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THE SUPPRESSION BY NULL CELLS OF FcM RECEPTOR SYNTHESIS BY HUMAN T CELLS IN VITRO

BY

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ABSTRACT

It has been previously demonstrated that receptors for FcM (FcM receptors) are not detected on any of the freshly isolated human circulating mononuclear cells but are only detected on T cells following culture for 24 hours in medium containing Fetal Calf Serum (FCS).

The objectives of this investigation were (i) to investigate the mechanism(s) responsible for the absence of FcM receptors on freshly isolated circulating T cells, (ii) to investigate the functional role of these receptors in immunoglobulin synthesis by B cells cultured with T helper cells, Null cells, monocytes and pokeweed mitogen.

It was unequivocally demonstrated that FcM receptors are only detected on T cells after 24 hours of culture in medium containing 20 percent FCS and that these receptors are synthesized by the T cells in culture. It was also demonstrated that circulating IgM and Null cells suppress the synthesis of FcM receptors by the T cells and that Null cells carry out this suppressor activity by secreting a factor referred to as receptor synthesis suppressor factor or RSSF. It was also found that FcM receptors are not required for the synthesis of immunoglobulins by the B cells in in vitro cultures.

It is concluded that both Null cells and IgM inhibit the synthesis of FcM receptors by the T cells and that these receptors are not required for the synthesis of immunoglobulins by cultured B cells.

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LIST OF ABBREVIATIONS

Ab	Antibody.
ADCC	Antibody-dependent cell-mediated cytotoxicity.
AFC	Antibody forming cell
AR	Autologous rosetting
CD3	Cluster of differentiation for Pan-T cells.
CD4	Cluster of differentiation for Helper/Inducer T cells.
C	Complement
C3b	Active constituent of third component of complement.
CMI	Cell-mediated immunity
E	Erythrocyte
EA	Erythrocyte complexed with antibodies (IgG or IgM)
EAG	IgG-coated ox erythrocytes
EAM	IgM-coated ox erythrocytes
EGME	Ethylene glycol monoethyl ether
ELISA	Enzyme linked immunosorbent assay
FCS	Fetal calf serum
FITC	Fluorescein isothiocyanate
FNCS	Filtered null cell sonicate
FcG	Fc of IgG

FcG receptor	Receptor for FcG
FcM	Fc of IgM
FcM receptor	Receptor for FcM
g	Gravity
HBSS	Hank's Balanced Salt Solution
HI	Humoral immunity
HLA	Human leukocyte antigen
hrs	hours
IF	Immunofluorescence
Ig	Immunoglobulin
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IU	International units
Leu M3	Becton-Dickson Laboratories monoclonal antibody marker for monocytes.
Mo	Monocytes
MHC	Major histocompatability complex
MNC	Mononuclear cells
MNC-Mo_1	MNC depleted once of monocytes
MNC-Mo_2	MNC depleted twice of monocytes
mw	molecular weight
NSE	Non-specific esterase
Leu12	Becton-Dickson Laboratories monoclonal antibody marker for B cells
Leu16	Becton-Dickson Laboratories monoclonal antibody marker for B cells
OKT3	Ortho Laboratories monoclonal antibody marker for Pan-T cells

ORBC	Ox red blood cells
PB	Peripheral blood
PBS	Phosphate buffer solution
PWM	Pokeweed mitogen
RSSF	Receptor Synthesis Suppressor Factor
SG	Specific gravity
SmIg	Surface membrane immunoglobulin
SRBC	Sheep red blood cells
T_O	Freshly isolated human T lymphocytes
T_{24}	Human T cells cultured for 24 hours
T_M	T cells with receptor for Fc of IgM
T_G	T cells with receptor for Fc of IgG
U.S.P.	United States Pharmacopia.

1.0 GENERAL INTRODUCTION

Today researchers take it for granted that cellular recognition, cellular interactions, and the reactions of lymphokines, hormones and other mediators with their target cells, are all dependent upon the presence of appropriate receptors on the cell. No one seriously questions the role of receptors in the initiation of an immunologic event. However, it is instructive to note that receptors in the immunological paradigm were unknown until as recently as the late-1960s when Mitchell and Miller presented evidence for the presence of receptors for antigens on clonally-selected precommitted T cells which were subsequently referred to as the antigen receptor bearing cells or ARC. It was confirmed by other investigators that the T ARC-antigen interaction constitutes one of the first events, if not the first event, leading to antibody synthesis by the antibody-forming B cells.

By the early 1970s, it had been shown that the circulating human T lymphocytes possess cell-surface receptors not present on the other circulating lymphocytes for a configuration on the sheep erythrocyte (SRBC). The B lymphocytes were shown to possess receptors for C3b as well as non-dissociable surface membrane immunoglobulins (smIg). It was also demonstrated that some of the circulating lymphocytes do not possess the surface markers characteristic of these cells and are therefore neither T cells nor B cells. Rather, they possess temperature labile smIg and receptors for the Fc of IgG antibody bound to

antigen and not for free, unreacted IgG. These cells were initially referred to as L (labile smIg) cells and were later called Null cells.

The receptors for FcG on the Null cells have functional relevance as they confer on the Null cell the capacity to carry out lysis of IgG-antibody-coated target cells by the ADCC (antibody-dependent cell-mediated cytotoxicity) pathway. The receptors for SRBC on the T cells and the receptors for C3b on the B cells appear to constitute non-functional markers of these cells.

In 1975, Moretta and his colleagues demonstrated that some of the circulating human T lymphocytes possess surface membrane receptors for FcG and that other circulating T lymphocytes possess surface membrane receptors for FcM. These T cells were referred to as T_G (or T_γ) and T_M (or T_μ) cells. These investigators subsequently demonstrated in 1977 that the T_M cells are obligatory participants in the synthesis of immunoglobulins (Ig) by the circulating human B lymphocytes cultured with the T_M cells, monocytes and pokeweed mitogen (PWM). They demonstrated that the T_G cells added to these cultures suppress Ig synthesis by the B cells. Moretta et al therefore referred to the T_M and T_G cells as helper and suppressor cells, respectively. They also attributed a functional role to the receptors themselves and claimed that the helper T_M cells and suppressor T_G cells only exhibit these immunological activities following activation of these cells as a result

of interaction of the receptors with their target immunoglobulin. These findings of helper and suppressor activity by the T_M and T_G cells had been verified by many investigators and is not seriously questioned today.

It is important to note that the receptors for FcM (FcM receptors) are generally not detected on the freshly isolated T cells nor on any cells following culture of the unfractionated mononuclear cells (MNC) in culture medium containing fetal calf serum (FCS) in a concentration of 20 percent. However, these receptors are detected on the T cells following their culture for 24 hours under the same culture conditions. It has been claimed by some investigators that the FcM receptors are newly synthesized by the freshly isolated T cells in vitro and by other investigators that the FcM receptors are unmasked by IgM immunoglobulins which dissociate from the receptors in culture thus freeing these receptors and permitting their detection by facilitating rosette formation with EAM indicator.

The absence of FcM receptors on cultured MNC, 70 percent of which are T cells, and their presence on a significant percentage of cultured T cells suggest that a non-T cell(s) inhibits the generation of FcM receptors by the cultured T cells. The investigations carried out in this thesis constitute an attempt to resolve these issues.

2. RATIONALE, HYPOTHESIS AND OBJECTIVES

2.1 RATIONALE

Neither freshly isolated unfractionated mononuclear cells (MNC) nor purified T cells exhibit surface membrane receptors for the Fc of IgM (FcM receptors). These receptors are only detected following culture of the purified T cells, but not the unfractionated MNC, in medium containing fetal calf serum (20%). These observations suggest that FcM receptors are synthesized de novo by the T cells in vitro and that one or more of the non-T cells constituting the MNC are capable of suppressing the synthesis of FcM receptors by T cells in vitro.

2.2. HYPOTHESIS

The hypothesis is that one or more of the non-T cells in the unfractionated MNC suppresses the generation of FcM receptors on T cells in culture and that this suppressor effect is mediated by a soluble factor(s) secreted by the suppressor cell(s).

2.3. OBJECTIVE

The objective of this thesis is to identify the cells which suppress the synthesis of FcM receptors by T cells in culture and the mechanism of this suppression.

3. HISTORICAL REVIEW.

3.1. THE FUNCTIONAL HETEROGENEITY OF LYMPHOCYTES.

Prior to 1960, it was considered that the immune response was the result of the activation of a single lymphocyte and its transformation into an antibody forming cell. However, in 1962 Warner et al (1) demonstrated that there are two classes of lymphocytes involved in the immune response in birds, one thymus-dependent and other thymus independent but bursal-dependent. This finding was followed by the milestone reports by Claman et al (2-5) that two ontogenically distinct lymphocytes, one a thymus derived (T) cell and the other a bone marrow derived (B) cell must interact in the antibody response to sheep erythrocyte antigen in the mouse. Their 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 thymic lymphocytes or bone marrow lymphocytes alone, irrespective of their number, did not confer to the irradiated recipients the capacity to respond with antibody synthesis. However, the injection into the irradiated recipients of a mixture of thymus and bone marrow lymphocytes restored the capacity to respond with antibody synthesis within the normal 5 days following immunization.

This finding by Claman et al (2-5) was quickly confirmed by a number of investigators (6-14).

The discovery of T and B cells in the 1960s stressed

the need for markers to permit the isolation of these two cell-types. T cells were known to be larger (15), more dense (16) and less adherent (to materials like glass, plastic, nylon) (16) than the B cells. T cells were also known to have a longer generation time (17). However, these properties were not useful in the separation of these cells.

3.2. SURFACE MARKERS WHICH DISTINGUISH THE B AND T CELLS .

Tallberg et al (18), working with human thymocytes, and Reif and Allen (19) working with rodent thymocytes, were the first to report that thymocytes carry organ-specific antigens on their cell membranes. Reif and Allen were also the first to refer to the mouse thymocyte specific surface membrane antigen as the theta (θ) antigen. Raff and his associates (21-23) carried out systematic studies of θ-antigen positive cells in different lymphoid and non-lymphoid organs of the mouse and demonstrated that cells in the central nervous system also possess the θ antigen. They established the presence of theta antigen bearing cells in the spleen and lymph nodes of the mouse. Simultaneous studies by numerous investigators in the human confirmed that human thymocytes also possess an organ specific surface membrane antigen similar to the theta antigen in the mouse (24-26).

de la Noue, Koperstych and Richter (27) were the first to demonstrate thymocyte-specific antigens in the

rabbit. This finding was later verified by Fradelizi et al (28).

B cells in the mouse (29) and the rabbit (30) were also found to possess specific surface membrane antigens. In addition, B cells were observed to possess surface membrane immunoglobulins and receptors for C 3b which could also be used to distinguish them from the T cells (29-34).

Thus by the early 1970s, it became established that B and T cells belonged to two distinct cell lineages which could be distinguished and seperated from each other on the basis of specific cell-surface markers.

3.3. EVIDENCE THAT NULL CELLS CONSTITUTE A DISTINCT CLASS OF LYMPHOCYTES.

3.3.1. SURFACE MARKERS WHICH DISTINGUISH NULL CELLS FROM B CELLS AND T CELLS .

Up to the early 1970s, the circulating lymphocytes were considered to consist of only two lineages of cells - T and B cells. Approximately 60 to 75 percent of the circulating lymphocytes rosette with sheep red blood cells (SRBC), and stain positive by immunofluorescence with the monoclonal pan anti-T cell antiserum, OKT3 or CD3. Both are universally accepted properties of T cells. The non-T cells were considered to be B cells since a high percentage were shown to possess surface membrane immunoglobulin (smIg) as demonstrated by immunofluorescence using FITC conjugated anti-human immunoglobulin (Ig).

In 1965, it was demonstrated that some circulating lymphocytes possess surface membrane receptors for the Fc of IgG (FcG receptors) by virtue of which they can carry out antibody-dependent cell-mediated cytotoxity (ADCC) (35). 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 (36-39). It was not until the early to mid-1970s that it was demonstrated that the FcG receptor-bearing "B" cells were, indeed, different from the non-FcG receptor bearing "B" cells since the former cells shed their smIg following incubation at 37°C for 30 to 60 minutes and did not regenerate it whereas the latter cells did not shed their smIg following even longer incubation at 37°C suggesting that the smIg is an integral constituent of the cell membrane (40-43). 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 (40-47). 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 FcG receptors, and the pseudo B or L cells which possess temperature labile smIg, cannot synthesize smIg, and do not bear surface

membrane FcG receptors. 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).

In recent experiments conducted on isolated cultured B and Null cells, neither investigations carried out in this laboratory (M.Richter, unpublished results) nor by Moretta et al (personal communications) 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 Leu12 or Leu16 monoclonal antibodies, do not generate smIg and do not lose their capacity to mediate ADCC. Similar findings have previously been reported by Ng et al (48). It must therefore be concluded that the circulating B and Null cells in the immunologically mature individual are distinct from each other and represent distinct lineages of cells (Table 1).

