 10<sup>5</sup> B cells, was added to the primary cultures.
- 3 Determined by immunofluorescence.
- 4 Determined by the ELISA technique.

5.3 The Null cells secrete a factor in culture, immunoglobulin synthesis/secretion facilitating factor or ISFF, which can replace the Null cells in the synthesis of Ig by the B cells cultured with T<sub>H</sub> helper cells, monocytes and PWM.

5.3.1 Rationale and objectives

The results presented in the previous section of this Chapter demonstrated that the Null cells have an obligatory participating role in the synthesis of Ig by the B cells. It was demonstrated that the B cells could synthesize (to some extent) and secrete (significant) quantities of Ig even if the Null cells were added as late as day 6 of the 7 day culture. These results suggest that the B cells require the signal provided by the Null cells only after they have attained a certain degree of maturity and that they do not require input by the Null cells prior to attaining that level of competence or maturity.

The objective of the following experiments was to demonstrate whether the Null cells secrete a factor in culture which can replace the Null cells in the synthesis of Ig by B cells cultured in the presence of T<sub>H</sub> cells, monocytes and PWM.

5.3.2 Experimental protocol.

The methods used to isolate the circulating MNC, the T cells, the T<sub>H</sub> helper cells, the non-T cells, the B cells and the Null cells from whole blood have been described in the previous section (Section 5.2.2). In order to demonstrate

whether the Null cells secrete a replacement factor, Null cells were cultured in the presence of  $T_H$  helper cells, monocytes, B cells and PWM (to mimic the conditions in which they are normally cultured for Ig synthesis) for up to 7 days and the cell-free culture supernatants were retained as described in Section 4.2.9. In other instances, the Null cells were cultured with only the  $T_H$  cells, monocytes and PWM for 7 days. The culture supernatant obtained after 7 days, of culture is referred to as the 7 day conditioned medium or 7DCM. The number in front of the "DCM" refers to the days of culture of the cells providing the DCM. These DCMs were used as culture media for the culture of B cells,  $T_H$  helper cells, monocytes and PWM for 7 days. The percent of Ig synthesizing cells was determined by immunofluorescence as described in Section 4.2.10 and the amounts of IgM secreted by the cultured cells was determined by the ELISA technique described in Section 4.2.11.

### 5.3.3 Results.

As can be seen in Table 13, B cells cultured for 7 days with  $T_H$  cells, monocytes, PWM and Null cells ( $T_H$  plus non-T cells) synthesized and secreted Ig, whereas the B cells cultured for 7 days with  $T_H$  cells, monocytes and PWM in culture medium could neither synthesize nor secrete Ig. However, when these latter cells were cultured in a 7DCM obtained from 7 day cultures of  $T_H$  and non-T cells in the presence of PWM, the B cells generated a normal percentage of IF positive cells and they secreted normal quantities of

IgM into the culture medium. These results suggested that a factor is present in the 7DCM of  $T_M$  plus non-T cell cultures which can replace the Null cells in Ig synthesis by the B cells. It should be noted that in 2 of the 3 experiments presented in Table 13, there was a heightened secretion of Ig when the  $T_M$  and non-T cells were cultured in 7DCM as compared to their culture in culture medium.

Can the B cells, cultured by themselves with PWM for 7 days in the 7DCM (obtained from 7 day cultures of  $T_M$  cells, non-T cells and PWM) synthesize and secrete Ig? As can be seen in Table 13A, the answer is no. The B cells require the presence of  $T_M$  cells in addition to the PWM in order to synthesize and secrete Ig.

A question which was then addressed was - what is the cell-source of this Null cell replacement factor referred to as ISFF? In order to resolve this question, different combinations of cells were cultured for 7 days and the cell-free culture supernatants were used as culture media for the 7 day cultures consisting of B cells,  $T_M$  cells, monocytes and PWM. As can be seen in Table 14, cultures of  $T_M$  cells and non-T cells, in the absence of PWM, did not secrete ISFF. ISFF was only detected in the 7 DCM obtained from Null cells cultured in the presence of PWM, PWM and  $T_M$  helper cells, or PWM and IL-2 (Table 14). Null cells cultured for 7 days with  $T_M$  helper cells, monocytes and PWM in these 7DCM were not able to synthesize or secrete Ig in any significant amounts.

Is ISFF actively synthesized and secreted by the Null cells or is it simply released following lysis of the cells? In order to resolve this question, cells were cultured in culture medium, 7DCM or filtered Null cell sonicates (FNCS). As can be seen in Table 15, the sonicates of the Null cells (FNCS) did not stimulate the B cells, cultured in the presence of  $T_M$  cells, monocytes, and PWM, to synthesize Ig. On the other hand, 7DCM obtained from the 7 day cultures of Null cells,  $T_M$  cells, monocytes and PWM facilitated the synthesis and secretion of Ig by the cultured B cells.

It was investigated whether the Null cells require FCS in the culture in order to secrete ISFF. As can be seen in Table 16, FCS was required in the culture in order for the Null cells to secrete a factor which facilitated the synthesis of Ig (detected by immunofluorescence) by the cultured B cells. However, in the absence of FCS, the Null cells secreted a factor which facilitated low but significant secretion of Ig by the cultured B cells (detected by ELISA) (Table 16).

It was investigated as to whether ISFF functions in an HLA restricted manner. 7DCM of cultures ( $T_M$  cells, Null cells and PWM) of 4 unrelated donors were used as culture media for the culture of B cells,  $T_M$  cells and PWM of each of the other donors. As can be seen in Table 17, the 7DCM of each of these donors could facilitate Ig synthesis by the B cells of the other donors, thus demonstrating that the ISFF is not HLA restricted in its activity.

Experiments were carried out to determine whether ISFF can be secreted by the Null cells in the absence of monocytes and whether the B cells require monocytes in culture in order to synthesize and secrete Ig in the presence of preformed ISFF. As can be seen in Table 18, the Null cells cultured for 7 days in the presence of  $T_M$  cells and PWM, but in the absence of monocytes, did not secrete ISFF into the culture medium as was demonstrated by the inability of the B cells, when cultured in this 7DCM, to synthesize and secrete Ig. Furthermore, the B cells cultured in the presence of  $T_M$  cells and PWM, but in the absence of monocytes, did not synthesize nor secrete Ig even though ISFF was present in the culture (Table 19). These results indicate that the monocytes play an accessory role in the synthesis of Ig by the B cells in addition to their requirement for the secretion of ISFF by the Null cells (Table 19 and Table 18, respectively).

What is the mechanism of action of ISFF? B cells were cultured in the presence of  $T_M$  cells, monocytes and PWM in a 7DCM (obtained from 7 day cultures of  $T_M$  cells, Null cells and PWM) containing ISFF. The 7DCM containing PWM was replaced by culture medium and PWM once on days 1 to 6 of the 7 day culture. As can be seen in Table 20, ISFF had to be present in the cultures up to day 6 in order for the B cells to secrete optimal (or near optimal) quantities of Ig. Replacement of the ISFF by culture medium prior to day 4 resulted in failure to synthesize and secrete Ig by day 7 of

culture (Table 20).

The above experiment was also carried out in reverse. The cells were cultured initially in culture medium which was replaced by 7DCM containing ISFF (obtained from a 7 day culture of Null cells and  $T_M$  cells and PWM) during the 7 day culture (days 1 to 6). As can be seen in Table 21, normal percentages of Ig synthesizing cells, and normal quantities of Ig secreted into the culture medium, were recorded providing the 7DCM was added to the cultured B cells and  $T_M$  cells in the presence of PWM prior to day 6 of culture. If the replacement of culture medium by the 7DCM was delayed until day 7 of the 7 day culture, then the synthesis and secretion of Ig decreased precipitously (Table 21). Thus, it appears that ISFF must be present with the B cells during the last two days of the 7 day culture period in order for optimal Ig synthesis and secretion to take place.

The question which was then addressed was - does ISFF facilitate more rapid synthesis and secretion of Ig by the cultured B cells? The results presented in Table 22 definitely imply that it does. Cultures of  $T_M$  and B cells in 7DCM synthesized and secreted Ig on day 5 as did cultures of  $T_M$  cells, B cells and Null cells. However, cultures of  $T_M$  cells, B cells and Null cells cultured in culture medium failed to synthesize and secrete Ig on day 5 of culture.

The question as to when during the culture period the Null cells secrete ISFF was investigated. As can be seen in Table 23, Null cells cultured with  $T_M$  cells and PWM secreted

functional quantities of ISFF by day 3 of culture.

However, maximum amounts of ISFF were not secreted until day 7, the last day of the culture.

#### 5.3.4 Discussion.

The results of the experiments carried out in the previous section (Chapter 5.2) demonstrated that the Null lymphocytes are obligatory participants in Ig synthesis by the B cells cultured in the presence of  $T_M$  helper cells, monocytes and PWM. However, the mechanism of action of the Null cell, that is whether it carries out its function via a secreted factor or via cell-cell contact, was not touched upon. The objective of this series of experiments was to demonstrate whether the Null cell secretes a soluble factor which can replace the Null cell in its ability to facilitate Ig synthesis by the B cells.

It was indeed shown that the Null cells secrete a factor(s) in culture, referred to as immunoglobulin synthesis/secretion facilitating factor or ISFF, which can replace Null cells in the culture. The factor is secreted in optimal functional amounts by the Null cells cultured for 7 days with  $T_M$  helper cells, monocytes and PWM or with IL-2 and PWM. Null cells cultured by themselves, or in the presence of only PWM, either did not secrete significant quantities of ISFF or did not secrete ISFF in a functional state.