3.3.2. FUNCTIONAL PROPERTIES OF NULL CELLS .

3.3.2.1. CAPACITY OF THE NULL CELLS TO UNDERGO BLASTOGENESIS IN VITRO AND PARTICIPATE IN THE ADCC REACTION .

Several functional properties distinguish the Null cells from T cells and B cells: (i) only the Null cells participate in the antibody-dependent cell-mediated cytotoxicity (ADCC) reaction; the true B cells and the T cells do not mediate the ADCC cytotoxic reaction (49-54), and (ii) Null cells do not undergo blastogenesis and

mitosis when stimulated with the conventional phytomitogens, PHA and CON-A (which stimulate T cells) and PWM (which stimulates T cells and B cells) in culture (48,55).

3.3.2.2. EVIDENCE THAT NULL CELLS ARE OBLIGATORY PARTICIPANTS IN IMMUNOGLOBULIN SYNTHESIS .

No in vitro system, using human or animal circulating or parenchymal cells, for the investigation of immunoglobulin synthesis was available until 1971, when Cooper, Lawton and Bocham (56) reported that human circulating mononuclear cells cultured in the presence of pokeweed mitogen (PWM) could synthesize immunoglobulins. This finding by Cooper et al (64) was quickly confirmed by numerous investigators (57-64). It was also shown that B lymphocytes in man could be identified by the presence of high density surface membrane-bound immunoglobulins (smIg) (65-71). It was further demonstrated that pokeweed mitogen induces transformation and differentiation of the B lymphocytes (57,60,61) and these cells are lacking in individuals presenting with the Bruton or X-linked agammaglobulinemia syndrome (56,71-74). As a result of these studies, it was generally concluded that it is the B lymphocytes which synthesize and secrete the immunoglobulins.

In the middle 1970s, 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 (62) and Keightley et al (60) 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 cells and the culture medium was fortified with decomplemented fetal calf serum (FCS) to a final concentration of 15-20 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. 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 i.e. (35S-methionine) into the newly synthesized immunoglobulins during the culture.

A further improvement of the culture conditions for the optimal synthesis of Ig was introduced by Moretta and his colleagues in mid 1970s. This group of investigators demonstrated that the T lymphocytes could be seperated into two subclasses of cells -one with receptors for Fc of the IgG (T_G or Tγ cells) and the other with receptors for the

Fc of IgM (T_M or $T\mu$ cells). These cells could be physically separated from each other by their ability to rosette with IgG antibody coated ox erythrocytes (EAG indicator cells) and IgM antibody coated ox erythrocytes (EAM indicator cells), respectively (75-79). They demonstrated that the T_M cells facilitate Ig synthesis by co-cultured B cells in the presence of PWM whereas the T_G cells inhibit Ig synthesis by the co-cultured T_M cells, B cells and PWM (80-86). The T_M and T_G were therefore referred to as helper and suppressor T cells respectively. These findings were quickly confirmed by others (87-89).

Gerrard and Fauci (92), Rosenberg and Lipsky (93) Thiele and Lipsky (94) and Shin and Choi (95) all demonstrated that monocytes had to be present in the cell cultures for immunoglobulin synthesis to take place since removal of the monocytes resulted in failure of Ig synthesis.

Thus, by the early 1980s, it appeared that the cells required in the cultures for the optimal synthesis of Ig are B cells, T_M cells and monocytes.

In all of the investigations referred to thus far, the B cells in the culture were actually the non-T cell. Investigators generally assumed that the non-T cells consisted of only B cells. However, in 1985, Richter and Jodouin (96) reported that B cells cultured with T_M 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_M helper cells and PWM, in the absence of B cells, also 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 Null cells could not be attributed to the absence of monocytes. Furthermore, Ig was synthesized by reconstituted cultures of purified B cells, Null cells, T_M helper cells, monocytes and PWM. These results demonstrated that B cells must be cultured with T_M helper cells, Null cells, monocytes and PWM in order for immunoglobulin synthesis to take place.

3.4. T CELLS WITH FcM RECEPTORS .

3.4.1. FcM RECEPTORS ON FRESHLY ISOLATED T CELLS .

3.4.1.1. EVIDENCE THAT FcM RECEPTORS EXIST ON FRESHLY ISOLATED T CELLS .

A number of investigators have detected FcM receptors on T cells in the freshly isolated MNC. Gupta and Good (97) reported that 40 to 48 percent of freshly-isolated bone marrow MNC exhibited surface membrane FcM receptors. Furthermore, the percent of FcM receptor bearing cells decreased following culture of the cells for 24 hours at 37°C in medium containing FCS(20%). In contradistinction, Romagnani et al (98) reported that the percent of EAM rosetting cells increased dramatically from

12 percent of the freshly isolated MNC to 34 percent following incubation of the cells for 24 hours in medium containing FCS (20%). Gmeling-Myeling et al (99) reported that about 46 percent of the circulating human MNC rosette with EAM indicator cells. This figure implies that 70 to 80 percent of the circulating T cells are T_M cells.

Bolhuis and Nooyen (100) also detected FcM receptors on approximately 30 percent of the freshly isolated lymphocytes. Gupta and Good (101) reported that 35 to 40 percent of freshly isolated MNC of elderly individuals rosette with EAM. Moretta et al (102) detected FcM receptors on 16 to 33 percent of freshly isolated MNC of patients with immunodeficiency disease. Similar results were obtained by other investigators (103-104).

3.4.1.2. <u>EVIDENCE THAT FcM RECEPTORS DO NOT EXIT ON FRESHLY ISOLATED T CELLS</u>.

It has been the experience of the overwhelming majority of investigators (only a few of which are referred to below) that freshly isolated T cells do not possess membrane FcM receptors since they do not rosette with EAM. However, following culture in medium containing FCS (20%), a significant percentage (20 to 50 percent) of the T cells generate FcM receptors and thereby attain the capacity to rosette with EAM (84,97,100,101,105,106,107,108). It is not possible at this time to reconcile the failure by the majority of investigators to detect FcM receptors on freshly isolated T cells with the findings by a minority of

investigators who detected significant numbers of FcM receptors bearing cells within freshly isolated T cells population.

3.4.2. T CELLS SYNTHESIZE FcM RECEPTORS IN CULTURE

The evidence to date is that T cells synthesize the surface membrane FcM receptors during in vitro culture in culture medium [medium RPMI-1640 containing FCS (20%)] Moretta et al (83) and Mingari et al (109) incubated T cells at 37°C in culture medium overnight to allow for the expression of FcM receptors on a proportion of the T cells. However, if the T cells were incubated in culture medium with cyclohexamide, an inhibitor of protein synthesis, in a non-toxic concentration for 24 hours, the cells did not rosette with EAM, indicating the absence of FcM receptors. They concluded that cyclohexamide had prevented the synthesis of FcM receptors by the cultured T cells.

Romagnani et al (110), Mingari et al (109) and Pichler and Knapp (111) incubated T cells in culture medium for 24 hours to facilitate the expression of FcM receptors on cells. They then exposed these T cells to trypsin and/or pronase for several hours following which the cells were washed and placed in culture. These cells were initially devoid of FcM receptors as they failed to rosette with EAM. However, within 2 hours of culture, FcM receptors began to reappear and resynthesis of these receptors was completed by 6 hours in culture. These results demonstrated that the

receptors for Fc of IgM can be synthesized by the T cells in vitro.

Further evidence that the FcM receptors are in a dynamic state of synthesis and degradation was provided by the findings of Mingari et al (109). These investigators cultured FcM receptor-bearing T cells (T_M cells) with cyclohexamide. The percent of T cells forming rosettes with EAM decreased by 40 percent within 2 hours of culture and no rosetting cells were detected after 6 hours of culture with cyclohexamide. These results suggested that the FcM receptors were degraded in the culture but could not be resynthesized due to the inhibitory activity of cyclohexamide and that the FcM receptors normally undergo continuous degradation and resynthesis. These findings were confirmed by other investigators (112-113).

3.4.3. THE EFFECT OF IgM ON THE EXPRESSION OF FcM RECEPTORS BY T CELLS .

3.4.3.1. SURFACE MEMBRANE FcM RECEPTORS PRESENT ON THE FRESHLY ISOLATED T CELLS ARE MASKED BY PASSIVELY ADSORBED IgM .

A number of investigators have attributed the failure to detect FcM receptors on freshly isolated T cells to binding of the receptors by circulating IgM (84,97,98,102,114,115). They have theorized that the bound IgM is released by or shed from the cells during a 24 hour incubation at 37°C, thus freeing the FcM receptors and

permitting their detection by facilitating rosette formation with EAM. However, none of these investigators actually looked for and/or detected any IgM in the cell-free supernatants of the cultures of freshly isolated T cells.

3.4.3.2. IgM INHIBITS THE SYNTHESIS OF FcM RECEPTORS BY T CELLS IN VITRO .

Moretta et al (83) incubated freshly isolated T cells for 24 hours in normal human serum containing IgM and in serum of immunodeficient patients devoid of IgM. The latter rosetted with EAM whereas the former did not rosette with EAM. One interpretation of these findings was that the IgM in the culture medium containing normal human serum inhibited the synthesis of FcM receptors by the T cells. However, it is also possible that the IgM in the medium became bound to the FcM receptors, thereby neutralizing them.

On the other hand, Romagnani et al (110) reported only a 20 percent drop in the number of T cells which rosette with EAM following their culture for 24 hours at 37°C in culture medium containing IgM at a concentration of 800 ug per ml. These results suggested that IgM does not inhibit or marginally inhibits the synthesis of FcM receptors.

3.4.3.3. IgM DOES NOT INHIBIT THE SYNTHESIS OF FcM RECEPTORS BY T CELLS IN VITRO .

Romagnani et al (98) incubated freshly isolated T cells for 24 hours at 37°C in medium containing either

normal human serum (20%) or FCS (20%). They observed an increased proportion of EAM rosetting T cells following their incubation in normal human serum as compaired to FCS (45% vs 34% respectively). They concluded that the FcM receptors on T cells are neither inhibited in their generation nor saturated in vitro by IgM.

In a subsequent investigation, Romagnani et al (110) incubated freshly isolated T cells in normal human serum containing IgM (approximately 200,000 ng IgM per ml) and in IgM-free human serum. The percent of the T cells which subsequently rosetted with EAM were almost identical, demonstrating that the presence of IgM in the culture did not affect the synthesis or expression of FcM receptors.

3.4.3.4. IgM INHIBITS THE ROSETTING OF FcM RECEPTOR-BEARING T CELLS (T_M CELLS) WITH THE EAM INDICATOR CELLS .

A not insignificant number of investigators have reported that IgM can inhibit the rosetting of T_M cells with the EAM indicator cells, presumably by reacting with and neutralizing the FcM receptors. Gmeling-Myeling et al (99) incubated T cells with IgM, in varying concentration, for 2 hours prior to rosetting with EAM. They observed that the capacity to rosette decreased as the concentration of IgM increased during the preincubation period. These results were confirmed by Romagnani et al (98) and Samarut and Revilland (116) who reported a 25 percent decrease in EAM rosettes by the T_M cells if IgM, in a concentration

of 25 ug per ml, was added to the T_M cells prior to rosetting with EAM.

Fanger and Lydyard (117) observed a 50 percent decrease in EAM rosettes following a 30 min incubation of the T_M cells with IgM in a concentration of 25 ug per ml and a total inhibition of rosetting following incubation of the T_M cells with IgM in a concentration of 100 ug per ml.

Ferrarini et al (118) and Bolhuis and Nooyen (100) also concluded that IgM, in a concentration of 125 ug per ml, can inhibit rosetting of the T_M cells with EAM.

Preud'homme et al (119) incubated T_M cells with IgM, in a concentration of 1 mg per ml, for 5 minutes prior to rosetting with EAM and observed total suppression of rosetting. They also reported that monomeric IgM was as good an inhibitor of EAM rosetting as was pentameric IgM. A similar conclusion was arrived at by Ferrarini et al (120). Conradi and Bubb (121) demonstrated that CH4 domain of IgM is responsible for this inhibition.

Romagnani et al (98), Preud'homme et al (119) and Bolhuis and Nooyen (100) all demonstrated that IgG could not inhibit the formation of rosettes between the T_M cells and the EAM indicator cells.

3.4.4. THE SYNTHESIS OF FcM RECEPTORS BY FRESHLY ISOLATED T CELLS IS A FUNCTION OF THEIR TIME IN CULTURE .

Romagnani et al (110) observed maximum rosetting

capacity of the T cells with EAM following culture of the cells in culture medium for 12 hours. Similar observations were made by Moretta et al (83) who concluded that incubation of T cells for 24 hours in culture medium ensures the maximum synthesis of FcM receptors.

3.4.5 IgG DOES NOT INHIBIT THE ROSETTING OF T_M CELLS WITH THE EAM INDICATOR CELLS .

In contradistinction to the results presented above of inhibition of EAM rosette formation by IgM, investigators have reported that IgG does not inhibit the formation of EAM rosettes by the T cells. Gmeling-Myeling (99), Moretta et al(83), Romagnani et al (98), Samarut and Revilland (116) and Ferrarini et al (118) all incubated T_M cells with IgG in concentration as high as 7 mg per ml for varying periods (114) of time and observed no effect on the capacity of the T_M cells to rosette with EAM. On the other hand, IgG very effectly inhibited rosetting of T_G cells with EAG (98-99).

3.5. FcM RECEPTORS ON B CELLS .

FcM receptors have been detected on B cells in addition to T cells. Like the T cells, the B cells (or non-T cells) must also be incubated in IgM-free culture medium in order to generate FcM receptors and rosette with EAM (111,114,118,122,123). Romagnani et al (96) reported that fresh non-T cells, incubated for 24 hours in medium fortified with IgM-containing normal human serum (20 percent) generated a high percentage (25 percent) of EAM

rosetting cells.

Romagnani et al (98) demonstrated that, in order for the B cells to rosette with the EAM indicator cells, the ox erythrocytes must be coated with more IgM antibodies than are required for the rosetting of these indicator cells by T_M cells. This may be one reason for the failure of most investigators to detect EAM rosetting by non-T cells. In general, the percent of non-T cells capable of rosetting with EAM is considerably lower than the percent of T cells capable of rosetting with EAM following culture for 24 hours (5 to 20 percent versus 40 to 60 percent).

In none of the above investigations was it determined which of the non-T cells (B cell, Null cell, monocyte) generates the FcM receptors in culture. The function, if any, of the FcM receptor bearing non-T cells is also not known at the present time.

3.6. SUMMARY .

Ever since the description of T cells bearing surface membrane FcM receptors by Moretta et al, numerous investigators have confirmed their presence and speculated upon their functional roles. The investigations carried out thus far on T_M cells have generated conflicting data and the following questions are still unanswered: (i) why are the FcM receptors not detected on freshly isolated but only on cultured T cells? (ii) what are the optimal culture conditions required for the maximum

expression/synthesis of the FcM receptors by the T cells? (iii) are the FcM receptors synthesized de novo by the T cells in culture; or are they actually present on the freshly isolated T cells but not detected as they are masked by IgM; and (iv) do the FcM receptors have a functional role in Ig synthesis by B cells in culture?

The objective of the investigations carried out in this thesis was to provide answers to these questions.

TABLE 1

PROPERTIES WHICH DISTINGUISH
NULL CELLS FROM T AND B CELLS

Circulating lymphocytes	Property									
	% in circulation	Blasto-genic 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 phytomitogens 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, Ontario and stored at 18-20°C.

Complement source was fresh human serum stored at -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 buffer (final concentration 2%).

Erythrocytes

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

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

Ficoll 400 - 500 g containers were obtained from Pharmacia Fine Chemicals AB, Upsala, 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.

Hank's Balanced Salt Solution (HBSS) - 500ml bottles were obtained from Media tech, Herdon, VA and stored at 4°C.

Heparin (1000 U.S.P. units/ml) - 20 ml vials were obtained from Organon Canada Ltd., Toronto, Ontario 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.

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

Hypaque sodium 50% (300 mg/ml diatrizoatesodium) - 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,aliquated and stored at -70°C.

Penicillin -streptomycin mixture (5000 units Potassium Penicillin G/ml, 5000 mgs. Streptomycin/ml) - 100 ml bottled 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 staining of lymphocytes

Alpha-Naphthyl acetate - obtained from Sigma Chemical Co., St. Louis, Missouri and stored at -20°C.

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

Methylene blue : was obtained from Sigma Chemical Co., St. Louis, Missouri and stored at 18-20°C.

Methyl green : was obtained from Sigma Chemical Co., St. Louis, Missouri and stored at 18-20°C.

Monoclonal antibodies(25 ug/ml) - Leu-M3 was obtained from Becton Dickinson Immunocytometry Systems, Mountain View, California and stored at 4°C.

Anti-mouse IgG-FITC conjugates were obtained from Ortho Diagnostics, Don Mills, Ontario and stored at 4°C.

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

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

4.1.1.3 Reagents for enzyme linked immunosorbent assay

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 (μ chain specific) or IgG (γ 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, Ontario and stored at 4°C.

Human IgG and IgM (unconjugated, chromatographically purified)(5 mg/ml) .

5 ml vials were obtained from Organon Tecknika, Westchester, PA. and stored at 4°C.

Magnesium Chloride - 1 lb bottles of $MgCl_2$ crystals were obtained from British Drug House, Montreal, Quebec 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 - 200 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 V-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, Ontario.

Culture tubes, sterile - 5 ml (12 x 75 mm) falcon 2003 and 15 ml (17 x 100mm) falcon 2001 round bottomed polystyrene tubes were obtained from Becton Dickinson Co., Lincoln Park, N.Y.

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 John Scientific, Toronto, Ontario.

Gauze sponges -(5.1 x 5.1) were purchased sterile from Kandall Canada, Toronto, Ontario.

Microscope slides (75 x 25 mm) - frosted one end on both sides, were obtained from Serum International Inc., Montreal, Quebec.

MSI syringe adaptable filters, sterile (0.2 Um) - were obtained from MSI, Westborough, MA.

Nalgene filters 0.22 Mm - were obtained from Nalgene Labware, Rochester, N.Y.

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

Pipettes - 1,2,5 and 10 ml pyrex sterile disposable

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, California.

4.1.3 Equipment

CO_2 incubator - cultures were maintained in a National incubator, NAPCO, Portland, Oregan. This incubator is fully automated with respect to temperature (37.5°C ± 0.5) and PCO_2 (5.0% ± 0.1).

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

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

Cytocentrifuge - (cytospin SCA-0030) - was obtained from Shandon Southern instrumetns, 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., Spring Field, Massachusetts.

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

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 medication, either for medical or recreational reasons. The volunteers were between 20 and 55 years of age.

4.2.2 Isolation of mononulear cells(MNC)

The procedure is that essentially described by Boyum (126). Heparinized (50 units heparin/ml drawn blood) venous blood of healthy volunteers (180 ml), diluted with an equal volume of Hank's 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 and collected 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. A 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 washed three times in HBSS at 450 450 g for 10 minutes at 18-20°C and resuspended in HBSS

to the desired cell concentration. The SRBC and ORBC suspended at cell concentration of 3×10^8 and 5×10^8 per ml constitutes 3% ad 5% suspensions of SRBC and ORBC, respectively.

4.2.4 Preparation of IgG sensitized ox red blood cells (5% 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 suspended in RPMI-1640, and again centrifuged at 450 g for 10 minutes. This procedure was repeated twice. The EAG indicator cells were suspended 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 3% SRBC suspension at 37° (ratio of MNC: SRBC was 1: 30) for 15 minutes centrifuged for 5 minutes & 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 (0.1 M) for 2 minutes in order to lyse

the SRBC present in either cell suspension. The cells were then washed three times in HBSS, resuspended in HBSS and a cell count was performed.

4.2.6 Isolation of T_M, T_G, T_C, B and Null lymphocytes - To isolate T_M cells, the T cells were incubated with 5% EAG suspension (ratio of T:EAG was 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 a discontinuous gradient of Ficoll Hypaque (S.G. 1.077) and centrifuged at 400 g for 30 minutes. The rosetted T_G cells sedimented to the bottom of the tube leaving the non-rosetted T cells which contained the T_M+T_C cells at the interphase. The rosetted(T_G) and non-rosetted(T_M + T_C) cells were collected from the pellet and interphase and washed in hypotonic ammonium chloride (0.1M) for 2 minutes in order to lyse the indicator cells in either cell suspension. To isolate T_C cells, (T_M + T_C) cells were incubated with 5% EAM suspension (ratio of T:EAM was 1:50) for 15 min. at 4°C. The cells were centrifuged at 200 g for 5 minutes and incubated for 1 hour at 4°C. Rosetted (T_M) and non-rosetted(T_C) cells were isolated by centrifuging on Ficoll hypaque discontinuous density gradient (S.G. 1.077) and latter treatment with hypotonic ammunium chloride chloride(0.1M). The cells were washed and resuspended in HBSS.

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_M 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 a cell count was performed.

4.2.7 Preparation of monocyte depleted cells

The mononuclear cells were suspended (2×10^6 cells per ml) in sterile disposable tubes in 5 ml serum - HBSS (serum: HBSS was 1:1) to 10 mg carbonyl iron were added he tubes were placed on a multipurpose rotator for 30 minutes at 37°C in an atmosphere of 5% CO_2 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 ($MNC-Mo_1$). 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 ($MNC-Mo_2$). The cells were stained

for non-specific esterase (NSE) and with Leu M3. Both of these markers are specific for monocytes, and examined by light microscopy and fluorescent microscopy respectively. It was consistently found that less than 1% of the cells in the MNC-Mo_2 could be identified as monocytes.

4.2.8. 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 (128).

4.2.9 Preparation of receptor synthesis suppressor factor (RSSF)

One million Null cells were incubated in RPMI-1640 for 24 hours at the end of which the cell free supernatant was used as RSSF either fresh or stored at -20°C.

4.2.10 Preparation of IgM sensitized Ox erythrocytes (EAM)

Equal volumes of a 1% suspension of ORBC (E) and rabbit IgM anti-ORBC antibodies (A), used in a subagglutinating concentration, were mixed and incubated at 4°C for 60 minutes. The resulting antibody sensitized ORBC (EA-IgM or EAM) were centrifuged at 400 g for 10 minutes at 4°C. The supernatant was aspirated, the cells were suspended in RPMI-1640 and again centrifuged at 400 g for 10 min at 4°C. This procedure was repeated twice. The EAM indicator cells were suspended in RPMI-1640 to a

concentration of 1% and kept at 4°C.

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 minutes 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 (Step 2). After another 60 minutes 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 Preparation of EAM rosettes

EAM indicator erythrocytes (5×10^6 cells in 0.1 ml) were mixed with the mononuclear cells (3×10^6 cells in 0.1 ml) and immediately centrifuged at 4°C for 5 min at 200 g. The rosetted cells were kept at 4°C for a minimum of 45 min before the addition of methylene blue and enumeration of the the rosetted cells.

4.2.13 Preparation of E(SRBC), EA(IgG) and EAC rosettes

The indicator erythrocytes (10^7 RBC in 0.1 ml) were incubated with the mononuclear cells (3×10^5 cells in 0.1 ml) at 37°C for 15 min. These cells were then centrifuged at 200 g for 5 minutes. In the case of E rosette, incubation was continued for 2 hours at 4°C.

rosette, incubation was continued for 2 hours at 4°C. One drop of 0.33% methylene blue solution was added, the cells were gently resuspended, and the rosettes were counted in a hemocytometer.

4.2.14 Assessment of T-cell viability was done by the conventional trypan blue dye exclusion test.

4.2.15 Dialysis - RSSF was obtained as mentioned in 4.2.9 and dialysed for 24 hours through dialysis membranes of exclusion m.wt. of 14,000 and 50,000. The RSSF was assayed for its capacity to inhibit the generation of FcM receptors on freshly isolated T cells.

4.2.16 Staining for non-specific esterase - Monocytes were identified by staining for non-specific esterase according to the method described by Yam et al (127).

The air dried cytocentrifuged cells were stained for 30 minutes with the staining reagent (see below). The slides were washed twice in distilled water, fixed in methanol for 60 seconds and dried in cold air. The slides were counter stained with 1% methyl green (see below) for 1 minute, washed twice with distilled water and air dried at 18°C.

The staining reagents were prepared as follows: 10 ml of distilled water was added to 0.4 g sodium nitrate, 10 ml of ethylene glycol monoethyl ether (EGME) was added to 0.2 gm alpha nephthyl acetate, 7.5 ml of Pararosanalin solution was added to 7.5 ml of 4% sodium nitrite solution following

exactly 1 minute, 12 ml of EGME solution was added. Following the addition of 178 ml of PBS, a buff coloured precipitate formed and was filtered. The pH was adjusted to 6.1 using HCl or 1 N NaOH.

Methyl green was prepared as follows: 100 ml of distilled water was added to 1.4 g sodium acetate and 1.0 g methyl green. The pH was adjusted to 4.2 with acetic acid.

4.2.17. Cell cultures for Ig synthesis .

(T_O-T_G), (T_{24}-T_G) were incubated with equal numbers of non-T, Null and/or B cells (2 x 10^5 cells of each) in 1 ml of culture medium. Pokeweed mitogen (0.05ml) was added in its optimal mitogenic concentration. The cultures were maintained in a stationary phase at 37°C in 5% CO_2 in air. After 7 days (unless otherwise stated), the tubes were centrifuged at 450 g for 10 minutes, the supernatants were carefully removed. The Ig present in the supernatant was measured by the ELISA assay.

5. RESULTS

5.1. THE OPTIMAL CONDITIONS REQUIRED FOR THE GENERATION OF RECEPTORS FOR FcM BY THE T CELLS.

5.1.1. RATIONALE AND OBJECTIVES.

Before embarking into experiments to define the mechanism and regulation of the synthesis of FcM receptors by the T cells, it was essential to confirm the cell type capable of expressing FcM receptors and to establish the optimal conditions required for the maximum expression/generation of these receptors using methods similar to previous investigators.

The objectives of this series of experiments were (i) to identify the cell(s) which exhibit the FcM receptors, and (ii) to determine the culture conditions required for the maximum expression of FcM receptors by these cells.