It is noteworthy that the B cells, cultured in the supernatant of a 7 day culture of  $T_M$  cells, non-T cells and

PWM (referred to as 7DCM which includes ISFF, PWM and interleukins secreted by the T<sub>M</sub> helper cells), nevertheless still require the presence of viable T<sub>M</sub> helper cells in order to synthesize and secrete Ig. B cells cultured alone in the presence of added interleukin cocktail (IL-2, BCDF, BCGF) and PWM did not synthesize and secrete Ig. These results indicate that the transformation, proliferation and differentiation of the B cells into Ig synthesizing and secreting cells requires physical contact with the T<sub>M</sub> helper cells, or that the 7DCM and/or the interleukin cocktail is deficient in one or more T<sub>M</sub> derived soluble mediators in the absence of which Ig synthesis and secretion cannot take place.

( ISFF activity is not HLA-restricted as it can restore Ig synthesis to allogeneic B cells in culture. ISFF need be in contact with the B cells, cultured in the presence of T<sub>M</sub> cells, monocytes and PWM, for only the last 1 or 2 days of the 7 day culture period. It appears that the B cells are not receptive to the Null cell signal until they have attained a certain level of maturity or functional state.

The Null cells require the presence of monocytes in order to secrete ISFF. Furthermore, even in the presence of ISFF, the monocytes must be present in the culture for the B cells to synthesize and secrete Ig. Thus, Ig synthesis is the result of a complex series of interactions which take place in an orderly sequence and which finally culminate in

Ig synthesis by the B cells. The series of interactions may be conceptualized as follows:

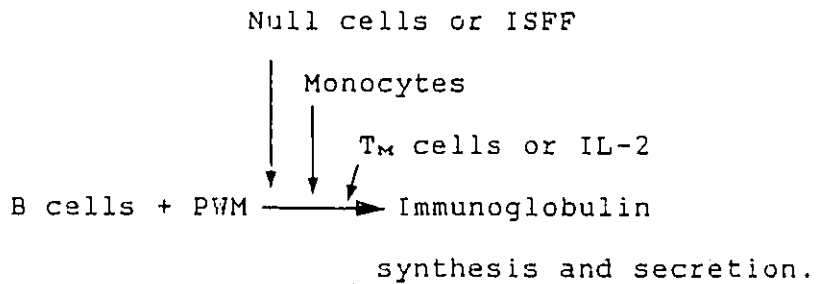
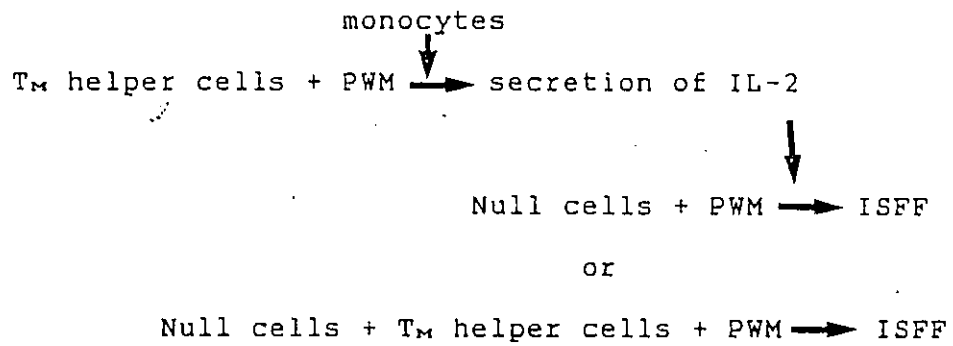


TABLE 13

A COMPARISON OF THE SYNTHESIS AND SECRETION OF IMMUNOGLOBULIN (Ig) BY THE B CELLS CULTURED IN CULTURE MEDIUM OR IN THE SUPERNATANTS OF 7 DAY CULTURES OF T AND NON-T CELLS IN THE PRESENCE OF PWM (7DCM).

Cells cultured in the presence of PWM for 7 days	Medium used		Ig synthesis and secretion by the cultured cells					
	culture medium	7DCM <sup>a</sup>	percent ig synthesizing cells <sup>b</sup>			ig secreted (ng per ml) <sup>c</sup>		
			Exp 1	Exp 2	Exp 3	Exp 1	Exp 2	Exp 3
T <sub>H</sub> + non-T	YES	NO	14	18	13	1875	1500	1276
T <sub>H</sub> + B	YES	NO	1	3	1	<56	<56	<56
T <sub>H</sub> + non-T	NO	YES	16	17	13	3111 <sup>d</sup>	2174 <sup>e</sup>	1314 <sup>f</sup>
T <sub>H</sub> + B	NO	YES	17	13	9	5250 <sup>d</sup>	2880 <sup>e</sup>	1217 <sup>f</sup>

<sup>a</sup> The cell-free supernatant of a 7 day culture of T<sub>H</sub> cells, non T cells and PWM.

<sup>b</sup> Determined by immunofluorescence.

<sup>c</sup> ng IgM per ml cell-free culture supernatant as determined by the ELISA technique.

<sup>d</sup> Includes IgM present in 7DCM after 7 days of culture (324 ng per ml).

<sup>e</sup> Includes IgM present in 7DCM after 7 days of culture (667 ng per ml).

<sup>f</sup> Includes IgM present in 7DCM after 7 days of culture (320 ng per ml).

TABLE 13A

B CELLS CULTURED IN 7 DCM (SUPERNATANT OF A 7 DAY CULTURE OF T<sub>H</sub> CELLS, NON-T CELLS AND PWM) REQUIRE T<sub>H</sub> HELPER CELLS IN ORDER TO SYNTHESIZE AND SECRETE I<sub>g</sub>.

Cells cultured <sup>a</sup> with PWM for 7 days in the 7DCM	I <sub>g</sub> synthesis and secretion by the cultured cells <sup>b</sup>			
	ISC <sup>c</sup>		I <sub>g</sub> M secreted <sup>d</sup> (ng per ml)	
	Exp.1	Exp.2	Exp.1	Exp.2
T <sub>H</sub> + NON-T	21	22	2032 <sup>e</sup>	2353 <sup>e</sup>
T <sub>H</sub> + B	18	15	1844	2048
T <sub>H</sub> + NULL	5	3	<56	<56
B + NULL	7	ND <sup>f</sup>	<56	ND
B	4	6	<56	<56
B + IL-2/BCDF/BCGF	6	ND	<56	ND
NULL	5	6	<56	<56
NULL + IL-2/BCDF/BCGF	4	ND	<56	ND

- a The concentration of monocytes in the cultures was invariably greater than 10%.
- b None of the cultures synthesized or secreted I<sub>g</sub> if PWM was absent from the culture.
- c Percent I<sub>g</sub> synthesizing cells as determined by immunofluorescence.
- d Determined by the ELISA technique.
- e Each value represents the ng I<sub>g</sub>M in the cell-free culture supernatant less the ng I<sub>g</sub>M in the 7DCM used in the culture.
- f Not done.

TABLE 14

THE ABILITY OF SUPERNATANTS OF SEVEN DAY CULTURES (7DCM) OF DIFFERENT CELLS TO FACILITATE Ig SYNTHESIS AND SECRETION BY B CELLS IN THE PRESENCE OF T<sub>H</sub> HELPER CELLS AND PWM.

Supernatants of 7 day cultures of the following cells (7DCM)	Ig synthesis and secretion by B cells in the presence of T <sub>H</sub> cells, monocytes and PWM after 7 days of culture in the 7DCM				Ig synthesis and secretion by Null cells in the presence of T <sub>H</sub> cell, monocytes and PWM after 7 days of culture in the 7DCM			
	percent Ig synthesizing cells <sup>a</sup>		IgM secreted (ng per ml) <sup>b</sup>		percent Ig synthesizing cells		IgM secreted (ng per ml)	
	Exp. 1	Exp. 2	Exp. 1	Exp. 2	Exp. 1	Exp. 2	Exp. 1	Exp. 2
T <sub>H</sub> + non-T	2	4	<56 <sup>c</sup>	<56 <sup>c</sup>	1	0	<56 <sup>c</sup>	<56 <sup>c</sup>
T <sub>H</sub> + non-T + PWM	17	14	1180	1078	5	2	<56	256
T <sub>H</sub> + B + Null + PWM	14	14	1024	1098	2	<1	<56	<56
T <sub>H</sub> + B + PWM	5	4	<56	<56	2	<1	104	<56
T <sub>H</sub> + Null + PWM	16	10	2127	1020	3	<1	264	<56
Null + PWM	8	3	1060	642	3	<1	94	98
Null + IL-2 + PWM	13	7	2057	850	2	<1	<56	110
Null + IL-2	2	<1	146	94	<1	<1	<56	<56
B + PWM	3	<1	<56	<56	1	<1	80	<56
T <sub>H</sub> + PWM	6	2	370	<56	1	<1	264	<56
Non-T + PWM	12	17	1057	1005	3	1	513	<56
-----								
B cells cultured in the presence of T <sub>H</sub> cells, Null cells, monocytes and PWM for 7 days in culture medium	20	18	2106	2148	2	2	<56	<56

<sup>a</sup> Determined by immunofluorescence.

<sup>b</sup> Determined by the ELISA technique.

<sup>c</sup> Each value represents the ng IgM in the cell-free culture supernatant less the ng IgM in the 7DCM used in the culture as determined by the ELISA technique.

TABLE 15

ISEE IS ACTIVELY SYNTHESIZED AND SECRETED BY THE NULL CELLS AND IS NOT SIMPLY RELEASED FOLLOWING LYSIS OF THE FRESHLY ISOLATED CELLS.