5.1.2. EXPERIMENTAL PROTOCOL.

Normal human volunteers without a history of recent illness and not on medication were bled from the anticubital vein into heparinized tubes as described in chapter 4.2.2. The blood was diluted 1:1 with HBSS and centrifuged through a Ficoll-Hypaque discontinuous density gradient (S.G. 1.077) as described in chapter 4.2.2. The mononuclear cells at the interphase were collected, washed and suspended in medium as described in chapter 4.2.2. MNC were then separated into T and non-T cells by rosetting with SRBC as described in chapter 4.2.5. Non-T cells were then separated

into B cells and Null cells as described in chapter 4.2.6. Aliquots of MNC, T and B cells were assayed for the presence of FcM, FcG and C3b receptors using EAM, EAG and EAC indicators, respectively, as described in chapter 4.2.12. and chapter 4.2.13. The viability of the cultured cells was determined by the trypan blue dye exclusion test as described in chapter 4.2.14. The culture medium used, except where stated otherwise, consisted of RPMI-1640 fortified with penicillin, streptomycin, and FCS (final concentration 20%) as described in chapter 4.1.1.1.

Each experiment was carried out in duplicate or triplicate and was repeated at least five times. The results presented in the tables are representative of the results obtained.

5.1.3. RESULTS.

Unfractionated mononuclear cells (MNC), Non-T cells, T cells, B cells and Null cells were assayed for FcM receptors by their ability to rosette with EAM indicator cells after culture for 24 hours. As can be seen from Table 2, none of the freshly isolated cells exhibited FcM receptors and only the T cells following culture for 24 hours exhibited the FcM receptors (20 to 30 percent of the cultured T cells).

T cells, both freshly isolated (T_0) and following 24 hours of culture (T_{24}), were assayed for FcM, FcG and C3b receptors by rosetting with EAM, EAG and EAC indicator cells, respectively (Table 3). As can be seen from the

results presented in Table 3, T_0 cells consistently exhibited FcG and C3b receptors on 8 to 20 percent of the cells. However, as stated above, they failed to exhibit FcM receptors. On the other hand, a significant percentage of T_{24} cells (25 to 35 percent) exhibited FcM receptors.

The time course for the appearance of FcM receptors on the cultured T_0 cells and the role of the concentration of fetal calf serum (FCS) in the culture medium was then systematically investigated. It was observed that T_0 cells after 24 hours in culture exhibited the highest percentage of cells which rosetted with EAM indicator cells. There was a slight drop in the percentage of EAM rosetting cells when the T_0 cells were cultured for 48 hours but a much larger drop when the T_0 cells were cultured for only 4 to 8 hours in culture medium. Very few T_0 cells cultured in medium devoid of FCS, irrespective of the period of culture, rosetted with EAM (Table 4). It was also found that FCS in a concentration of 20 percent facilitated the generation of the maximum percentage of EAM rosetting cells after 24 hours of culture (Table 5). FCS in a concentration of 10 or 40 percent in the culture medium provided inferior conditons for the generation of EAM rosetting cells after 24 hours of culture. The absence of EAM rosetting cells in culture medium with a low or high concentration of FCS could not be attributed to the death of the cultured T_0 cells as the viability of the cells remained high even in the absence of FCS (Table 4 and 5).

5.1.4. COMMENTARY.

Although it is generally agreed that FcM receptors are only detected on T cells after 24 hours of culture in medium containing fetal calf serum in a concentration of 20 percent (84,97,100,101,105,106,107,108), a few investigators have also reported the presence of FcM receptors on freshly isolated T cells (97-101). Still others have demonstrated the presence of these receptors on freshly isolated and cultured B cells (111,114,118,122,123). However, the culture conditons for the generation of FcM receptors by T cells varied in all of these investigations. It was therefore important to reassess these seemingly conflicting findings before continuing this investigation. In these series of experiments, it was unequivocally demonstrated that (i) freshly isolated T cells, B cells, Null cells and MNC do not express FcM receptors (Tables 2 and 3), (ii) T cells, but not unfractionated MNC, B cells or Null cells, exhibit FcM receptors following culture for 24 hours in medium containing fetal calf serum (20%) (Tables 2 and 3), (iii) the culture medium should contain FCS in a final concentration of 20 percent to facilitate the maximum generation/expression of the FcM receptors (Table 5), and (iv) the optimal time required for the maximum generation of FcM receptors on cultured T cells is 24 hours (Table 4).

This consistent finding of FcM receptors on T cells following culture for 24 hours in culture medium does not in itself indicate whether these receptors are synthesized de

novo or whether they are masked by a "covering protein" (presumably IgM) which is shed in culture allowing the detection of the FcM receptors. The experiments in the following two sections were designed to resolve these questions.

TABLE 2

T CELLS, BUT NOT B CELLS OR NULL CELLS, EXHIBIT FcM RECEPTORS ONLY FOLLOWING CULTURE FOR 24 HOURS

Cells assayed	Percent of cells which rosette with EAM					
	Exp. 1		Exp. 2		Exp. 3	
	0hr	24hr*	0hr	24hr*	0hr	24hr*
Unfractionated MNC	0	0	0	0	0	0
Non-T cells	0	0	0	0	0	0
T cells	0	21	0	28	0	18
B cells	0	0	0	0	0	0
Null cells	0	0	0	0	0	0

* These cells were cultured in culture medium for 24 hours prior to assay.

TABLE 3

FRESHLY ISOLATED CIRCULATING HUMAN T LYMPHOCYTES (T_O) DO NOT ROSETTE WITH THE EAM INDICATOR CELLS

T cells assayed	Percent of T cells which rosette with the following indicators EAM	EAG	EAC
Volunteer No. 1			
T_O* cells	0	10	18
T_{24}** cells	27	6	16
Volunteer No. 2			
T_O cells	0	12	14
T_{24} cells	30	8	14
Volunteer No. 3			
T_O cells	0	8	20
T_{24} cells	29	4	16

* T_O are freshly isolated T lymphocytes.
** T_{24} are freshly isolated T lymphocytes cultured for 24 hours in culture medium.

TABLE 4

THE SYNTHESIS OF THE FcM RECEPTORS BY THE T_O LYMPHOCYTES IS A FUNCTION OF THEIR TIME IN CULTURE

T_O lymphocytes cultured for the following period of time (hrs)	Culture medium RPMI-1640	Culture medium RPMI-1640 plus FCS*	Percent viable cells at the termination of culture**	Percent of cells which rosette with EAM Exp 1	Exp 2	Exp 3
0	-	+	97	0	1	0
	+	-	94	0	0	0
4	+	-	97	1	2	0
	-	+	92	10	13	12
8	+	-	95	3	2	3
	-	+	91	17	19	17
12	+	-	95	4	5	5
	-	+	89	22	23	21
20-24	+	-	92	4	4	5
	-	+	88	28	26	25
48	+	-	92	7	6	6
	-	+	86	25	24	23

* Concentration of FCS is 20 percent.
** The mean of the three experiments.

TABLE 7

THE SYNTHESIS OF THE FcM RECEPTORS BY THE T_O LYMPHOCYTES IS A FUNCTION OF THE CONCENTRATION OF FCS IN THE CULTURE MEDIUM

T_O lymphocytes cultured for 24 hr in medium RPMI-1640 fortified with FCS in the following concentration	Percent viable cells at the termination of culture*			Percent EAM rosetting cells at the termination of culture		
	Exp 1	Exp 2	Exp 3	Exp 1	Exp 2	Exp 3
0	95	94	93	3	3	4
2.5 percent	98	95	95	10	5	8
5 percent	89	88	90	12	8	12
10 percent	89	90	93	15	12	14
20 percent	95	95	93	22	20	25
40 percent	78	80	79	10	8	17

* Determined by the trypan blue dye exclusion test.

5.2. T CELLS CULTURED WITH CYCLOHEXAMIDE FAIL TO EXHIBIT FcM RECEPTORS.

5.2.1. RATIONALE AND OBJECTIVES.

Investigators who failed to detect FcM receptors on freshly isolated T cells (87,97,100-108) have speculated that FcM receptors exist on freshly isolated T_0 cells but are not detected in vitro since they had interacted with and been neutralized by circulating IgM in vivo. However, it has also been suggested that the FcM receptors are, in fact, absent on freshly isolated T_0 cells and are generated de novo in culture in the presence of FCS (which does not contain IgM). It was therefore important to address the question whether or not FcM receptors are synthesized in culture by the circulating T cells.

The objective of this series of experiments was to investigate the capacity of circulating T cells to exhibit FcM receptors in culture in the presence of cyclohexamide, a well documented inhibitor of protein synthesis.

5.2.2. EXPERIMENTAL PROTOCOL.

Normal human volunteers without a history of recent illness and not on medication were bled from the anticubital vein into heparinized tubes as described in chapter 4.2.2. The blood was then diluted 1:1 with HBSS and centrifuged through a Ficoll-Hypaque discontinuous density gradient (S.G. 1.077) as described in chapter 4.2.2. The mononuclear cells at the interphase were collected, washed and resuspended in medium as described in chapter 4.2.2.

The MNC were then separated into T and non-T cells by rosetting T cells with SRBC as described in chapter 4.2.5. The T_0 cells were then cultured for 24 hours in culture medium containing cyclohexamide in concentrations ranging from 0.25 ug per ml to 320 ug per ml. At the end of the culture period, the T cells were washed and assayed for EAM rosetting cells. Viability of the cultured cells was determined by the trypan blue dye exclusion test as described in chapter 4.2.14.

5.2.3. RESULTS.

As can be seen in Table 6, T_0 cells cultured for 24 hours with cyclohexamide in a concentration range of 0.25ug to 4.0 ug per ml were not markedly affected in their capacity to rosette with EAM. However, only 5 percent or less of the T_0 cells cultured with cyclohexamide in a concentration of 5 ug per ml and none of the T_0 cells cultured with cyclohexamide in a concentration of 10 ug per ml rosetted with EAM indicator cells. It is important to stress that the majority of the cultured cells were viable at the end of the culture period.

5.2.4. COMMENTARY.

Investigations carried out by Moretta et al (83) and Mingari et al (109) using cyclohexamide in their culture media provided evidence that the FcM receptors are newly synthesized by the cultured T cells since the cells cultured in the presence of cyclohexamide did not rosette with EAM

indicator cells. A number of other investigators (84,87,98,102,114,115) have attributed the failure to detect FcM receptors on freshly isolated T cells to binding of the receptors by circulating IgM. The experiments carried out in this section were designed to resolve this conflicting issue.

The results of the experiments presented in Table 6 strongly suggest that T cells actively synthesize FcM receptors in culture since cyclohexamide, in a non cytotoxic concentration (5 ug per ml), was able to inhibit their synthesis. However, these experiments do not unequivocally prove that these receptors are not unmasked by a "covering protein" (presumably IgM) which is shed by these cells in culture.

The experiments in the next section were carried out to investigate the role of IgM in the generation of FcM receptors by T cells.

TABLE 6

FRESHLY ISOLATED T LYMPHOCYTES (T_O CELLS) DO NOT SYNTHESIZE FcM RECEPTORS WHEN CULTURED FOR 24 HOURS WITH CYCLOHEXAMIDE IN A NON-CYTOTOXIC CONCENTRATION

T_O cells(3×10^6) cultured for 24 hr in culture medium in the presence of cyclohexamide	Percent viable cells at the termination of culture*			Percent EAM rosetting cells at the termination of culture		
	Exp 1	Exp 2	Exp 3	Exp 1	Exp 2	Exp 3
(ug cyclohexamide per ml)						
0	95	83	87	34	28	26
0.25	85	82	78	30	25	24
1	79	78	72	20	20	20
2	85	89	79	23	26	24
4	83	80	64	16	19	20
5	85	80	74	5	0	0
10	78	70	68	0	0	0
20	70	68	60	0	0	0
40	45	56	42	0	0	0
80	20	28	30	0	0	0
160	6	9	10	0	0	0
320	0	0	0	0	0	0

* Determined by the trypan blue dye exclusion test.

5.3. IgM INHIBITS THE SYNTHESIS OF FcM RECEPTORS BY THE CO-CULTURED T CELLS BUT DOES NOT REACT WITH AND NEUTRALIZE THEM.

5.3.1. RATIONALE AND OBJECTIVES.

A number of investigators have attributed the failure to detect FcM receptors on freshly isolated T cells to binding of the receptors by circulating IgM (84,97,98,102,114,114). They have theorized that bound IgM is released by or is shed from T cells during 24 hour incubation at 37°C, thus freeing the FcM receptors and permitting their detection by facilitating rosette formation with EAM. However, none of these investigators actually looked for and/or detected any IgM in the cell-free supernatants of the cultures of freshly isolated T cells. There are also conflicting reports on the issue of whether or not the presence of IgM in culture medium is capable of inhibiting the formation of EAM rosettes.

The objective of this series of experiments was to investigate the role IgM plays in the generation and/or detection of FcM receptors on T cells.

5.3.2. EXPERIMENTAL PROTOCOL.

Normal human volunteers without a history of recent illness and not on medication were bled from the median cubital vein into heparinized tubes as described in chapter 4.2.2. The blood was then diluted 1:1 with HBSS and centrifuged through a Ficoll-Hypaque discontinuous density gradient (S.G. 1.077) as described in chapter 4.2.2. The

mononuclear cells at the interphase were collected, washed and resuspended in medium as described in chapter 4.2.2. The MNC were then separated into T and non-T cells by rosetting the cells with SRBC, as described in chapter 4.2.5. The T cells were cultured for 24 hours in medium containing chromatographically pure human IgM, normal human serum, or chromatographically pure human IgG. The T_0 and T_{24} cells were assayed for FcM receptors by rosetting with EAM indicator cells as described in chapter 4.2.12. T_{24} cells were cultured for another 24 hours in medium containing human IgM, IgG or normal human serum. They were then assayed for FcM receptors by rosetting with EAM indicator cells as described in chapter 4.2.12.

T_0 cells were cultured for 24 hours at 37°C. The cell-free supernatants were collected and assayed for IgM using the ELISA technique described in chapter 4.2.11.

5.3.3. RESULTS.

As can be seen from the results presented in Table 7, IgM in low concentrations (<1,000 ng per ml) was unable to affect the synthesis of the FcM receptors by these cells. However, when the concentration of IgM in the culture medium was increased to 100,000 ng per ml, which is one tenth the physiological (circulating) concentration (10^6 ng per ml), the synthesis of FcM receptors by the cultured T_0 cells was markedly diminished; EAM rosetting cells decreased by 20 to 30 percent from the control cultures (Table 7). However, it should be noted that IgM, even in physiological

concentrations (10^6 ng per ml), failed to interact with and neutralize the FcM receptors since T_{24} cells incubated for 24 hours with IgM did not lose their capacity to rosette with EAM (Table 8). Normal human serum which contained IgM at a concentration of 116 mg percent (1.16×10^6 ng per ml) behaved as did purified IgM (Tables 7 and 8). On the other hand, IgG, irrespective of its concentration in the culture medium, neither inhibited the synthesis of FcM receptors (Table 7) nor neutralized the FcM receptors (Table 8). These results strongly indicate that IgM inhibits the synthesis of FcM receptors by the cultured T cells.

It can also be concluded, from the data presented in Table 9, that IgM is not bound to the freshly isolated T cells since the cell-free supernatants of the cultured T cells did not contain any IgM.

5.3.4 COMMENTARY.

It was demonstrated in the previous section that FcM receptors are synthesized by cultured T_0 cells de novo (chapter 5.2.). It was, however, necessary to investigate the role of IgM in the synthesis/expression of FcM receptors in view of conflicting findings of previous investigators. Evidence that IgM is not normally bound to presumed FcM receptors on freshly isolated T_0 cells is provided by the fact that the cell-free supernatants of 24 hour cultures of T_0 cells (3×10^6, 2×10^7) did not contain detectable quantities of IgM (<56 ng per ml) (Table 9). These results support the stand that IgM is not bound to FcM receptors on

T_0 cells. If IgM were bound to these cells from which it would dissociate in culture, it should be detected by the ELISA assay.

In a manner not yet understood, IgM in physiological concentration is capable of suppressing the synthesis of FcM receptors by the cultured T_0 cells (Table 7). The failure to detect these receptors on cultured T cells must be attributed to the failure of synthesis rather than neutralization of the synthesized FcM recptors, since IgM, in a physiological concentration, is not capable of interacting with and neutralizing the FcM receptors (Table 8). However, it should be noted that IgM cannot totally inhibit the synthesis of FcM receptors by the cultured T_0 cells. The synthesis of approximately 25 percent of the FcM receptors is resistant to the suppression mediated by IgM.

TABLE 7

T_O LYMPHOCYTES INCUBATED FOR 24 HOURS WITH IgM IN HIGH CONCENTRATION FAIL TO SYNTHESIZE FcM RECEPTORS

T_O cells (3×10^6) cultured for 24 hours in the presence of	Percent of T cells which rosette with the EAM indicator cells Exp. 1	Exp. 2	Exp. 3
Nil	28	27	28
IgM, 25 ng per ml	27	28	26
IgM, 100 ng per ml	28	26	28
IgM, 1,000 ng per ml	26	27	24
IgM, 10,000 ng per ml	11	12	11
IgM, 100,000 ng per ml	7	4	4
Serum* diluted 10,000X	28	21	13
Serum diluted 1,000X	12	10	9
Serum diluted 100X	9	8	ND**
Serum diluted 10X	6	6	7
Serum, undiluted	ND	4	6
IgG, 25 ng per ml	26	26	ND
IgG, 100 ng per ml	29	28	ND
IgG, 1,000 ng per ml	27	26	23
IgG, 10,000 ng per ml	26	23	25
IgG, 100,000 ng per ml	30	25	26

* The concentration of IgM was 1.16×10^6 ng per ml.
** ND = Not done.

TABLE 8

IgM, IgG AND NORMAL HUMAN SERUM FAIL TO NEUTRALIZE THE RECEPTOR FOR IgM FOLLOWING INCUBATION WITH T_{24} CELLS FOR 24 HOURS

T_{24} lymphocytes ($3x10^6$) incubated for 24 hr in culture medium with the following	Percent of T cells which rosette with the EAM indicator cells		
	Exp. 1	Exp. 2	Exp. 3
Nil	26	24	25
IgM, 25 ng per ml	26	25	25
IgM, 100 ng per ml	28	23	24
IgM, 1000 ng per ml	26	25	23
IgM, 10,000 ng per ml	27	23	20
IgM, 100,000 ng per ml	23	20	28
Serum, diluted 10,000X	25	22	18
Serum, diluted 1,000X	26	20	22
Serum, diluted 100X	29	ND*	ND
Serum, diluted 10X	25	20	25
Serum, undiluted	ND	22	20
IgG, 25 ng per ml	26	ND	ND
IgG, 100 ng per ml	24	ND	ND
IgG, 1000 ng per ml	28	24	20
IgG, 10,000 ng per ml	26	22	21
IgG, 100,000 ng per ml	28	23	20

*ND = Not done.

TABLE 9

CELL-FREE CULTURE SUPERNATANTS OF T_O CELLS CULTURED FOR 24 HOURS DO NOT CONTAIN DETECTABLE IgM

Number of T_O cells cultured for 24 hours	ng IgM detected in the cell-free culture supernatants	
	Exp. 1	Exp. 2
3×10^6	<56	<56
2×10^7	<56	<56

5.4. CIRCULATING NULL LYMPHOCYTES, BUT NOT B LYMPHOCYTES OR MONOCYTES, INHIBIT THE SYNTHESIS OF FcM RECEPTORS BY T LYMPHOCYTES IN CULTURE.

5.4.1. RATIONALE AND OBJECTIVE.

The results presented in section 5.1 of this chapter demonstrated that pure T_0 cells but not unfractionated MNC_0 cells (70 percent of which are T_0 cells) incubated for 24 hours in culture medium generated cells with FcM receptors capable of rosetting with EAM indicator cells (Tables 2,3). These results suggested that one or more of the cells within the non-T cell population can suppress the synthesis of FcM receptors by T_0 cells.

The objectives of the following experiments were to ascertain whether (i) any of the non-T cells (T, B, Null) in the non-T cells inhibits the synthesis of FcM receptors on the co-cultured T_0 cells, and (ii) whether bystander T cells are required for the synthesis of FcM receptors by the T_M cells.

5.4.2. EXPERIMENTAL PROTOCOL.

The methods used to isolate the circulating MNC, T cells and B cells from blood have been described in the section 5.1.2. of this chapter. Null cells were isolated using rosetting methods described in chapter 4.2.6. T cells were separated into T_G, T_C and T_M cells using the method described in chapter 4.2.6. T and non-T cells were depleted of monocytes by using carbonyl iron, as described in chapter 4.2.7. The presence or absence of monocytes in

the T and non-T cell populations was determined using monoclonal Leu-M3 and staining for nonspecific esterase (NSE) as described in chapters 4.2.8 and 4.2.16, respectively.

In order to ascertain whether non-T cells (Null cells, B cells, monocytes) inhibit the synthesis of FcM receptors by the co-cultured T_0 cells, T_0 cells were co-cultured with non-T cells, Null cells or B cells in varying ratios. In order to determine whether the non-T_M T cells affect the synthesis of FcM receptors by the T_M cells, T_0 cells depleted of T_G cells and/or T_C cells were incubated with the non-T cells, B cells or Null cells.

5.4.3. RESULTS.

It can be seen from the data presented in Table 10, the non-T cells were capable of inhibiting the synthesis of FcM receptors by cocultured T cells. Total suppression of the synthesis of FcM receptors was attained when the non-T cells were present in a concentration of 30 percent of the T cells (Table 10). However, it should be noted that the non-T cells even in a concentration as low as 2 percent of the cultured T cells suppressed the synthesis of FcM receptors to an extent of 60 to 65 percent (Table 10). Total suppression of FcM receptor synthesis was observed only when the non-T cells were added to the T_0 cells at the beginning of the 24 hour culture (Table 11). Significant suppression of FcM receptor synthesis was observed when the non-T cells were added to the T_0 cells

during the first 4 to 12 hours of culture. The addition of non-T cells to T_0 cells after 20 hours of culture resulted in only marginal suppression of FcM receptor synthesis by the T_0 cells (Table 11).

To investigate whether the suppressive effect of non-T cells on FcM receptor synthesis by the T cells is HLA restricted, T_0 cells were cultured with autologous or allogeneic non-T cells for 24 hours followed by rosetting with EAM indicator cells. As can be seen from the data presented in Table 12, the cultures of all of the T_0 and non-T cells resulted in total suppression of FcM receptor synthesis, indicating that this activity by the non-T cells is HLA independent.

The role of monocytes in the suppression of FcM receptor synthesis by the T_0 cells was investigated. T_0 and non-T_0 cells were prepared from monocyte-depleted and non-depleted MNC_0 and co-cultured for 24 hours. No monocytes were detected in the monocyte-depleted cell suspensions either at the beginning or the end of culture. As can be seen from data presented in Table 13, non-T cells depleted of monocytes were as effective as non-T cells containing monocytes in the their ability to suppress FcM receptor synthesis by the co-cultured T_0 cells. These results indicate that monocytes are not involved in the non-T cell mediated suppression of FcM receptor synthesis by T_0 cells.

The purified B or Null cells were then co-cultured with

the autologous T_0 cells for 24 hours. As can be seen from the results presented in Table 14, the Null cells present in a concentration of 30 percent of the cultured T_0 cells totally suppressed the synthesis of FcM receptors by the cultured T_0 cells. In fact, the Null cells suppressed the synthesis of FcM receptors by greater than 60 perecant even when they were present in a concentration of 1 percent of the cultured cells. In contradistinction, the B cells present in as high a concentration as 50 percent of the cultured cells were consistently incapable of suppressing the synthesis of FcM receptors by the cultured T_0 cells (Table 14).

It can be concluded by the data presented in Table 15 that removal of the T_G and/or the T_C cells from the T_0 cells did not affect the Null cell mediated suppression of FcM receptor synthesis.

5.4.4. COMMENTARY.

Freshly isolated unfractionated MNC_0 cells and reconstituted MNC_0 cells consisting of freshly isolated T_0 and non-T cells failed to generate cells with FcM receptors when cultured for 24 hours in culture medium. These results suggested that the non-T cells exerted a suppressive effect on the T_0 cells resulting in the inhibition of FcM receptor synthesis. Since the non-T cells consist of monocytes, B and Null cells, the suppressive activity of each of these cells was systematically investigated.

MNC_0 were depleted of monocytes twice over a period of 24 hours (N. Kazaninowsky, PhD thesis, University of Ottawa, 1986). It was demonstrated that the monocyte-depleted non-T cells were as effective in suppressing FcM receptor synthesis by co-cultured monocyte-depleted T_0 cells as were the monocyte containing non-T cells co-cultured with monocyte-containing T_0 cells. It was therefore concluded that monocytes do not play a role in the non-T cell mediated suppression of FcM receptor synthesis by T_0 cells.

The Null cells, isolated from the non-T cells, totally suppressed the synthesis of FcM receptors by the co-cultured T_0 cells whereas the B cells displayed no suppressive activity. In fact, the Null cells exhibited very significant suppression of FcM receptor synthesis when they constituted only 0.1 percent of the cells (T_0 plus Null) cultured, It was therefore concluded that only the Null cells, and not the B cells or the monocytes, are capable of suppressing the synthesis of FcM receptors by freshly isolated T_0 cells in culture. It was also demonstrated that the suppressive activity by the Null cells is HLA independent as non-T cells added to allogeneic T_0 cells in culture were just as effective as autologous non-T cells in suppressing the synthesis of FcM receptors by cultured T_0 cells.

It was also demonstrated that the suppressive activity of Null cells on FcM receptor synthesis is not dependent

upon the presence of non-T_M T cells (T_G,T_C) in culture. It would therefore appear that the Null cells act directly on the T_M cells to inhibit FcM receptor synthesis.

TABLE 10

NON-T LYMPHOCYTES INHIBIT THE SYNTHESIS OF RECEPTORS FOR FcM BY AUTOLOGOUS T LYMPHOCYTES

Cells cultured for 24 hr			Percent EAM rosetting cells		
T cells	Non-T cells	Non-T cells as a percentage of the T cells	Exp. 1	Exp. 2	Exp. 3
3.0×10^6	Nil	0	23(0)*	19(0)	25(0)
3.0×10^6	0.06×10^6	2	9(61)	7(63)	9(64)
3.0×10^6	0.15×10^6	5	8(65)	7(63)	7(72)
3.0×10^6	0.30×10^6	10	3(87)	4(79)	3(88)
3.0×10^6	0.90×10^6	30	0(100)	3(84)	0(100)
3.0×10^6	1.50×10^6	50	0(100)	0(100)	0(100)

* The figures outside and inside the brackets represent the percent of EAM rosetting cells observed and the percent suppression of EAM rosetting cells, respectively.