Cells cultured in the presence of PWM for 7 days <sup>a</sup>	Medium used in the culture	Ig synthesis and secretion by the cultured B cells					
		Percent Ig synthesizing cells <sup>b</sup>			IgM secreted (ng per ml) <sup>c</sup>		
		Exp 1	Exp 2	Exp 3	Exp 1	Exp 2	Exp 3
T <sub>H</sub> + non-T	culture medium	21	18	20	1567	2200	1367
T <sub>H</sub> + B + Null	culture medium	23	21	20	1443	2145	1516
T <sub>H</sub> + B	culture medium	2	0	3	<56	<56	117
T <sub>H</sub> + non-T	7DCH <sup>d</sup>	18	18	22	2196	2020	1620
T <sub>H</sub> + B + Null	7DCH	19	18	16	1982	1944	1563
T <sub>H</sub> + B	7DCH	24	15	16	1648	1700	1335
T <sub>H</sub> + non-T	FNCS <sup>e</sup>	20	17	21	1280	1510	1442
T <sub>H</sub> + B + Null	FNCS	20	18	19	1263	1263	1325
T <sub>H</sub> + B	FNCS	1	1	0	<56	96	<56

- a The concentration of monocytes in the cultures was invariably greater than 10 percent.
- b Determined by Immunofluorescence.
- c Determined by the ELISA technique. The values for IgM presented are the ng IgM per ml cell-free culture supernatant less the ng IgM in the different media used.
- d The cell-free supernatant of a 7 day culture of T<sub>H</sub> cells, Null cells and PWM.
- e The filtered Null cell sonicate prepared from a suspension 2 x 10<sup>6</sup> Null cells per ml. This is 10 times the concentration of Null cells used to prepare the 7DCH.

TABLE 16

THE NULL CELLS REQUIRE FETAL CALF SERUM (FCS) IN CULTURE FOR OPTIMAL SYNTHESIS AND SECRETION OF ISFF.

7DCH(ISFF) obtained from 7 day cultures of the following cells in the presence of PWM	Cells cultured in the presence(+) or absence(-) of FCS (final conc. 20 percent)	The synthesis and secretion of Ig by the autologous B cells cultured in the 7DCH in the presence of T <sub>H</sub> cells and PWM percent ISCa IgM secreted (ng per ml) <sup>b</sup>
Exp. 1		
T <sub>H</sub> + Null	+	18
T <sub>H</sub> + Null	- <sup>c</sup>	2
		2822 822
Exp. 2		
T <sub>H</sub> + Null	+	21
T <sub>H</sub> + Null	-	1
		2740 630

<sup>a</sup> Percent Ig synthesizing cells determined by immunofluorescence.

<sup>b</sup> Determined by the ELISA technique.

<sup>c</sup> The medium was fortified with FCS (final conc. 20 percent) prior to its use as culture medium for the B cells and T<sub>H</sub> cells in the presence of PWM.

TABLE 17

ISFF FACILITATES Ig SYNTHESIS BY THE B CELLS IN CULTURE IN THE PRESENCE OF T<sub>H</sub> CELLS IN A HLA INDEPENDENT MANNER.

7DCM (ISFF) obtained from cultures of T <sub>H</sub> cells + Null cells + PWM of donor	Ig synthesis and secretion by B cells of the different donors in the presence of T <sub>H</sub> cells, monocytes, and PWM, cultured for 7 days in 7DCM.							
	B cells of donor A		B cells of donor B		B cells of donor C		B cells of donor D	
	Percent ISC <sup>a</sup>	IgM secreted <sup>b</sup>	Percent ISC	IgM secreted	Percent ISC	IgM secreted	Percent ISC	IgM secreted
A	7	1010	21	1870	18	1635	14	2176
B	7	1867	22	1927	21	1336	20	2013
C	ND <sup>c</sup>	ND	ND	ND	19	2763	17	2518
D	ND	ND	ND	ND	22	2106	26	3067

<sup>a</sup> Percent Ig synthesizing cells determined by immunofluorescence.

<sup>b</sup> ng IgM per ml of cell-free culture supernatant determined by the ELISA technique.

<sup>c</sup> Not done.

TABLE 18

NULL CELLS IN CULTURE REQUIRE MONOCYTES  
IN ORDER TO SECRET ISFF.

7DCM OBTAINED FROM CULTURES OF THE FOLLOWING CELLS	THE SYNTHESIS AND SECRETION OF Ig BY AUTOLOGOUS B CELLS, T <sub>M</sub> CELLS, MONOCYTES AND PWM CULTURED FOR 7 DAYS IN 7DCM.	PERCENT ISC <sup>a</sup>	IgM SECRETED <sup>b</sup>
Exp. 1			
T <sub>M</sub> + Null + PWM (<56) <sup>c</sup>		13	1002
T <sub>M</sub> (MD) <sup>d</sup> + Null(MD) + PWM (<56)		8	118
Exp. 2			
T <sub>M</sub> + Null + PWM (<56)		18	2822
T <sub>M</sub> (MD) + Null(MD) + PWM (<56)		1	56
Exp. 3			
T <sub>M</sub> + Null + PWM (<56)		19	2794
T <sub>M</sub> (MD) + Null(MD) + PWM (<56)		3	120

a Percent Ig synthesizing cells determined by immunofluorescence.  
b ng IgM per ml of cell-free supernatant determined by the ELISA technique.  
c The figures within the brackets are the ng IgM per ml of the 7DCM.  
d MD= monocyte depleted.

TABLE 19

B CELLS IN CULTURE REQUIRE MONOCYTES  
IN ORDER TO RESPOND TO THE ISEF SIGNAL  
TO SYNTHESIZE AND SECRETE Ig.

7DCM (ISFF) OBTAINED FROM CULTURES OF T <sub>M</sub> CELLS, NULL CELLS AND PWM	Ig SYNTHESIS AND SECRETION BY B CELLS IN AUTOLOGOUS 7DCM FOR 7 DAYS WITH T <sub>M</sub> CELLS AND PWM			
	IN THE PRESENCE OF AUTOLOGOUS MONOCYTES		IN THE ABSENCE OF MONOCYTES	
	PERCENT ISC <sup>a</sup>	IgM SECRETED <sup>b</sup>	PERCENT ISC	IgM SECRETED
Exp. 1	.13	1002	1	140
Exp. 2	18	2822	2	83
Exp. 3	19	2794	1	70

a Percent Ig synthesizing cells determined by immunofluorescence.

b ng IgM per ml of cell-free supernatant determined by the ELISA technique.

TABLE 20

THE EFFECT OF REMOVING ISFF FROM THE CULTURE MEDIUM AT DIFFERENT TIMES FOLLOWING THE INITIATION OF CULTURE OF B CELLS, T<sub>H</sub> CELLS AND MONOCYTES IN THE PRESENCE OF PWM.

B cells and T <sub>H</sub> cells - cultured in 7DCH(containing ISFF) <sup>a</sup> in the presence of PWM from the beginning of the 7 day culture. The medium(7DCH containing ISFF) was replaced by culture medium and PWM on following day of the culture	The synthesis and secretion of Ig by the B cells			
	percent Ig synthesizing cells <sup>b</sup>		IgM secreted(ng per ml) <sup>c</sup>	
	Exp. 1	Exp. 2	Exp. 1	Exp. 2
day 1	1	4	205	<56
day 2	2	2	278	<56
day 3	1	4	277	156
day 4	6	4	479	150
day 5	9	7	423	197
day 6	13	8	711	567
day 7(7DCH not replaced)	16	14	923	1145

a The 7DCH is the supernatant of T<sub>H</sub> and Null cells cultured for 7 days in the presence of PWM. The IgM concentration in the 7DCH was less than 56 ng per ml.

b Determined by immunofluorescence.

c Determined by the ELISA technique.

TABLE 21

THE EFFECT, ON Ig SYNTHESIS BY THE B CELLS, OF ADDING ISFF AT DIFFERENT TIMES TO THE CULTURES OF B CELLS, T<sub>H</sub> CELLS, AND MONOCYTES IN THE PRESENCE OF PWM.

Culture medium and PWM replaced by 70CM(containing ISFF) <sup>a</sup> and PWM on the following day of the 7 day culture of B cells and T <sub>H</sub> cells in the presence of PWM	The synthesis and secretion of Ig by the B cells			
	percentage Ig synthesizing cells <sup>b</sup>		IgM secreted(ng per ml) <sup>c</sup>	
	Exp. 1	Exp. 2	Exp. 1	Exp. 2
Day 0 <sup>d</sup>	22	24	2716	2683
Day 2	17	19	2927(176) <sup>e</sup>	2727(118)
Day 3	23	24	2705(107)	2650(296)
Day 4	18	ND	2517(151)	2783(418)
Day 5	22	11	3505(98)	2628(163)
Day 6	10	12	2109(96)	1184(140)
Day 7	5	6	1029(96)	185(121)
-----				
CONTROL CULTURES (7 days)				
T <sub>H</sub> + B + PWM	2	1	108	88
T <sub>H</sub> + B + Null + PWM	20	22	2657	2256

<sup>a</sup> The 70CM is the supernatant of T<sub>H</sub> and Null cells cultured for 7 days in the presence of PWM. The IgM concentration in the 70CM was 256 ng per ml.

<sup>b</sup> Determined by immunofluorescence.

<sup>c</sup> Determined by the ELISA technique.

<sup>d</sup> The cells were cultured in 70CM and PWM for the entire 7 days of culture.

<sup>e</sup> The figures outside and inside the brackets are the conc. of IgM after 7 days of culture and at the time of addition of the ISFF to the cultures, respectively.

TABLE 22

ISFF FACILITATES MORE RAPID SYNTHESIS AND SECRETION OF Ig BY B CELLS CULTURED WITH T<sub>H</sub> CELLS AND PWM.