TABLE 11

THE INHIBITION BY AUTOLOGOUS NON-T CELLS OF FcM RECEPTOR SYNTHESIS BY THE T_O LYMPHOCYTES IN CULTURE IS A FUNCTION OF THE TIME OF ADDITION OF THE NON-T CELLS TO THE CULTURES

Non-T cells (0.90×10^6) added to the T_O cells (3.0×10^6) at the following time of the 24 hr culture	Percent EAM rosetting cells		
	Exp 1	Exp 2	Exp 3
Control (no non-T cells added)	22(0)*	27(0)	30(0)
0 hr	0(100)	0(100)	0(100)
2 hr	4(82)	5(80)	3(90)
4 hr	6(74)	8(67)	8(75)
8 hr	8(64)	10(60)	10(67)
12 hr	10(55)	12(52)	15(50)
20 hr	16(28)	20(20)	22(24)

* The figures outside and inside the brackets represent the percent of EAM rosetting cells observed and the percent suppression of EAM rosetting cells, respectively.

TABLE 12

THE SUPPRESSION BY NON-T CELLS OF THE SYNTHESIS OF FcM RECEPTORS BY T_O CELLS IS NOT HLA RESTRICTED

T_O cells (3×10^6) cultured with autologous or allogenic non-T cells (0.9×10^6) for 24 hrs*	Percent EAM rosetting cells		
	Exp 1	Exp 2	Exp 3
T_1	27(0)**	20(0)	29(0)
T_2	24(0)	22(0)	30(0)
T_3	20(0)	30(0)	28(0)
T_1 + Non-T_1	0(100)	0(100)	0(100)
T_1 + Non-T_2	0(100)	0(100)	0(100)
T_1 + Non-T_3	0(100)	0(100)	0(100)
T_2 + Non-T_1	0(100)	0(100)	0(100)
T_2 + Non-T_2	0(100)	0(100)	0(100)
T_2 + Non-T_3	0(100)	0(100)	0(100)
T_3 + Non-T_1	0(100)	0(100)	0(100)
T_3 + Non-T_2	0(100)	0(100)	0(100)
T_3 + Non-T_3	0(100)	0(100)	0(100)

* The cells were obtained from three unrelated donors.
** The figures outside and inside the brackets represent the percent of EAM rosetting cells observed and the percent suppression of EAM rosetting cells, respectively.

TABLE 13

MONOCYTES DO NOT PLAY A ROLE IN THE SYNTHESIS OF RECEPTORS FOR FcM BY AUTOLOGOUS T LYMPHOCYTES

Cells cultured for 24 hrs	Percent non-T cell	Percent EAM rosetting cells		Percent monocytes in the culture	
		Exp 1	Exp 2	Exp 1	Exp 2
$T(3.0\times10^6)$	0	28(0)*	30(0)	2(1)**	1(1)
$T(3.0\times10^6)$+non-$T(0.15\times10^6)$	5	6(79)	8(74)	2(2)	1(2)
$T(3.0\times10^6)$+non-$T(0.30\times10^6)$	10	3(81)	5(83)	4(4)	2(3)
$T(3.0\times10^6)$+non-$T(0.90\times10^6)$	30	0(100)	0(100)	11(13)	5(10)
T_{MD}***(3.0×10^6)	0	26(0)	29(0)	<1(<1)	<1(<1)
$T_{MD}(3.0\times10^6)$+non-$T_{MD}(0.15\times10^6)$	5	8(70)	7(75)	<1(<1)	<1(<1)
$T_{MD}(3.0\times10^6)$+non-$T_{MD}(0.30\times10^6)$	10	5(81)	3(90)	<1(<1)	<1(<1)
$T_{MD}(3.0\times10^6)$+non-$T_{MD}(0.90\times10^6)$	30	0(100)	0(100)	<1(<1)	<1(<1)

* The figures outside and within the brackets represent the percent EAM rosetting cells observed and the percent suppression of EAM rosetting cells, respectively.

** The figures outside and within the brackets represent the percent monocytes at the beginning of the culture and at the end of the 24 hour culture period, respectively.

*** MD = monocyte depleted.

TABLE 14

NULL LYMPHOCYTES, NOT B LYMPHOCYTES, INHIBIT THE SYNTHESIS OF RECEPTORS FOR FcM BY AUTOLOGOUS T LYMPHOCYTES

Cells cultured for 24 hours*	Non-T, B, or Null cells as a percentage of the T cells	Percent EAM rosetting cells		
		Exp 1	Exp 2	Exp 3
T	0	25(0)**	30(0)	25(0)
T + Non-T	30	0(100)	0(100)	0(100)
T + Null	0.1	16(36)	17(44)	16(36)
T + Null	0.2	13(48)	16(47)	13(48)
T + Null	0.3	12(52)	15(50)	11(56)
T + Null	0.5	10(60)	14(53)	9(64)
T + Null	1.0	9(64)	13(59)	8(68)
T + Null	5	5(80)	8(74)	8(68)
T + B	5	22(8)	23(18)	22(8)
T + Null	10	3(83)	7(77)	6(74)
T + B	10	18(18)	22(18)	20(13)
T + Null	30	0(100)	0(100)	0(100)
T + B	30	17(5)	20(0)	18(0)
T + Null	50	0(100)	0(100)	0(100)
T + B	50	17(0)	18(0)	16(0)

* Constant number of T_o cells cultured (3×10^6)
** The figures outside and inside the brackets represent the percent EAM rosetting cells observed and percent suppression of EAM rosetting cells, respectively.

TABLE 15

THE T_G AND T_C CELLS ARE NOT REQUIRED FOR THE NULL CELL-MEDIATED SUPPRESSION OF THE SYNTHESIS OF RECEPTORS FOR FcM BY THE T_O CELLS

T_o cells (3×10^6) cultured for 24 hours	Null cells(10^6) added to the T_o cell cultures	Percent EAM rosetting cells	
		Exp. 1	Exp. 2
T_o	NO	28(0)*	32(0)
	YES	0(100)	0(100)
T_o depleted of T_G cells	NO	25(0)	27(0)
	YES	0(100)	0(100)
T_o depleted of T_C cells	NO	22(0)	18(0)
	YES	0(100)	0(100)
T_o depleted of T_G and T_C cells	NO	26(0)	22(0)
	YES	0(100)	0(100)

* The figures outside and within the brackets represent the percent of EAM rosetting cells observed and the percent suppression of EAM rosetting cells, respectively.

5.5. THE NULL LYMPHOCYTES SECRETE A FACTOR(S) IN CULTURE, RECEPTOR SYNTHESIZING SUPPRESSOR FACTOR OR RSSF, WHICH INHIBITS THE SYNTHESIS OF FcM RECEPTORS BY AUTOLOGOUS T LYMPHOCYTES.

5.5.1. RATIONALE AND OBJECTIVE.

In the previous chapter, it was demonstrated that Null lymphocytes, but not B lymphocytes or monocytes, are capable of suppressing the synthesis of FcM receptors by the T cells following co-culture of Null cells and T cells. Null cells in a concentration as low as 0.1 percent of the cultured cells (T:Null=1000:1) resulted in greater than 50 percent suppression of FcM receptor synthesis.

The objective of this series of experiments was to define the mechanism(s) whereby null cells suppress the synthesis of FcM receptors by co-cultured T_0 cells .

5.5.2. EXPERIMENTAL PROTOCOL.

The methods used to isolate the circulating MNC, T cells, and B cells from blood have been described in section 5.1.2. of this chapter. Null cells were isolated as described in section 5.4.2. of this chapter. In order to demonstrate whether Null cells secrete a factor which is capable of suppressing the synthesis of FcM receptors by T cells, Null cells were cultured in culture medium for 24 hours. The cell-free culture supernatant was then filtered and used as culture medium for the culture of T_0 cells for

24 hours, at the end of which these T cells were assayed for EAM rosetting cells. The viability of the cells was determined by the conventional trypan blue dye exclusion test as described in chapter 4.2.14. Ultrasonicates of null cells were obtained by subjecting these cells to 60,000 K cycles per minute at 4°C for 1 minute.

5.5.3.RESULTS.

As can be seen from the results presented in Table 16, Null cells but not B cells or T cells, cultured for 24 hours in the presence or absence of fetal calf serum, secreted a factor(s) capable of inhibiting the synthesis of FcM receptors by freshly isolated T_0 cells in culture. The factor was named receptor synthesizing suppressor factor (RSSF). B cells failed to secrete RSSF even when they were cultured with T_0 cells.

The Null cell ultrasonicates, prepared from three times the number of Null cells required to totally suppress FcM receptor synthesis in culture for 24 hours, were devoid of suppressor activity (Table 17).

The cell-free culture supernatants of 2 hour cultures of Null cells inhibited FcM receptor synthesis by about 70 percent and were almost as suppressive as the supernatants of 24 hour cultures of Null cells. However, only the cell-free culture supernatants of 24 hour cultures of Null cells were able to totally suppress FcM receptor synthesis (Table 18). These results indicate that RSSF was secreted very rapidly by the null cells in culture.

The Null cells were cultured for up to 5 days in culture medium.The cell - free supernatants were replaced every 24 hours and assayed for suppressor activity.As can be seen from the data presented in Table 19, the Null cells secreted highly potent RSSF during the first 2 days in culture. They secreted very little RSSF on day 3 of culture and no RSSF was secreted on the 4th and 5th days of culture. The viability of the cells was very high for the first 4 days of culture but decreased significantly by day 5 of culture (Table 19).

The Null cells were then preincubated in culture medium for 24 hours at 37°C with cyclohexamide in a concentration range (0.5 ug to 4.0 ug per ml) which does not affect the synthesis of FcM receptors by the T_0 cells (Table 6 section 5.2.2). The cells were then washed twice and co-cultured with T_0 cells for an additional 24 hours. These Null cells were unable to suppress FcM receptor synthesis by the co-cultured T_0 cells (Table 20). Aliquots of the washed Null cells (previously incubated with cyclohexamide) were also cultured alone for an additional 24 hours in culture medium and cell-free culture supernatants were assayed for suppressor activity. These supernatants displayed no suppressor activity (Table 21).

RSSF was incubated with T_0 cells, Null cells or B cells for 2 hours at 37°C. The cell-free supernatants were then assayed for the capacity to suppress FcM receptor synthesis by co-cultured T_0 cells. The suppressor

activity of RSSF was not absorbed out of solution following co-incubation with T_0, B or Null cells for 2 hours since they were still capable of totally suppressing FcM receptor synthesis (Table 22).

As can be seen from the data presented in Table 23, RSSF did not interact with the FcM receptors on the T_{24} cells. The T_{24} cells following incubation with RSSF for 24 hours at 37°C rosetted with EAM to the same extent as did the the T_{24} cells incubated for 24 hours in culture medium in the absence of RSSF.

5.5.4. COMMENTARY.

In this investigation, it was demonstrated that the Null cells cultured in the presence or absence of FCS for 24 hours secreted a factor(s), RSSF, which inhibited the synthesis of FcM receptors by the cultured T_0 cells.

RSSF was secreted rapidly by the Null cells since the cell-free supernatants of 2 hour Null cell cultures inhibited FcM receptor synthesis by the cultured T_0 cells by 70 to 75 percent. RSSF was not present in the freshly isolated Null cells since the soluble, filtered ultrasonicates of Null cells exhibited no suppressor activity. Furthermore, RSSF detected in the supernatants of Null cell cultures was not simply released from degenerating or dying Null cells since the Null cells remained viable (> 70 percent) for at least 3 days in culture. In addition, Null cells incubated with an inhibitor of protein synthesis, cyclohexamide, in low non-cytotoxic concentration, failed to

synthesize RSSF although the viability of the cells was greater than 85 percent. It must be emphasized that the concentration of cyclohexamide which inhibited the synthesis of RSSF, 0.5 ug per ml, was at least 10 times lower than the concentration of cyclohexamide required to directly inhibit the synthesis of FcM receptors by the T_0 cells (Table 6 section 5.5.2.). Therefore, the inhibition of FcM receptor synthesis by RSSF cannot be attributed to the residual cyclohexamide carried over into the T_0 cell cultures. It was therefore concluded that RSSF is synthesized and secreted by the Null cells in culture. The failure to detect FcM receptors on T cells after culture with RSSF can only be attributed to the suppression of the synthesis of these receptors since RSSF was unable to interact with and therefore neutralize already generated FcM receptors on T_{24} cells even after co-culture for 24 hours (Table 23).

TABLE 16

THE NULL LYMPHOCYTES SECRETE A FACTOR(S) IN CULTURE, RECEPTOR SYNTHESIZING SUPPRESSOR FACTOR OR RSSF, WHICH INHIBITS THE SYNTHESIS OF THE FcM RECEPTOR BY AUTOLOGOUS T LYMPHOCYTES

Cell-free supernatants obtained from the following 24 hr cultures*	Percent EAM rosetting cells following culture of the T_o cells for 24 hours in the cell-free culture supernatants		
	Exp 1	Exp 2	Exp 3
T_o cells (3×10^6)	24(0)**	20(0)	22(0)
Null cells (10^6)	0(100)	0(100)	0(100)
B cells (10^6)	21(13)	18(10)	16(20)
T_o (3×10^6)+Null cells(10^6)	0(100)	0(100)	0(100)
T_o(3×10^6)+B(10^6)	19(20)	16(20)	16(20)

* The medium used was culture medium. Identical results were obtained when fetal calf serum was omitted from the medium.