Cells cultured for 7 days in the presence of PWM	Cells cultured in	Ig synthesis and secretion on the following days during the 7 day culture period							
		2		3		5		7	
		ISC <sup>a</sup>	IgH <sup>b</sup>	ISC	IgH	ISC	IgH	ISC	IgH
T <sub>H</sub> + B	7DCH	3	118	3	318	7	2275	16	2563
T <sub>H</sub> + B	culture medium	1	<56	2	<56	2	<56	2	<56
T <sub>H</sub> + B + Null	7DCH	5	<56	3	272	6	1516	22	2216
T <sub>H</sub> + B + Null	culture medium	3	96	2	<56	2	112	17	1906
T <sub>H</sub> + non-T	7DCH	2	59	2	603	6	1428	19	2208
T <sub>H</sub> + non-T	culture medium	2	<56	0	<56	1	206	19	1384
T <sub>H</sub> + B	7DCH	0	191	0	316	11	1067	17	1774
T <sub>H</sub> + B	culture medium	0	<56	1	<56	3	<56	3	213
T <sub>H</sub> + B + Null	7DCH	2	125	2	<56	14	1008	20	2002
T <sub>H</sub> + B + Null	culture medium	2	<56	0	<56	4	203	21	1605
T <sub>H</sub> + non-T	7DCH	2	<56	3	191	10	832	20	1900
T <sub>H</sub> + non-T	culture medium	1	<56	1	<56	3	280	23	1280
T <sub>H</sub> + B	7DCH	1	84	1	88	5	985	18	2175
T <sub>H</sub> + B	culture medium	1	<56	1	<56	1	<56	1	<56
T <sub>H</sub> + B + Null	culture medium	1	<56	1	<56	5	120	17	2078
T <sub>H</sub> + B	7DCH	2	68	9	<56	8	900	16	3702
T <sub>H</sub> + B	culture medium	3	<56	1	<56	1	255	1	120
T <sub>H</sub> + B + Null	culture medium	2	<56	2	<56	5	145	17	3790

<sup>a</sup> The percent ISC (Immunoglobulin synthesizing cells) determined by immunofluorescence.

<sup>b</sup> ng IgH secreted per ml of cell-free culture medium determined by the ELISA technique (ng IgH per ml of cell-free culture medium less the ng IgH in the 7DCH or culture medium used).

TABLE 23

THE SECRETION OF ISEF BY THE NULL CELLS  
INCREASES WITH THEIR TIME IN CULTURE.

Supernatants obtained. from cultures of T <sub>H</sub> cells + Null cells + PWM on the following day of culture	Ig synthesis and secretion by B cells <sup>a</sup> , cultured for 7 days in the 70CM in the presence of T <sub>H</sub> cells and PWM					
	percent Ig synthesizing cells <sup>b</sup>			IgM secreted (ng per ml) <sup>c</sup>		
	1	2	3	1	2	3
day 1	2	6	<1	<56	300	<56
day 3	1	8	<1	436	712	<56
day 5	18	9	10	844	968	696
day 7	22	22	18	1790	1549	1457

<sup>a</sup> These B cells, cultured with T<sub>H</sub> cells, Null cells and PWM for 7 days in culture medium, generated 21 percent Ig synthesizing cells and secreted 2144 ng IgM per ml of culture supernatant.

<sup>b</sup> Determined by immunofluorescence.

<sup>c</sup> Determined by the ELISA technique.

#### 5.4 Some physicochemical properties of ISFF

##### 5.4.1 Rationale and objective.

In the experiments presented in the previous section, it was demonstrated that the Null cells secrete a factor in culture which can replace the Null cells in the synthesis of Ig by the B cells cultured in the presence of the  $T_H$  helper cells, monocytes and PWM. This factor was referred to as immunoglobulin synthesis/secretion facilitating factor or ISFF.

The objective of the following experiments was to characterize ISFF.

##### 5.4.2 Experimental protocol

The method used for the preparation of the 7DCM containing ISFF was described in the previous section (section 5.3.2). The ISFF was dialyzed for 24 hours against medium RPMI-1640 through dialysis tubing with mol. wt. cut-offs of 14,000 and 50,000. ISFF in 7DCM was also kept at 4°C, 37°C, 56°C or 63°C for varying periods of time (up to 120 hrs). In each experiment, whether the ISFF was dialyzed or kept at the various temperatures, the ISFF was assayed for ISFF activity, which is its capacity to restore Ig synthesizing and secreting capability to the B cells cultured in the presence of  $T_H$  cells, monocytes and PWM (as described in the previous section).

##### 5.4.3 Results

As can be seen from Table 24, when ISFF was dialyzed for 24 hrs through dialysis tubing with mol. wt. cut-offs

of 14,000 or 50,000, ISFF retained its ability to facilitate immunoglobulin synthesis by the cultured B cells, at levels only slightly reduced from those of the control cultures (20%-30% reduction as compared to control). However, the amounts of Ig secreted by these same B cells were very markedly reduced as compared to the control (70%-80% reduction).

ISFF did not lose its activity when cultured for up to 72 hours at either 4°C or 37°C (Table 25). However, ISFF incubated for 120 hours at 37°C lost essentially all its activity whereas the ISFF incubated for 120 hours at 4°C retained its ability to facilitate Ig synthesis by the cultured B cells and was minimally able to stimulate the secretion of Ig from these same cells. This experiment was only carried out once and must definitely be repeated.

As can be seen from Table 25, ISFF exposed to 56°C for even 15 minutes resulted in a major loss of activity. ISFF was totally inactivated following exposure to 56°C for 2 hours. Similarly, heating the ISFF at 63°C for only 15 minutes resulted in a total loss of activity.

#### 5.4.4 Discussion.

It was demonstrated, on the basis of dialysis and temperature stability experiments, that ISFF may be composed of 2 functional factors - one factor which is capable of facilitating intracellular Ig synthesis as detected by immunofluorescence microscopy, and a second factor which facilitates the secretion of Ig by the

cultured B cells. The former factor is not lost following dialysis for 24 hours through the dialysis tubing with mol. wt. cut-offs of 14,000 or 50,000 , nor is it lost following incubation at 4°C for 120 hours. The second factor, however, is lost following dialysis through the dialysis tubing with a cut-off mol. wt. of 14,000 and is greatly reduced following incubation at 4°C for 120 hours.

It may be concluded that ISFF is stable when incubated for 3 days at either 4°C or 37°C. However, ISFF is not stable at temperatures higher than 37°C as it loses essentially all activity following exposure to 56°C for 2 hours or to 63°C for 15 minutes. It is therefore very susceptible to denaturation by high temperature.

TABLE 24

THE ABILITY OF DIALYZED ISFF TO RESTORE THE CAPACITY FOR IMMUNOGLOBULIN SYNTHESIS AND SECRETION TO B CELLS CULTURED IN THE PRESENCE OF T<sub>H</sub> CELLS, MONOCYTES AND PWM.

ISFF dialyzed through dialysis tubing with a molecular weight cut-off of	Ig synthesis and secretion by the B cells, cultured in the presence of T <sub>H</sub> cells, monocytes, PWM and the dialyzed ISFF, after 7 days of culture.			
	percent ISC <sup>a</sup>	Exp. 1 ng IgM/ml <sup>b</sup>	percent ISC	Exp. 2 ng IgM/ml
14,000	16	238	13	412
50,000	14	281	16	87
-----				
Control - not dialyzed	22	1807	18	1395

<sup>a</sup> Percent Ig synthesizing cells, as determined by immunofluorescence.  
<sup>b</sup> ng IgM per ml of culture supernatant, as determined by the ELISA technique.

TABLE 25

THE STABILITY OF ISFF FOLLOWING INCUBATION AT 4°C, 37°C, 56°C OR 63°C FOR VARYING PERIODS OF TIME.

ISFF incubated at the following temperature for the following period of time		Ig synthesis and secretion by the B cells, cultured in the presence of T <sub>H</sub> cells, monocytes, PWM and the treated ISFF, after 7 days of culture	
		Percent ISC <sup>a</sup>	ng Ig/ml <sup>b</sup>
37°C	24 hr	21	2308
37°C	48 hr	23	1123
37°C	72 hr	15	1153
37°C	120 hr	6	120
4°C	24 hr	24	3291
4°C	48 hr	20	2749
4°C	72 hr	22	2452
4°C	120 hr	18	509
56°C	15 min	11	1102
56°C	2 hr	2	<56
63°C	15 min	4	<56
63°C	2 hr	<1	<56

<sup>a</sup> Percent Ig synthesizing cells, as determined by immunofluorescence.

<sup>b</sup> ng IgM per ml culture supernatant, as determined by the ELISA technique.

## 6. GENERAL DISCUSSION

Following the demonstration in the early 1970s by a number of investigators that Ig synthesis by B cells can be induced in in vitro culture (64,65), the primary emphasis in subsequent research was the establishment of the optimal in vitro conditions for Ig synthesis - the physical conditions and the cell composition. It was quickly established (68-70) that the optimal physical conditions are  $2 \times 10^5$  to  $10^6$  cells per ml of culture medium fortified with fetal calf serum (FCS) to a final conc. of 20 percent, the requirement of pokeweed mitogen (PWM) in the culture, and a culture period of 7 days. It was also demonstrated that the maximum percent of immunoglobulin - synthesizing cells is attained if equal numbers of  $T_H$  helper cells and non-T (B) cells are cultured. Investigators did not culture beyond 7 days and appeared to be satisfied that optimal Ig synthesis, as detected by immunofluorescence (IF), is optimal after 7 days in culture. This assumption was based on the fact that the percent of IF positive cells, that is the percent of cultured cells actively synthesizing immunoglobulin (Ig), is about equal to the number of B cells cultured. It should be recalled that approximately 60 to 70 percent of circulating lymphocytes are T cells, 10 to 20 percent are B cells and 15 to 25 percent are Null cells. However, the percent of T, B and Null cells does not remain static during the culture period since both the T cells (by day 3 of culture) and the B cells (by day 6 of culture)

undergo blastogenesis, mitosis and proliferation in response to the PWM. It may be just a coincidence that the percent of B cells in the cultures after 7 days of culture is the same as at the initiation of culture. Nevertheless, it is true that the percent of IF positive cells is maximum around day 7 of culture. However, the assumption that there is an absolute relationship between IF and Ig synthesis is not correct. As was shown in this investigation, the cultured cells secreted more Ig between days 14 to 21 than around day 7. Yet, the percent IF positive cells is negligible between days 14 to 21. One explanation for this finding is that, initially, the B cells attain the capacity to synthesize Ig faster than the capacity to secrete it; therefore, there will initially be a build-up of Ig in the cytoplasm which can be detected by the immunofluorescence assay. However, within a short period of time, the B cells learn to secrete the Ig as quickly as it is synthesized. Therefore, no Ig (immunofluorescent positivity) will be detected in the cells. It is therefore necessary to assess Ig synthesis not just by immunofluorescence which detects intracellular Ig, but also by ELISA to determine the quantity of Ig secreted by the cells.

Up to 1985, investigators were unanimous in their assertion that Ig synthesis involves the participation of only two lymphocyte lines - the T cells and the B cells (see Chapter 3). This conclusion was based on the assumption that non-T cells are B cells, and vice versa. As

was discussed in Chapter 3.6, the non-T cells consist of at least two cell types or lineages - the B cells and the Null cells. These two cell populations exhibit different response profiles to stimulants in vitro and they possess different surface "antigens" and receptors which distinguish one cell type from the other (Table 1). The confusion in the minds of investigators which prompted them, in the early to mid 1970s to consider all non-T cells to be B cells was due to the observation that a very high percentage, sometimes greater than 80 percent, of the freshly isolated circulating non-T cells possess surface membrane Ig (smIg). However, following incubation of the non-T cells for 1 hour at 37°C, the Null cells shed their smIg while the B cells do not. The smIg on the Null cell is not, therefore, a constituent of the surface membrane but is rather a dissociable protein, one which is normally bound to the receptor for FcG on the Null cell surface which is not present on B cells.

In 1985, a publication from this laboratory (144) stressed the role of the Null cell as an obligatory participant in the synthesis of Ig by the B cells. It was demonstrated that, irrespective of the numbers of T<sub>H</sub> helper cells, B cells or monocytes in the culture, or the concentration of PWM, no Ig synthesis took place in the absence of the Null cells. However, this publication has two short-comings: (1) only the immunofluorescence assay was utilized to detect Ig synthesis, and (ii) the cultures

were only maintained for the conventional 7 days.

It was subsequently considered quite possible that the B cells may simply be delayed in their capacity to synthesize Ig in the absence of Null cells and that the cells should be cultured for periods longer than 7 days. Furthermore, it is also possible that Ig synthesis is disturbed or deranged in the absence of the Null cells so that the Ig may be expelled by the B cell as quickly as it is synthesized and would therefore not be detected by immunofluorescence. However, Ig would then be detected in the culture supernatant using the very sensitive ELISA assay.

The objectives of this investigation therefore were (i) to demonstrate unequivocally that B cells, cultured in the presence of  $T_M$  cells, monocytes and PWM, neither synthesize nor secrete Ig irrespective of the duration of the culture period, (ii) to identify the mechanism of participation of the Null cell in Ig synthesis by the B cells, that is, whether the Null cell secretes a factor which can replace it or whether cell-cell contact is essential, and (iii) to characterize the secreted factor, if a factor is secreted, in terms of its physicochemical properties.

It was indeed demonstrated that the Null cells secrete a factor, after 7 days of incubation at 37°C, which can replace these cells in the synthesis of Ig by the B cells cultured in the presence of the  $T_M$  helper cells, monocytes and PWM. This factor, referred to as

immunoglobulin synthesis/secretion facilitating factor or ISFF is maximally secreted by the Null cells providing  $T_M$  cells or IL-2, monocytes, and PWM are present. However, Null cells in the absence of detectable  $T_M$  cells can secrete lesser amounts of ISFF, providing monocytes and PWM are present. Since the Null cells appear to be invariably contaminated with small numbers of  $T_M$  cells (<3%), it is possible that these contaminating  $T_M$  cells provide sufficient signal to the Null cells to facilitate the secretion of some ISFF. What is more certain is that the secretion of ISFF is monocyte and PWM dependent since no detectable ISFF is secreted in the absence of either monocytes or PWM in the culture. It is therefore obvious that the secretion of ISFF by the Null cells is the response to at least 3 signals. However, it must be determined whether these signals are delivered simultaneously to the Null cells and must be in the medium for the duration of culture, or whether they are delivered sequentially to the Null cells.

It is interesting to note that the B cells do not respond to the Null cell-derived signal, ISFF, until day 5 to 6 of the 7 day culture period. Thus, B cells cultured for 7 days in the presence of  $T_M$  helper cells and PWM will synthesize and secrete almost maximum Ig as long as the ISFF is added to the cultures by day 5 or 6 of culture. Thus, the B cells must undergo maturational changes in culture, under the influence of the  $T_M$  cells, monocytes and

PWM signals, before they can respond to ISFF and synthesize and secrete Ig. These results support the hypothesis that ISFF is the signal delivered by the Null cells to the B cells. Null cells must be added to the cultures of B cells, T<sub>H</sub> cells and PWM prior to day 4 of the culture; yet ISFF can be added to the cultures as late as day 6. Since it takes 2 to 3 days for the Null cells to secrete detectable quantities of ISFF, it is necessary for the Null cells to be added to the cultured B cells several days earlier than the ISFF in order to achieve the same results - the synthesis and secretion of Ig by the cultured B cells.

The monocyte appears to play a very important role, if not a pivotal role, in Ig synthesis by the B cells since it is required for the secretion of ISFF by the Null cells and for the response of the B cells to the ISFF signal. Whether these activities by the monocytes can be attributed to IL-1 secreted by the monocytes remains to be determined.

The composition of ISFF is not known at the present time. It is stable for only several days in vitro since it loses its B cell stimulating activity if it is kept at 37°C for more than 72 hours. However, ISFF loses most of its activity if exposed to 56°C for 15 minutes and it becomes totally inactive if exposed to 63°C for 15 minutes. Results of preliminary experiments suggest that ISFF consists of two, not one, functional constituents since ISFF dialyzed through dialysis tubing with exclusion mol. wt. 50,000 or 14,000 is still capable of stimulating B cells to

synthesize Ig. However, it is not capable of inducing secretion of Ig by these B cells. One factor, that which facilitates Ig synthesis by the B cells, would appear to have a mol. wt. in excess of 50,000 whereas the other factor, the one which facilitates Ig secretion by the B cells, would appear to have a mol. wt. lower than 14,000. However, these are preliminary experiments and must be repeated.

7. CONCLUSION

Circulating human B cells begin to synthesize and secrete immunoglobulins beginning on the fifth day of culture in the presence of  $T_M$  helper cells, Null cells, monocytes and PWM. Immunoglobulin synthesis reaches a plateau on day 7 of culture. The Null cells are obligatory participants in the synthesis of immunoglobulins by the B cells, as are the  $T_M$  cells, the monocytes and PWM. No immunoglobulin synthesis nor secretion takes place in the absence of the Null cells. The B cells can synthesize and secrete immunoglobulins by day 7 of culture, in the presence of  $T_M$  cells, monocytes and PWM providing the Null cells are added to the cultures by day 4.

The Null cells secrete a factor(s), referred to as immunoglobulin synthesis/secretion facilitating factor(s) or ISFF, following 5 to 7 days in culture with  $T_M$  helper cells, monocytes and PWM. The Null cells can secrete lesser amounts of ISFF in the absence of the  $T_M$  helper cells. The B cells can synthesize and secrete immunoglobulins by day 7 of culture, in the presence of  $T_M$  cells, monocytes and PWM, providing the ISFF is added to the cultures by day 6.

The secretion of ISFF by the Null cells is dependent upon the presence of monocytes and PWM in the culture. Furthermore, the B cells, cultured in the presence of  $T_M$  cells and PWM, can respond to the ISFF signal and synthesize and secrete immunoglobulins providing monocytes are present in the culture.

It is concluded that the synthesis and secretion of immunoglobulins by the B cells is the result of a complex series of sequential interactions between the B cells and the T<sub>M</sub> helper cells, monocytes, Null cells and PWM. In the case of the Null cells, it has been demonstrated in this investigation that the Null cells can be replaced by a factor, ISFF, secreted by the Null cells during their culture with the T<sub>M</sub> cells, monocytes and PWM.

REFERENCES.

1. Shoenheimer, R. The Dynamic State of the Body Constituents. Harvard University Press. 1942.
2. Stiehm, E.R. and V.A. Fulginiti. 1980. Immunologic Disorders in Infants and Children. W.B. Saunders Company, pp.65.
3. Hong, R. 1972. The immunoglobulins. Clin. Immunobiol. 1: 29-34.
4. Stevenson, G.T. 1974. Immunoglobulins, in Structure and Function of the Plasma Proteins. Allison, A.C., Editor. Plenum Press, 1: 223-263.
5. Avrameas, S. and E.H. Leduc. 1970. A receptor for Ab on lymphocytes. J. Exp. Med. 131: 1137-1145.
6. White, R.G. 1960. The relation of the cellular responses in germinal or lymphocytopoietic centers of lymph nodes to the production of antibody, in Mechanisms of Antibody Formation, Holub, M. and L. Jaroskovam, Editors. Prague Publishing House of the Czechoslovak Academy of Sciences, pp. 25-29.
7. Vasquez, J.J. 1961. Antibody or gamma globulin forming cells as observed by the fluorescent antibody technique. Lab. Invest. 10: 1110-1125.
8. Van Furth, R., H.R.E. Schuit and W. Hijmans. 1966. The formation of immunoglobulins by human tissues in vitro. III. Spleen, lymph nodes, bone marrow and thymus. Immunol. 11: 19-26.
9. Van Furth, R. R.H.E. Schuit and W. Hijmans. 1966. The formation of Immunoglobulins by Tissues in vitro. Immunol. 11: 29-40.
10. Yokoyama, M., Y. Koga, K. Taniguchi, H. Nakano and K. Nomoto. 1988. T lymphocytes emigrating from the thymus to the spleen during post-partum regulate serum immunoglobulin levels in mice. Immunol. 63: 151-156.
11. Choi, Y.S. 1978. Biosynthesis and secretion of immunoglobulins, in Comprehensive Immunology, Good, R.A. and S.B. Day, Editors, 5: 345-355.
12. Alley, C.D. 1987. Human bone marrow-derived IgA is produced by IgA-committed B cells in vitro. J. Clin. Immunol. 7: 151-158.