**The figures outside and inside the brackets represent the percent of EAM rosetting cells observed and the percent suppression of the EAM rosetting cells, respectively.

TABLE 17

ULTRASONICATES OF NULL CELLS DO NOT EXHIBIT RSSF ACTIVITY

T_o cells(3×10^6) incubated for 24 hrs with	Percent EAM rosetting cells	
	Exp 1	Exp 2
Nil	26	28
Untreated Null cells(10^6 cells)	0	0
Supernatant of a 24 hr Null cell culture (10^6 cells per ml of culture medium)	0	0
Null cell sonicate (prepared from 3×10^6 cells)	25	27

TABLE 18

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

Supernatants of Null cell cultures(10^6 per ml) after the following period of culture	Percent EAM rosetting cells following culture of the T_o cells for 24 hours in the Null cell culture supernatants		
	Exp. 1	Exp. 2	Exp. 3
0 hr	20(0)*	23(0)	24
2 hr	5(75)	8(66)	6(76)
8 hr	4(80)	7(70)	5(80)
20 hr	3(85)	5(79)	3(88)
24 hr	0(100)	0(100)	0(100)
T_{24} Control	20(0)	24(0)	25(0)

* The figures outside and inside the brackets represent the percent of EAM rosetting cells observed and the percent suppression of EAM rosetting cells, respectively.

TABLE 19

THE NULL LYMPHOCYTES LOSE THE CAPACITY TO SECRETE RSSF AFTER 2 DAYS IN CULTURE

Supernatants of Null cell cultures (10^6 per ml) after the following periods of culture	Percent viable Null cells at the end of the culture period*		Percent EAM rosetting cells following culture of T_o cells for 24 hours in the Null cell culture supernatants	
	Exp 1	Exp 2	Exp 1	Exp 2
Nil	88	87	26(0)**	28(0)
0 to 24 hours	80	78	0(100)	0(100)
24 to 48 hours	70	80	0(100)	0(100)
48 to 72 hours	77	84	24(8)	25(14)
72 to 96 hours	70	65	26(0)	27(3)
96 to 120 hours	50	48	28(0)	29(0)

* Determined by the trypan blue dye exclusion test.

** The figures outside and inside the brackets represent the percent of EAM rosetting cells observed and the percent suppression of the EAM rosetting cells, respectively.

TABLE 20

NULL CELLS PREINCUBATED FOR 24 HOURS WITH CYCLOHEXAMIDE FAIL TO INHIBIT THE SYNTHESIS OF FcM RECEPTORS BY T_O CELLS IN CULTURE

Null cells preincubated for 24 hours with cyclohexamide in the following concentration (ug cyclohexamide per ml)	Mean percent viable Null cells following incubation with cyclohexamide*	Percent EAM rosetting cells following culture of the T_O cells for 24 hours with the cyclohexamide-treated Null cells	
		Exp. 1	Exp. 2
Nil(Null cells not preincubated with cyclohexamide)	80	0	0
0.5	78	23	19
1.0	70	19	18
2.0	72	22	16
3.0	66	20	17
4.0	62	23	20
5.0	55	18	23
T_O cells incubated in culture medium for 24 hours	88	25	22

* Determined by the trypan blue dye exclusion test.

TABLE 21

NULL CELLS CULTURED FOR 24 HOURS WITH CYCLOHEXAMIDE FAIL TO SECRETE RSSF

Null cells cultured for 24 hours with cyclohexamide, washed, and cultured for an additional 24 hours in culture medium	Percent viable cells at the termination of culture*		Percent EAM rosetting cells following culture of the T_O cells for 24 hours in the Null cell culture supernatants	
	Exp. 1	Exp. 2	Exp. 1	Exp. 2
(ug cyclohexamide per ml)				
0	84	86	0	0
0.5	83	79	24	20
1.0	78	80	23	18
2.0	79	84	20	19
4.0	85	80	18	20
T_O cells incubated in culture medium for 24 hours	88	81	24	26

* Determined by the trypan blue dye exclusion test.

TABLE 22

RSSF IS NOT ABSORBED BY FRESHLY ISOLATED T, B OR NULL CELLS

RSSF incubated with the following cells*	Percent EAM rosetting cells following culture of the T_o cells for 24 hours with the absorbed RSSF		
	Exp. 1	Exp. 2	Exp. 3
Nil	0(100)**	0(100)	0(100)
T_o cells (2×10^7)	2(98)	1(99)	0(100)
Null cells (10^7)	0(100)	0(100)	0(100)
B cells (5×10^6)	0(100)	0(100)	0(100)
Control (T_o cells incubated for 24 hours in culture medium)	23(0)	25(0)	19(0)

* RSSF was incubated with T_o, Null or B cells for 2 hours at 37°C. The tubes were shaken every 15 minutes.

** The figures outside and inside the brackets represent percent of EAM rosetting cells observed and the percent suppression of EAM rosetting cells, respectively.

TABLE 23

RSSF DOES NOT NEUTRALIZE THE FcM RECEPTORS ON THE T_{24} CELLS

T_{24} cells* were incubated in culture medium for the following period of time prior to rosetting with the EAM indicator cells	RSSF added to the T_{24} cultures	Percent EAM rosetting cells	
		Exp. 1	Exp. 2
0 hours	NO	33	22
24 hours	YES	29	21
24 hours	NO	32	20

* T_{24} cells are T_o cells cultured for 24 hrs in culture medium at 37°C.

5.6. SOME PHYSCOCHEMICAL PROPERTIES OF RSSF.

5.6.1. RATIONALE AND OBJECTIVE.

In the experiments presented in the previous section, it was demonstrated that Null cells secrete a factor in culture which is capable of suppressing the synthesis of FcM receptors by T cells. This factor was referred to as receptor synthesis suppressor factor or RSSF.

The objective of the following experiments was to characterize RSSF.

5.6.2. EXPERIMENTAL PROTOCOL.

Null cells were cultured in medium RPMI-1640 (10^6 in 1ml) for 24 hours and the cell-free culture supernatants, referred to as RSSF or RSSF conditioned medium, were used either in the fresh state or were kept at -20°C until used.

RSSF was incubated at 4°C, 37°C and 56°C for varying periods of time (up to 120 hours). The RSSF was dialysed for 24 hours against medium RPMI-1640 through dialysis tubing with molecular weight cut-offs of 14,000 and 50,000. The RSSF conditioned medium and regular medium were incubated with a variety of enzymes overnight*.

All of the treated RSSF preparations were reconstituted

* I wish to thank Dr. Harvey Kaplan, Department of Chemistry, for his assistance in this part of the investigation.

with FCS(20 percent) and used as culture media for T_0 cells for 24 hours. The cells were then assayed for FcM receptors.

5.6.3. RESULTS.

As can be seen from the results presented in Table 24, RSSF did not lose its suppressive activity when cultured for up to 48 hours at either 4°C or 37°C. However, RSSF incubated for 120 hours at 37°C lost essentially all its activity whereas the RSSF kept at 4°C for 120 hours retained considerable suppressive activity (60 to 68 percent). It can also be seen that RSSF exposed to 56°C for even 15 minutes retained only marginal suppressor activity. RSSF was totally inactivated following exposure to 56°C for 2 hours (Table 24).

As can be seen from Table 25, RSSF dialyzed for 24 hours through dialysis tubing with a molecular weight cut-off of 50,000 lost its ability to suppress the synthesis of FcM receptors by cultured T_0 cells. However, RSSF retained its suppressive activity following dialysis through a dialysis tubing with a molecular weight cut-off of 14,000.

RSSF subjected to different enzymes retained its capacity to suppress the synthesis of FcM receptors when it was used as culture medium for T_0 cells for 24 hours (Table 26).

5.6.4. COMMENTARY.

It was demonstrated, on the basis of experiments presented in Table 25, that the molecular weight of RSSF is

greater than 14,000 but less than 50,000. RSSF is a stable compound as it retains its activity after culture at 37°C for 48 hours or incubation at 4°C for 120 hours. It was also demonstrated that exposure of RSSF to various enzymes did not result in the loss of suppressive activity, suggesting that RSSF is not a conventional protein, although the rapid loss of activity following incubation for only 15 minutes at 56°C suggests that it is a protein.

TABLE 24

THE STABILITY OF RSSF FOLLOWING INCUBATION AT 4°C, 37°C, 56°C FOR VARYING PERIODS OF TIME

RSSF incubated at the following temperature for the following period of time(hr)		Percent EAM rosetting cells following incubation of T_0 cells for 24 hr in the preincubated RSSF	
		Exp. 1	Exp. 2
37°C	24 hr	0(100)*	0(100)
37°C	48 hr	0(100)	0(100)
37°C	72 hr	9(68)	7(67)
37°C	120 hr	19(30)	20(24)
4°C	24 hr	0(100)	0(100)
4°C	48 hr	0(100)	0(100)
4°C	72 hr	2(93)	5(83)
4°C	120 hr	9(68)	12(60)
56°C	15 min	20(30)	22(29)
56°C	2 hr	28(0)	29(0)
$(T)_{24}$(Control)		28(0)	30(0)

* The figures outside and inside the brackets represent the percent of EAM rosetting cells observed and the percent suppression of EAM rosetting cells, respectively.

TABLE 25

THE ABILITY OF DIALYZED RSSF TO INHIBIT THE GENERATION OF FcM RECEPTORS BY T_O CELLS

RSSF dialyzed through dialysis tubing with a molecular weight cut-off of	Percent and percent suppression of EAM rosetting cells following culture of T_O cells in dialyzed RSSF	
	Exp. 1	Exp. 2
14,000	0(100)*	0(100)
50,000	27(4)	30(6)
Control - not dialyzed	0(100)	0(100)
T_{24}	28(0)	32(0)

* The figures outside and inside the brackets denote the percent of EAM rosetting cells observed and the percent suppression of EAM rosetting cells, respectively.

TABLE 26

RSSF IS NOT SUSCEPTIBLE TO
ENZYMATIC DEGRADATION IN VITRO

RSSF[a] or medium alone[b] incubated for 24 hours with the following enyzme	The viability of the T_o cells incubated for 24 hours in the		The percent EAM rosetting cells following incubation of the T_o cells for 24 hours in the	
	enzyme-treated RSSF[c]	enzyme-treated medium[c]	enzyme-treated RSSF[c]	enzyme-treated medium[c]
Nil	57	70	0	23
Papain	70	62	0	17
Pronase	63	56	0	21
Trypsin	60	70	1	19
Lysozyme	68	65	0	20
Elastase	71	56	2	18
RNAase	57	48	1	22
Thermolysin	43	40	0	18
α-chymotrypsin	53	66	0	17

[a] RSSF-conditioned medium (RPMI-1640).

[b] RPMI-1640.

[c] RSSF and the medium were supplemented with fetal calf serum to a concentration of 20 percent prior to the addition of the T_o cells.

5.7. THE FUNCTIONAL ROLE OF THE FcM RECEPTORS ON T CELLS.

5.7.1. RATIONALE AND OBJECTIVE.

The T cells bearing FcM receptors, referred to as T_M cells, are considered to be essential for Ig synthesis by the B cells cultured with monocytes, Null cells and PWM for 7 days (56-64,96). However, it has been demonstrated in the previous chapters that the freshly isolated T cells are devoid of FcM receptors. One may therefore question whether the FcM receptors on the T_M helper cells serve a functional role or whether they are simply innocuous markers on the T_M helper cell generated artifactually in vitro.

The objective of the experiments carried out in this section was to investigate the functional role of FcM receptors on T_M cells in Ig synthesis in in vitro cultures.

5.7.2. EXPERIMENTAL PROTOCOL. The methods used to isolate the circulating MNC, T cells, T_O-T_G and non-T cells from blood have been described in section 5.1.2. of this chapter. The T_G-depleted T_O cells were cultured for 24 hours in culture medium for the generation of FcM receptors and are referred to as T_{24} cells. The T_O and T_{24} cells depleted of T_G cells were added to in vitro cultures of B cells, Null cells, monocytes and PWM as described in chapter 4.2.17. Cell-free culture supernatants were assayed for IgM on the 1st, 3rd, 5th and 7th day of culture by the ELISA assay. Cultured cells were also assayed for EAM

rosetting cells on the 1st, 3rd, 5th and 7th day of culture.

5.7.3 RESULTS.

As can be seen from the results presented in Table 27, Ig synthesis was detected by the 3rd day and reached a peak level on the 7th day of culture (it was lower by day 10, results not shown in Table). The percent of EAM rosetting cells in the cultures in which T_{24} cells were used decreased significantly on the third day of culture and no EAM rosetting cells were detected on days 5 or 7. It is noteworthy that no EAM rosetting cells were detected in the cultures to which were added T_0 cells.

5.7.4. COMMENTARY.

It is obvious from the results presented in Table 27 that immunoglobulin secretion by the cultures containing either the T_0 or the T_{24} cells was identical. However, it is important to note that no FcM receptor-bearing cells could be detected in the cultures consisting of the T_0 cells. These results indicate that FcM receptors are not required for immunoglobulin synthesis and secretion.