13. Watanabe, S., Y. Yagi. and D. Pressman. 1973. Immunoglobulin production in synchronized cultures of human hematopoietic cell lines. II. Variation of synthetic and secretion activities, during the cell cycle. *J. Immunol.* 111: 797-804.
14. Alley, C.D., R.P. Macdermott, G.S. Nash and M.J. Bartovich. 1983. Suppression of in vitro antibody production by pokeweed mitogen-stimulated human marrow mononuclear cells. Evidence for a soluble suppressor substance. *Immunol.* 50: 387-395.
15. Engle, R.L. and L.A. Willis. 1969. *Immunoglobulinopathies.* Charles C. Thomas, publisher.
16. Claman, H.N., E.A. Chaperon and R.F. Triplett. 1966. Thymus-marrow cell combinations. *Proc. Soc. Exp. Biol. Med.* 122: 1167-1171.
17. Chaperon, E.A., J.C. Selner and H.N. Claman. 1968. Migration of antibody-forming cells and antigen-sensitive precursors between spleen, thymus and bone-marrow. *Immunol.* 14: 553-561.
18. Claman, H.N., E.A. Chaperon and J.C. Selner. 1968. Thymus-derived immunocompetence. III. The requirement for living thymus cells. *Proc. Soc. Exp. Biol. Med.* 127: 462-466.
19. Claman, H.N., E.A. Chaperon and R.F. Triplett. 1966. Immunocompetence of transferred thymus-marrow cell combinations. *Immunol.* 97: 828-832.
20. Mitchell, G.F. and J.A.F.P. Miller. 1968. Cell to cell interaction in the immune response. II. The source of hemolysin-forming cells in irradiated mice given bone marrow and thymus or thoracic duct lymphocytes. *J. Exp. Med.* 128: 821-837.
21. Nossal, G.J.V., A. Cunningham, G.F. Mitchell and J.A. Miller. 1968. Cell to cell interaction in the immune response. III. Chromosomal marker analysis of single antibody-forming cells by reconstituted, irradiated or thymectomized mice. *J. Exp. Med.* 128:839-853.
22. Miller, J.A.P. and G.F. Mitchell. 1967. The thymus and the precursors of antigen reactive cells. *Nature* 216: 659-667.
23. Martin, W.I. and J. Miller. 1968. Cell to cell interaction in the immune response. IV. Site of action of antilymphocyte globulin. *J. Exp. Med.* 128: 855-872.

24. Miller, J.A.F.P., G.F. Miller and N.S. Weiss. 1968. Cellular basis of the immunological defects in thymectomized mice. *Nature* 214: 992-997.
25. Miller, J.A.F.P. and G.F. Mitchell. 1968. Cell to cell interaction in the immune response. I. Hemolysin-forming cells in neonatally thymectomized mice reconstituted with thymus or thoracic duct lymphocytes. *J. Exp. Med.* 128: 801-820.
26. Singal, S.F. and M. Richter. 1968. Cells involved in the immune response. I. The response of normal rabbit bone marrow cells to antigens in vitro.. *Int. Arch. Allergy Appl. Immunol.* 33: 493-500.
27. Singhal, S.K. and M. Richter. 1968. Cells involved in the immune response. IV. The response of normal and immune rabbit bone marrow and lymphoid tissue lymphocytes to antigens in vitro. *J. Exp. Med.* 128: 1099-1128.
28. Abdou, N.I. and M. Richter. 1969. Cells involved in the immune response. V. The migration of antigen-reactive immunocompetent cells of the bone marrow following antigen administration. *Int. Arch. Allergy Appl. Immunol.* 35: 330-334.
29. Richter, M. and N.I. Abdou. 1969. Cells involved in the immune response. VII. The demonstration, using allotypic markers of antibody formation by irradiation-resistant cells of irradiated rabbits injected with allogeneic bone marrow cells and sheep erythrocytes. *Exp. Med.* 129: 1261-1273.
30. Abdou, N.I., B. Rose and M. Richter. 1969. Cells involved in the immune response. VIII. The relationship between the loss and reappearance of antigen-reactive cells and immune responsiveness after irradiation of normal adult rabbits. *J. Exp. Med.* 23: 867-876.
31. Richter, M., B. Rose and N.I. Abdou. 1970. Cells involved in the immune response. XV. The organ source of the antigen-reactive cell(ARC) in the normal rabbit. *Int. Arch Allergy Appl. Immunol.* 38: 269-281.
32. Richter, M., M.E. Berry and P. Barron. 1986. Cells involved in the immune response. XXXI. The role of the spleen in the primary and secondary immune responses in the normal adult outbred rabbit. The initial localization of memory cells to the spleen and their subsequent dissemination to the thymus and the peripheral lymph nodes. *Clin. Immunol. Immunopath.* 38: 101-110.

33. Abdou, N.I. and M. Richter. 1969. Cells involved in the immune response . X. The transfer of antibody-forming capacity to irradiated rabbits by antigen-reactive cells isolated from normal allogeneic rabbit bone marrow after passage through antigen-sensitized glass bead columns. *J. Exp. Med.* 130: 141-163.
34. Humphrey, J.H. and R.G. White. 1970. *Immunology for Students of Medicine.* Blackwell Scientific Publications.
35. Sabet, T., C. Newlin and H. Friedman. 1968. The effect of RES Blocade on cellular antibody formation to sheep erythrocytes. *Proc. Soc. Exp. Biol. Med.* 128: 274-278.
36. Askonas, B.A. and J.M. Rodes. 1965. Immunogenicity of antigen-containing ribonucleic acid preparations from macrophages. *Nature* 205: 470-483.
37. Garvey, J.S. and D.H. Campbell. 1957. The retention of  $S^{35}$ -labelled bovine serum in normal and immunized rabbit liver tissue. *J. Exp. Med.* 105: 361-372.
38. Uhr, J.W. and G. Weisman. 1965. Intracellular distribution and degradation of bacteriophage in mammalian tissues. *J. Immunol.* 94: 544-550.
39. Feldman, M. and J. Palmer. 1971. The requirement for macrophages in the secondary immune response to antigens of small and large molecular weights in vitro. *Immunol.* 21: 685-699.
40. Pierce, C.W., J.A. Kapp, D.D. Wood and B. Benacerraf. 1974. Immune responses in vitro. X. Functions of macrophages. *J. Immunol.* 112: 1181-1194.
41. Kornfeld, L. and W.W.H. Weyzen. 1971. Antibody formation by transformed peritoneal cells and spleen cells from immunized irradiated donors to syngeneic recipients with and without antigens. *J. Ret. Med.* 9: 162-173.
42. Ehrenreich, B.A. and Z.A. Cohn. 1967. The uptake and digestion of iodinated human serum albumin by macrophages in vitro. *J. Exp. Med.* 126: 941-961.
43. Di Luzio, N.R. and W.R. Wooles. 1964. Depression of phagocytic activity and immune response by methyl palmitate. *Am. J. Physiol.* 206: 939-946.
44. Fujiwara, H. and J.J. Elliner. 1986. Spontaneous production of a suppressor factor by the human macrophage-like cell line U937. *J. Immunol.* 136: 181-185.

45. Oppenheim, J.J., S.B. Mizel and M.S. Meltzer. 1979. Comparison of lymphocyte and mononuclear phagocyte derived mitogenic "amplification" factors in biology of lymphokines. Cohen, S., E. Pick and J.J. Oppenheim, Editors. Academic Press, New York, pp. 291-323.
46. Smith, K.A., L.B. Lachman, J.J. Oppenheim and M.F. Favata. 1980. The functional relationship of the interleukins. J. Exp. Med. 151: 1551-1562.
47. Unanue, E.R. and P.M. Allen. 1987. The basis for the immunoregulatory role of macrophages and other accessory cells. Science 236 :551-557.
48. Feldmann, M., A. Rosenthal and P.E. Erb. 1979. Macrophage-lymphocyte interactions in immune induction. Int. Rev. Cytol. 60: 149-156.
49. Zeigler, H.K. and E.R. Unanue. 1982. Decrease in macrophage antigen catabolism caused by ammonia and chloroquine is associated with inhibition of antigen presentation to T cells. Proc. Natl. Acad. Sci. USA 79: 175-194.
50. Zeigler, H.K. and E.R. Unanue. 1981. Identification of a macrophage antigen processing event required for I-region-restricted antigen presentation to T lymphocytes. J. Immunol. 127: 1869-1872.
51. Allen, P.M. 1985. Specificity of the T cell receptor: two different determinants are generated by the same peptide and the I-A\* molecule. J. Immunol. 135: 368-376.
52. Babbit, B.P. et al 1986. Antigenic competition at the level of peptide-Ia binding. Proc. Natl. Acad. Sci. USA 83: 4509-4521.
53. Klaus, G.G.B., J.H. Humphrey, A. Kunkl and D.W. Dongworth. 1980: The follicular dendritic cell: Its role in antigen presentation in the generation of immunological memory. Immunol. Rev. 53: 3-28.
54. Mandel, T.E., R.P. Phipps, A. Abbot and J.G. Tew. 1980. The follicular dendritic cell: Long term antigen retention during immunity. Immunol. Rev. 53: 29-59.
55. Tew, J.G., R.P. Phipps and T.E. Mandel. 1980. The maintenance and regulation of the humoral immune response: Persisting antigen and the role of follicular antigen-binding dendritic cells as accessory cells. Immunol. Rev. 53: 175-201.

56. Guidos, C., A.A. Sinha and K.C. Lee. 1987. Functional differences and complementation between dendritic cells and macrophages in T-cell activation. *Immunol.* 61: 269-276.
57. Silberberg-Sinakin, I., L. Baer and G.J. Thorbecke. 1978. Langerhans Cells. A review of their nature with emphasis on their immunologic functions. *Prog. Allergy.* 24: 268-294.
58. Abdou, N.I. and M. Richter. 1969. Cells involved in the immune response. VI. The immune response to sheep red blood cells in irradiated rabbits following administration of normal, primed or immune allogeneic rabbit bone marrow cells. *J. Exp. Med.* 129: 757-774.
59. Rock, K.L., B. Benaceraff and A.K. Asabbas. 1984. Antigen presentation by hapten-specific B lymphocytes. I. Role of surface immunoglobulin receptors. *J. Exp. Med.* 160: 1002-1113.
60. Chesnut, R.W. and H.M. Grey. 1981. Studies on the capacity of B cells to serve as antigen-presenting cells. *J. Immunol.* 126: 1075-1079.
61. Jones, B. 1987. Cooperation between T and B cells. A minimal model. *Immunol. Rev.* 99: 5-18.
62. Swain, S.C. and R.W. Dutton. 1987. Consequences of the direct interaction of helper T cells with B cells presenting antigen. *Immunol. Rev.* 99:263-280.
63. Wigzell, H. and B. Anderson. 1969. Cell separation on antigen-coated columns. Elimination of high rate antibody forming cells and immunological memory cells. *J. Exp. Med.* 129: 23-26.
64. Cooper, M.D., A.R. Lawton and M.D. Bochman. 1971. Aggamaglobulinaemia with B lymphocytes. Specific defect of plasma-cell differentiation. *Lancet* 2: 791-794.
65. Wu, L.Y.F., A.R. Lawton and M.D. Cooper. 1973. Differentiation capacity of cultured B lymphocytes from immunodeficient patients. *J. Clin. Invest.* 52: 3180-3189.
66. Waldman, T.A., M. Durm, S. Broderm, M. Blackman, R.M. Blaese and W. Strober. 1974. Role of suppressor T cells in pathogenesis of common variable hypogammaglobulinaemia. *J. Exp. Med.* 191: 84-96.

67. Janossy G. and M. Greaves, 1975. Functional analysis of murine and human B lymphocyte subsets. *Transp. Rev.* 24: 177-236.
68. Keightley, R.G., M.D. Cooper and A.R. Lawton. 1976. The T cell dependence of B cell differentiation induced by pokeweed mitogen. *J. Immunol.* 117: 1538-1544.
69. Wu, L.Y.F., A. Blanco, M.D. Cooper and A.R. Lawton. 1976. Ontogeny of B lymphocyte differentiation induced by pokeweed mitogen. *Clin. Immunol. Immunopath.* 5: 208-217.
70. Saxon, A., R.H. Stevens and R.F. Ashman. 1977. Regulation of immunoglobulin production in human peripheral blood leukocytes. *Cellular interactions. J. Immunol.* 118: 1872-1879.
71. Fauci, A.S. and R.E. Ballieux, Editors. 1979. *Antibody Production in Man: In vitro Synthesis and Clinical Implications.* Academic Press.
72. Fauci, A.S. and R.E. Ballieux, Editors. 1982. *Human B lymphocyte Function: Activation and Immunoregulation.* Raven Press.
73. Coombs, R.R.A., A. Freinstein and A.B. Wilson. 1969. Immunoglobulin determinants on the surface of human lymphocytes in mice distinguishable by immunofluorescence. *Immunol.* 19: 637-648.
74. Raff, M.C. 1970. Two distinct populations of peripheral lymphocytes. *Lancet* 2: 1157-1161.
75. Pernis, B., L. Forni and L. Amante. 1970. Immunoglobulin spots on the surface of rabbit lymphocytes. *J. Exp. Med.* 132: 1001-1028.
76. Rabelino, E., S. Colon, H.M. Grey and E.R. Unanue. 1971. Immunoglobulins on the surface of lymphocytes. I. Distribution and quantitation. *J. Exp. Med.* 133: 156-189.
77. Kincade, P.W., A.R. Lawton and M.D. Cooper. 1971. Restriction of surface immunoglobulin determinants to lymphocytes of the plasma cell line. *J. Immunol.* 106: 1421-1438.
78. Froland, S., J.B. Natvig and P. Berdal. 1971. Surface bound immunoglobulin as a marker of B lymphocytes in man. *Nature New Biol.* 234: 251-276.

79. Grey, H.M., E. Rabellino and B. Pirofsky. 1971. Immunoglobulins on the surface of lymphocytes. IV. Distribution in hypogammaglobulinaemia, cellular immunodeficiency. *J. Clin. Invest.* 50: 2368-2378.
  80. Cooper, M.D. and A.R. Lawton. 1972. Circulating B cells in patients with immunodeficiency. *Am. J. Path.* 69: 513-536.
  81. Aiuti, F., L. Fontana and R.A. Gatti. 1973. Membrane bound immunoglobulin (Ig) and in vitro production of Ig by lymphoid cells from patients with primary immunodeficiencies. *Scand. J. Immunol.* 2: 9-12.
  82. Cooper, M.D., R.G. Keightley, L.Y.F. Wu and A.R. Lawton. 1973. Developmental defects of T and B cell lines in humans. *Am. J. Path.* 84: 210-235.
  83. Gerrad, T.L. and A/S/ Fauci. 1981. Activation and immunoregulation of antigen specific human B lymphocyte responses. Multifaceted role of the monocyte. *J. Immunol.* 128: 2367-2372.
  84. Rosenberg, S.A. and P.E. Lipsky. 1981. The role of monocytes in pokeweed mitogen stimulated human B cell activation: separate requirements for intact monocytes and a soluble monocyte factor. *J. Immunol.* 126: 1341-1345.
  85. Theille, D.L. and P.E. Lipsky. 1982. The accessory function of phagocytic cells in human T cell and B cell responses. *J. Immunol.* 129: 1033-1040.
  86. Shin, H.S. and Y.S. Choi. 1981. Role of monocytes in pokeweed mitogen-induced differentiation of human peripheral blood lymphocytes. *Cell. Immunol.* 58: 323-332.
  87. Fernandez, L.A. and J.M. MacSween. 1977. Effects of removal of monocytes by iron filling phagocytosis on mononuclear subpopulations. *J. Immunol. Meth.* 18: 193-196.
  88. Rook, G.A.W. and J. King. 1978. Is the adherent non-specific "suppressor" cell from rodent spleens an in vitro artefact? *Clin. Exp. Immunol.* 32: 466-470.
  89. Musson, R.A. 1983. Human serum induces maturation of human monocytes in vitro. Changes in cytolytic activity intracellular lysosomal enzymes and non-specific esterase activity. *Am. J. Path.* 111: 331-340.
  90. Moretta, L., M. Ferrarini, M.L. Durante and M.C. Mingari. 1975. Expression of a receptor for IgM by human T cells in vitro. *Europ. J. Immunol.* 5: 565-569.
- A

91. Moretta, L., S.R. Webb, C.F. Grossi, P.M. Lydyard and M.D. Cooper. 1977. Functional analysis of two human T-cell subpopulations: help and suppression of B-cell responses by T cells bearing receptors for IgM and IgG. *J. Exp. Med.* 146: 184-200.
92. Moretta, L., M. Ferrarini, A. Mingari, A. Moretta and S.R. Webb. 1976. Subpopulations of human T cells identified by receptors for immunoglobulins and mitogen responsiveness. *J. Immunol.* 117: 2171-2177.
93. Moretta, L., M.C. Mingari, A. Moretta and M.D. Cooper. 1979. Human T lymphocyte subpopulations. Studies of the mechanism by which T cells bearing receptors for IgG suppress T-dependent B cell differentiation induced by pokeweed mitogen. *J. Immunol.* 122: 984-990.
94. Rich, R.R. and C.W. Pierce. 1974. Biological expressions of lymphocyte activation III: Suppression of plaque-forming cell responses in vitro by supernatant fluids from concanavalin-A activated spleen cell cultures. *J. Immunol.* 112: 1360-1366.
95. Zambala, M., G.L. Asherson, B. Mayhew and J. Krefold. 1975. In vitro absorption and molecular weight of specific T cell suppressor factors. *Nature* 353. 72-98.
96. Thieze, J., J.A. Kapp and B. Benacerraf. 1977. Immunosuppressive factor(s) extracted from lymphoid cells of non-responder mice with L-glutamine acid 60-L-alanine 30-L-tyrosine 10 (GAT) III. Immunochemical properties of the GAT-specific suppressive factor. *J. Exp. Med.* 145: 839-840.
97. Gisler, R.H. and W.H. Fridman. 1976. Inhibition of the in vitro 19S and 7S antibody response by immunoglobulin-binding factor (IBS) from alloantigen-activated T cells. *Cell. Immunol.* 23: 99-107.
98. McGarry, R.C., R. Anderson and S.K. Singhal. 1982. Regulation of humoral responses by a bone-marrow-derived glycolipid-like molecule. *Cell. Immunol.* 71: 293-302.
99. Richter, M. and E. Talor. 1986. Cells involved in the immune response. XXXIV. Suppressor cells in the thymus of the immunized rabbit capable of secreting a factor which can suppress the secretion of antibodies from antibody forming cells in vitro. *Clin. Immunol. and Immunopath.* 41: 461-478.