These results strongly suggest that FcM receptors on the T_M lymphocytes do not functionally participate in Ig synthesis.

TABLE 27

THE RELATIVE EFFICACY OF T_0 AND T_{24} CELLS DEPLETED TO T_G CELLS TO FACILITATE IMMUNOGLOBULIN SYNTHESIS BY B CELLS IN THE PRESENCE OF NULL CELLS, MONOCYTES AND PWM MITOGEN

Cells incubated for 7 days in culture medium in the presence of PWM	Percent EAM rosetting cells on following days of culture					ng IgM secreted by the cultured B cells on the following days of culture			
	0	1	3	5	7	1	3	5	7
T_0* + Non-T(B,Null,monocyte)	0	0	0	0	0	<56	323	641	1030
T_{24}* + Non-T(B,Null,monocyte)	22	18	12	0	0	<56	383	684	1054
T_{24}*	26	25	18	6	0	-	-	-	-

* The T cells were depleted of T_G cells by rosetting the freshly isolated T cells with EAG indicator cells.

6. DISCUSSION

The background findings which formed the basis of this investigation were that freshly isolated circulating human T cells do not possess detectable FcM receptors whereas a significant percentage of these T cells possess easily detectable FcM receptors following culture for 24 hours since they rosette with EAM indicator cells. These results suggested that either FcM receptor synthesis is inhibited in vivo but not in vitro in culture or that FcM receptors are present on the circulating T cells but are bound by circulating IgM.

The objectives of this investigation were (i) to investigate whether or not FcM receptors are synthesized by the circulating T cells in culture, (ii) to investigate the role IgM plays in the generation and/or expression of FcM receptors by T cells, (iii) to ascertain whether or not any of the non-T cells (B cells, Null cells, monocytes) suppress the synthesis of FcM receptors by co-cultured T cells, (iv) to investigate whether suppression of FcM receptor synthesis is carried out by a soluble mediator, and (v) to investigate whether FcM receptors on T cells have a functional role in the synthesis of Ig by cultured B cells.

It was unequivocally demonstrated that (i) freshly isolated T cells, B cells, Null cells and MNC do not express FcM receptors (ii) T cells, but not unfractionated MNC, B cells and Null cells, generate FcM receptors

following culture for 24 hours (iii) the optimal time required for the maximum generation of FcM receptors by cultured T cells is 24 hours (iv) the culture medium should contain FCS in a final concentration of 20 percent to facilitate the maximum generation/expression of the FcM receptors (v) Null cells secrete a factor capable of inhibiting FcM receptor synthesis by T cells and (vi) the FcM receptors on the T_M helper cells do not play a role in Ig synthesis by cultured B cells.

In offering an interpretation for the absence of FcM receptors on freshly isolated T_O cells, it is necessary not to confuse their absence on the cells with our failure to detect them. It may be that these receptors in fact exist on freshly isolated T_O cells but are not detected in vitro as they had interacted with and were neutralized by circulating IgM in vivo (84,97,98,102,114,115). However, it has also been suggested that the FcM receptors are, in fact, absent on freshly isolated T_O cells and that they are generated de novo by the T_O cells following culture for 24 hours in medium containing FCS which does not contain IgM but not in medium fortified with serum which contains IgM (83,109,110).

The results of this investigation demonstrate in unambiguous and unequivocal fashion that the FcM receptors on the cultured T cells are, in fact, synthesized de novo. It was consistently observed that T_O cells incubated for 24 hours with cyclohexamide in a low non-toxic

concentration failed to exhibit FcM receptors. It was also demonstrated that T_0 cells cultured for 24 hours with IgM in a physiological concentration also failed to exhibit FcM receptors, suggesting that IgM binds to the newly synthesized FcM receptors preventing their detection. However, since IgM could not react with and neutralize preformed FcM receptors present on T_{24} cells, it is logical to attribute the absence of FcM receptors on T cells cultured with IgM as a failure of FcM receptor synthesis and not a failure of detection.

Freshly isolated mononuclear cells (MNC_0) and reconstituted MNC_0 cells consisting of freshly isolated T_0 (70 percent) and non-T (30 percent) cells failed to generate FcM receptors when cultured for 24 hours in culture medium. Only the freshly isolated pure T_0 cells generated FcM receptors in culture. These results suggested that the non-T cells exerted a suppressive effect on the T_0 cells resulting in the inhibition of FcM receptor synthesis.

It was indeed found that the non-T cells were capable of actively suppressing the synthesis of FcM receptors by cultured T cells. Since the non-T cells consist of monocytes, B cells and Null cells, the suppressive activity of each of these cells was systematically investigated. Only the Null cells were capable of suppressing the synthesis of FcM receptors on co-cultured T cells. It was also demonstrated that the suppressor effect of the Null

cells was mediated by a factor, RSSF, which is actively synthesized and secreted by Null cells. The molecular weight of RSSF is in the range of 14,000 to 50,000. If the circulating Null cells are as capable of suppressing the synthesis of FcM receptors by the T cells in vivo as they are in vitro, they would preclude the detection of FcM receptors on freshly isolated T_0 cells.

A second mechanism which appears to be operating to inhibit the synthesis of FcM receptors is an IgM dependent one. In a manner not yet understood, IgM in a physiological concentration is capable of suppressing the synthesis of FcM receptors by the cultured T_0 cells. The failure to detect these receptors on the cultured T_{24} cells must be attributed to failure of synthesis rather than neutralization of the synthesized FcM receptors since IgM, in a physiological concentration, is not capable of interacting with and neutralizing preformed FcM receptors. However, it should be noted that IgM was unable to totally inhibit the synthesis of FcM receptors by the cultured T_0 cells. The synthesis of approximately 25 percent of the FcM receptors was resistant to the suppression mediated by IgM whereas the Null cells totally inhibited the synthesis of FcM receptors. Therefore, there appear to be two physiological mechanisms which regulate the synthesis of FcM receptors by T_0 cells - circulating IgM and the circulating Null cells. Furthermore, there appear to be two types of FcM receptors - those whose synthesis can be

inhibited by both IgM and Null cells and those whose synthesis can be inhibited only by Null cells.

In 1975 Moretta et al demonstrated that the human circulating T cells can be segregated into two classes of cells, the T_G and T_M cells on the basis of their ability to rosette with EAG and EAM indicator erythrocytes, respectively, (75-87). This finding by itself was a milestone as it demonstrated the heterogeneous nature of human circulating T cells. However, this finding was shown to have a greater ramification when Moretta et al (87-89) demonstrated that the T_M cells function as helper or facilitating cells for immunoglobulin (Ig) synthesis by the B cells in culture whereas the T_G cells function as suppressor cells capable of inhibiting Ig synthesis by the B cells in the presence of T_M helper cells.

Moretta et al, defining the functional role of FcG and FcM receptors on T cells, stated that freshly isolated T_G suppressor cells become activated following the interaction of their FcG receptors with FcG on the antigen-antibody complex (84-87). The functional importance of FcM receptors on T cells was accepted as fact and has not been questioned.

FcM receptors were not detected on freshly isolated T_O cells and they were not synthesized in vitro when cultured with B cells, Null cells, monocytes and PWM which results in Ig synthesis and secretion by day 7 of culture. The question of a functional role for the FcM receptor was

therefore addressed. Freshly isolated T_M cells deficient in detectable FcM receptors and T_M cells which had been cultured for 24 hours in culture medium containing FCS to facilitate the synthesis of FcM receptors were cultured for 10 days with B cells, Null cells, monocytes and PWM. Ig synthesis and secretion were identical in both cultures. Since FcM receptors were not detected on any cells in the cultures consisting of T_O cells, B cells, Null cells and monocytes, it would appear that the FcM receptor does not have a functional role to play in Ig synthesis. Rather, it appears to be an innocuous, incidental marker on the T_M helper cell. This revelation should not be surprising since other similar situations exist. For example, the CD4 (OKT4) monoclonal antibodies react with not only the helper T cells, designated T4 cells, among the circulating MNC but they also react with many parenchymal non-circulating cells such as brain cells, liver cells and kidney cells. These cells have nothing to do with the immune system but they carry a surface constituent common with the T_M helper cells which is antigenic when injected into the mouse to generate the CD4 monoclonal antibodies.

It is necessary, however, to be cautious in rejecting outright a functional role for FcM receptors. Since rosetting is certainly not the most sensitive assay for the detection of FcM receptors (it is not known how many FcM receptors are required to enable a T_M cells to rosette with EAM although speculation is that they must number in

the thousands), it is very possible that FcM receptors may be present on the cultured T_M cells in sufficient numbers to participate in Ig synthesis by the co-cultured B cells but in insufficient numbers to allow rosetting to take place. In order to resolve this dilemma, it will be necessary to purify the FcM receptors, obtain antibodies to the FcM receptors, label the antibodies with a radioactive isotope, incubate these labelled antibodies with the T_M cells and determine the number of anti-FcM receptor antibodies bound per T_M cell. The number of FcM receptors per cell should be similar to the number of antibody molecules bound per cell.

A point which requires elaboration is the extent to which results in in vitro culture using circulating cells can be extrapolated to the in vitro situation in the lymph nodes and spleen where Ig synthesis normally takes place. It is possible that all of the accessory cells required to facilitate Ig synthesis by the B cells in vitro - the T_M helper cells, the Null cells and the monocytes, are not obligatory participants in the synthesis of Ig in vivo. However, very few investigations have been conducted with parenchymal lymphoid cells, either in vivo or in vitro, to permit any conclusions.

The chemical composition of RSSF is not known as only preliminary experiments have been carried out. It was demonstrated that the molecular weight of RSSF is between 14,000 and 50,000 since it was retained following dialysis

through a dialysis membrane with a molecular weight cut-off of 14,000 but was dialyzed following dialysis through a dialysis membrane with a molecular weight cut-off of 50,000. Furthermore, RSSF is quite stable at 37°C but loses its activity following exposure to 56°C for only 15 minutes, suggesting that RSSF is a protein. However, not one of a battery of 8 proteolytic enzymes was able to degrade RSSF over a 24 hour incubation period. The amount of each enzyme used per ml was 5 to 10 times than that which is normally required to degrade the substrate. On the basis of these preliminary data, it may be concluded that RSSF is either a protein resistant to conventional enzyme action or that it is not a protein.

In summary, it has been demonstrated that both Null lymphocytes and IgM can inhibit the synthesis of FcM receptors by freshly isolated T cells following culture at 37°C in culture medium for 24 hours. The Null cells secreted a factor, referred to as receptor synthesis suppressor factor or RSSF, which inhibited the synthesis of the FcM receptors when it was added to T cells in culture. It was also demonstrated that the FcM receptors do not appear to have a functional role in immunoglobulin synthesis by the B cells in culture since FcM receptors could not be detected during the 7 day culture of T_M cells, non-T cells and PWM.

7. CONCLUSIONS

It was demonstrated in this investigation that the FcM receptors detected on T cells cultured for 24 hours at 37°C were newly synthesized in vitro. The addition of cyclohexamide, an inhibitor of protein synthesis, to the cultured T cells in a low non-toxic concentration inhibited the synthesis of the FcM receptors.

The failure of the T cells cultured in the presence of the other circulating mononuclear cells to exhibit FcM receptors has been shown to be due to the suppressive activity of the Null cells. B cells and monocytes did not suppress FcM receptor synthesis by the T cells.

The Null cells secreted a factor in culture capable of suppressing FcM receptor synthesis by cultured T cells. This factor is referred to as receptor synthesis suppressor factor or RSSF. It is synthesized and secreted by cultured Null cells and its synthesis can be inhibited by the addition of cyclohexamide, in low non-toxic concentration, to the cultured Null cells. The concentration of cyclohexamide required to inhibit the synthesis and secretion of RSSF by the Null cells is much lower than that required to inhibit the synthesis of FcM receptors by cultured T cells. RSSF does not react with, or neutralize, preformed FcM receptors.

RSSF has a molecular weight between 14,000 and 50,000. RSSF loses its activity following incubation at 56°C for more than 15 minutes. However, it resists enzymatic

degradation following overnight culture with a battery of proteolytic enzymes. RSSF is therefore not a conventional protein or else it is a protein which is resistant to enzymatic degradation in its native state.

It was demonstrated that circulating IgM inhibited FcM receptor synthesis by cultured T cells. As was the case with RSSF, IgM did not react with, or neutralize, preformed FcM receptors.

The FcM receptors on the T_M helper cells do not appear to possess a functional role in the synthesis of immunoglobulins by cultured B cells in the presence of PWM and the obligatory accessory cells - the T_M helper cells, the Null cells and the monocytes. FcM receptors were not detected at any time during the conventional 7 day culture of T_M helper cells (T cell depleted of T_G suppressor cells), non-T cells and PWM. However, the T_M cells functioned as helper cells since immunoglobulin synthesis and secretion took place. It therefore appears that the FcM receptor is an incidental, non-functional marker on the T_M helper cells.

It is concluded that two mechanisms exist in vivo to inhibit the synthesis of FcM receptors by T cells. These are (i) the Null cells which act by secreting a suppressor factor, RSSF, and (ii) circulating IgM. The mechanism of suppression of FcM receptor synthesis by these two distinct suppressor agents can only be speculated upon at the present time.

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