100. Uytdehaag, F., C.J. Heijnen, K.H. Pot and R.E. Ballieux, 1981. Antigen-specific human T cell suppressor factor. Biological properties. *J. Immunol.* 126 : 503-507.
101. Lethibichthay, A., C. Samarut, J. Brochier, W.H. Fridman and J.P. Revillard. 1980. Suppression of mitogen-induced B cell differentiation by soluble Fcy receptors released from lymphocytes. *Eur. J. Immunol.* 10: 894-898.
102. Jelinek, D.F. and P.E. Lipsky. 1983. The role of B cell proliferation in the generation of immunoglobulin-secreting cells in man. *J. Immunol.* 130: 2597-2603.
103. Brenner, M.K., M.E. North, H.R. Chadda and J. Farrant. 1984. Non-specific factor enhancement of human in vitro antigen-dependent antibody-synthesis: Role of B cell activation and T cell help. *Immunol.* 51: 783-787.
104. Chiorazzi, N., S.M. Fu and H.G. Kundel. 1979. Induction of polyclonal antibody synthesis by human allogeneic and autologous helper factors. *J. Exp. Med.* 149: 1543-1548.
105. Reinherz, E.L. and S.F. Schlossman. 1982. Human inducer and suppressor T cell-subset regulation of B-cell immunoglobulin production, in human B-lymphocyte function: Activation and immunoregulation. Fauci, A.S. and R.E. Ballieux - Editors. Raven Press, pp. 201-212.
106. Reinherz, E.L. and S.E. Schlossman. 1980. Current concepts in immunology. Regulation of the immune response - inducer and suppressor T-lymphocyte subsets in human beings. *New Eng. J. Med.* 303: 370-373.
107. Reinherz, E.L., P.C. Kung, G. Goldstein and S.F. Schlossman. 1979. Further characterization of the human inducer cell subset defined by monoclonal antibody. *J. Immunol.* 123: 2894-2896.
108. Reinherz, E.L., C. Morimoto, A.C. Penta and S.F. Schlossman. 1980. Regulation of B cell immunoglobulin secretion by functional subsets of T lymphocytes in man. *Europ. J. Immunol.* 10: 570-572.
109. Reinherz, E.L., P.C. Kung, G. Goldstein and S.F. Schlossman. 1980. A monoclonal antibody reactive with the human cytotoxic/suppressor T cell subset previously defined by a heteroantiserum termed TH<sub>2</sub>. *J. Immunol.* 124: 1301-1307.

110. Reinherz, E.L., L. Moretta, M. Roper, J.M. Breard, M.C. Mingari, M.D. Cooper and S.F. Schlossman. 1980. Human T lymphocyte subpopulations defined by Fc receptors and monoclonal antibodies. A comparison. *J. Exp. Med.* 151: 969-974.
111. Richter, M., G. Ettin, S. Sklar, M. Richter, H. Hamdy, Jodouin, C.A. and N. Kazaniwsky. 1983. Surface receptors and immune activity of purified human circulating mononuclear cells. IV. The demonstration of seven subclasses of T cells in the circulation of the normal individual: The cytotoxic activities of these cells. *Cell. Immunol.* 82: 352-369.
112. Heijnen, C.J., K.H. Pot and R.E. Ballieux. 1982. Characterization of human T suppressor-inducer-precursor and effector lymphocytes in the antigen specific plaque-forming cell response. *Europ. J. Immunol.* 12: 860-866.
113. Heijnen, C.J. and R.E. Ballieux. 1983. Suppressor T-cell circuit in man. *Birth Defects: Original Article Series*, 19: 15-20.
114. Morimoto, C., N.L. Letvin, J.A. Distaso, W.A. Aldrich and S.F. Schlossman. 1985. The isolation and characterization of the human suppressor inducer T cell subset. *J. Immunol.* 134: 1508-1515.
115. Morimoto, C., N.L. Letvin, A.W. Boyd, M. Hagan, H.M. Brown, M.M. Kornacki and S.F. Schlossman. 1985. The isolation and characterization of the human helper inducer T cell subset. *J. Immunol.* 134: 3762-3769.
116. Calvo, C. et al. 1986. Regulation of immunoglobulin synthesis by human T cell subsets as defined by anti-D44 monoclonal antibody within the CD4+ and CD8+ subpopulations. *J. Immunol.* 136: 1144-1149.
117. Dinarello, C.A. 1985. An update on human interleukin-1: from molecular biology to clinical relevance. *J. Immunol.* 5: 287-297.
118. Smith, K.A. and F.W. Ruscetti. 1981. T cell growth factor and the culture of cloned functional T cells. *Ad. Immunol.* 31: 137-175.
119. Durum, S.K., J.A. Schmidt and J.J. Oppenheim. 1985. Interleukin 1: An immunological perspective. *Ann. Rev. Immunol.* 263-281.
120. Dinarello, C. 1984. Interleukin-1. *Reviews of Infectious Diseases* 6: 51-95.

121. Sarin, P.S. and R.C. Gallo. 1984. Human T-cell growth factor (TCGF). *Critical Reviews in Immunol.* 4: 279-333.
122. Howard, M. and W.E. Paul. 1983. Regulation of B-cell growth and differentiation by soluble factors. *Ann. Rev. Immunol.* 1:307-333.
123. Kashimoto, T. 1985. Factors affecting B-cell growth and differentiation. *Ann. Rev. Immunol.* 3: 133-157.
124. Paul, W.E. and J. Ohara. 1987. B-cell stimulatory factor-1/interleukin 4. *Ann.Rev. Immunol.* 5:429-459.
125. Melchers, F. 1986. Factors controlling the B-cell cycle. *Ann. Rev. Immunol.* 4: 13-36.
126. Kishimoto, T. and T. Hirano. 1988. Molecular regulation of B lymphocyte response. *Ann. Rev. Immunol.* 6: 485-512.
127. Moller, F. 1965. Contact-induced cytotoxicity by lymphoid cells containing foreign isoantigens. *Science* 147: 873-884.
128. Basten, A., J.F.A.P. Miller, J. Sprent and J.A. Pye. 1972. A receptor for antibody on B lymphocytes. *J. Exp. Med.* 135: 610-626.
129. Paraskevas, F., S.T. Lee, K.B. Orr. 1971. Cell surface associated gamma globulins in lymphocytes. *J. Immunol.* 106: 160-171.
130. Froland, S.S. and Natvig. 1973. Functional and Biochemical characteristics of human "null" lymphoid cells. *Trans. Rev.* 16: 114-117.
131. Froland, S.S., F. Wisloff and T.E. Michaelson. 1974. Human lymphocytes with receptors for IgG. A population of cells distinct from T and B lymphocytes. *Int. Arch. Allergy Appl. Immunol.* 47: 124-132.
132. Lobo, P., B. Westervelt and D.A. Horwitz. 1975. Identification of two populations of immunoglobulin bearing lymphocytes in man. *J. Immunol.* 114: 116-119.
133. Horwitz, D.A. and M.A. Garret. 1977. Distinctive functional properties of human blood L lymphocytes. A comparison with T lymphocytes, B lymphocytes, and monocytes. *J. Immunol.* 118:1712-1721.
134. Lobo, P.I. 1981. Characterization of a non-T, non-B human lymphocyte (L cell) with use of monoclonal antibodies. Its regulatory role on B lymphocyte function. *J. Clin. Invest.* 68: 431-438.

135. Horwitz, D.A. and P.I. Lobo. 1975. Characterization of two populations of human lymphocytes bearing detectable surface immunoglobulins. *J. Clin. Invest.* 56: 1464-1472.
136. Keder, E., M. Ortiz de Landazuri and L. Fahey. 1974. Comparative studies of immunoglobulin receptors and antibody-dependent cell cytotoxicity (ADCC) and rat lymphoid organs. *J. Immunol.* 112: 37-46.
137. MacLennan, E.C.M. 1972. Antibody in the induction and inhibition of lymphocyte cytotoxicity. *Transp. Rev.* 13: 67-90.
138. Perlmann, P. 1976. Cellular immunity: antibody dependent cytotoxicity (K-cell activity). *Clin. Immunobiol.* 3:107-112.
139. Perlmann, P. and J.C. Cerottini. 1979. "Cytotoxic lymphocytes" in the Antigens. Michael Sela Editor. Academic Press 5: 173-281.
140. Perlmann, P., H. Perlmann and H. Wigzell. 1972. Lymphocyte mediated cytotoxicity in vitro. Induction and inhibition by humoral antibody and nature of effector cells. *Transpl. Rev.* 13: 91-114.
141. Wisloff, F., T.E. Michaelson and S.S. Frøland. 1974. Inhibition of Ab-dependent human lymphocyte-mediated cytotoxicity by Ig classes, IgG subclasses and IgG fragments. *Scand. J. Immunol.* 3 : 29-38.
142. Despont, J.P., F. Steimer and E. Bandevet. 1981. Functional and biochemical characterization of human Null lymphoid cells. *Transpl.* 31: 25-30.
143. Ng, A.K., K. Indiveri, C. Russo, V. Quaranta, M. Pellergrini and S. Ferrone. 1981. Characterization of human Null cells isolated from peripheral lymphocytes by simultaneous double rosetting procedure. *Scand. J. Immunol.* 14: 225-231.
144. Richter, M. and C.A. Jodouin. 195. Cells and mediators which participate in immunoglobulin synthesis by human mononuclear cells. The obligatory requirement of Null lymphocytes in vitro. *Cell. Immunol.* 94: 614-618.
145. Boyum, A. 1969. Isolation of MNC and granulocytes from human blood. *Scand. J. Clin. Lab. Invest.* 21 (suppl. 97), 77-81.
146. Yam, L.T., Li, Cy and W.H. Crosby. 1971. Cytochemical identification of monocytes and granulocytes. *Am. J. Clin. Pathol.* 55: 283-291.

147. Jackson, D. and M.A. Weber. 1986. Manual of Clinical Laboratory Immunology. 3rd Edition, pp. 212-255.