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SOLUBLE MEDIATORS IN THE IMMUNE RESPONSES OF MICE. IMMUNOGLOBULIN CYTOPHILICITY ENHANCING FACTOR

by

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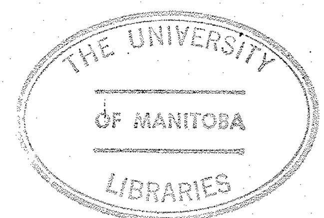

SOLUBLE MEDIATORS IN THE IMMUNE RESPONSES OF MICE. IMMUNOGLOBULIN CYTOPHILICITY ENHANCING FACTOR

BY

WILLIAM JOHN KERR

A dissertation submitted to the Faculty of Graduate Studies of the University of Manitoba in partial fulfillment of the requirements of the degree of

MASTER OF SCIENCE

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To Mom, Dad and Linnie, whose love and direction stimulated my sometimes faltering perseverance.

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Histry is somethin' you can't never finish, so
you might jist as well git it started at the beginnin'.
- Charlie Farquharson, 1972.

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ABSTRACT

A soluble mediator, immunoglobulin cytophilicity enhancing factor or ICEF, has been observed in the 3 hour culture supernates of thymocytes pretreated with the early immune serum of mice immunized with fibrinogen in Freund's complete adjuvant. This factor is capable of increasing the amount of surface immunoglobulin on normal spleen cells. Since nylon wool nonadherent, but not anti-θ plus complement-treated, spleen cells demonstrated an increase in surface Ig, the increase for normal cells was likely due to Ig uptake by T cells. Furthermore, ICEF could significantly enhance the anti-sheep erythrocyte response in the presence, but not the absence, of T cells *in vivo*. In this respect, it has been implicated in an amplification mechanism involving T-T cell cooperation. Also, its ability to reduce the number of Ig-bearing spleen cells in the absence of T cells suggests that it may also act in a regulatory capacity for the humoral antibody response.

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TABLE OF ABBREVIATIONS

Ab ≡ antibody

AFC ≡ antibody-forming cell

Ag ≡ antigen

anti-F,G ≡ anti-(γF+γG)

Asc ≡ extract of Ascaris suum

ATC ≡ activated T cell

ATS ≡ anti-thymocyte serum

ATxXB ≡ adult, thymectomized, X-irradiated, bone marrow reconstituted (mouse)

BCG ≡ bacille Calmette-Guẽrin strain of Mycobacterium tuberculosis

BGG ≡ bovine gamma globulin

BSA ≡ bovine serum albumin

BSA ≡ BSA-coated SRBC

C3 ≡ third component of complement

Con A ≡ concanavalin A

C-terminal ≡ carboxy terminal

DDW ≡ deionized distilled water

DNA ≡ deoxyribonucleic acid

DNP ≡ dinitrophenol

DNP-Asc ≡ dinitrophenylated Asc

DNP-GPA ≡ dinitrophenylated guinea pig albumin

DSS ≡ Dulbecco's phosphate-buffered saline solution

DSS-10 FCS ≡ DSS + 10% fetal calf serum

DTH ≡ delayed-type hypersensitivity

EA ≡ hen egg albumin

FCA ≡ Freund's complete adjuvant

FCS ≡ fetal calf serum

FIB	≡ human fibrinogen
γF	≡ mouse 7S γ_1 globulin
γG	≡ mouse 7S γ_{2a} globulin
GEP	≡ guinea pig encephalitogenic protein
GP-C	≡ guinea pig complement
GRF	≡ genetically restricted factor
GVH	≡ graft versus host (reaction)
H-2	≡ murine major histocompatibility locus
HBSS	≡ Hank's balanced salt solution
HoRBC	≡ horse erythrocytes
Ia	≡ I region-associated
IACF	≡ immunoglobulin-antigen complexing factor
IBF	≡ immunoglobulin binding factor
ICEF	≡ immunoglobulin cytophilicity enhancing factor
Ig	≡ immunoglobulin
i-RNA	≡ instructional RNA
i.p.	≡ intraperitoneally
IRA	≡ immunoregulatory alpha globulin
i.v.	≡ intravenously
KLH	≡ keyhole limpet hemocyanin
MBLA	≡ mouse B lymphocyte antigen
MEM	≡ Eagle's minimal essential medium
MHC	≡ major histocompatibility complex
MIF	≡ migration inhibitory factor
NMS	≡ normal mouse serum
NNA	≡ nylon (wool) nonadherent (lymphocytes)
NSC	≡ normal spleen cells
NSS	≡ normal serum-stimulated supernate
N-terminal	≡ amino-terminal

OD_{280}	≡ optical density at wavelength 280 nm
O-F	≡ oxidized ferrodoxin
PBS	≡ phosphate-buffered saline
PFC	≡ plaque-forming cell
R	≡ receptor
RAMIg	≡ rabbit anti-mouse immunoglobulin
RBC	≡ erythrocytes
RFC	≡ rosette-forming cells
RICA	≡ reverse immune cytoadherence
RNA	≡ ribonucleic acid
RNAse	≡ RNA-digesting enzyme
SC	≡ spleen cells
SE	≡ standard error
SIRS	≡ soluble immune response suppressor
SMIg	≡ surface membrane-bound immunoglobulin
SRBC	≡ sheep erythrocytes
θ	≡ theta antigen
TL	≡ thymic leukemia antigen
UM-10-FIL	≡ suppressor factor of Lee and Paraskevas (1976a; b)
x^+	≡ possessing any marker "x"
x^-	≡ not possessing marker "x"

INTRODUCTION AND LITERATURE REVIEW

Developments in immunology in recent years have led to the discovery of a number of soluble compounds which are released from lymphoid and other cells, and appear to be involved in several aspects of immune response control. They have been observed in both cell-mediated and humoral responses and have been implicated in suppressive as well as augmentative activities (reviewed in Waksman and Namba, 1976; Friedman 1975a).

Work described in this thesis concerns itself with a thymocyte-derived soluble mediator which can enhance the amount of extraneous immunoglobulin binding to lymphocyte surfaces. In this respect, evidence supporting the presence of such a factor and its biological significance within an immune response will be presented.

However, before one begins to discuss the production and functions of such a mediator, a general overview of the cells and reactions involved in various aspects of the immune response is in order. In view of this, the following literature review will be divided into several sections concerned with these areas.

I. Cells of the Immune System

(1) B Lymphocytes

There appear to be basically four populations of "immunologically competent" (Medawar, 1963) cells. One of these is the so-called "B" lymphocyte (Roitt et al., 1969) which was originally shown, in the avian system, to differentiate from stem cells in the bursa of Fabricius (Moore and Owen, 1966).

In experiments where chickens have been neonatally bursectomized, the birds fail to develop the capacity for serum antibody production following antigenic stimulation (Glick et al., 1956). When testosterone

was used to inhibit bursal development, similar results were obtained (Mueller et al., 1960; Warner et al., 1962), indicating that the bursal cells were important in humoral antibody (Ab) production. The capacity for homograft rejection or graft-versus-host (GVH) reactivity, on the other hand, remained unaltered (Warner et al., 1962).

Furthermore, while no antibody-forming cells (AFC) were detected in the bursa itself (Dent and Good, 1965), bursal cells could transfer the capability of Ab synthesis to irradiated secondary hosts (Gilmour et al., 1970). This demonstrated that the bursa contains AFC precursors. It is now generally believed that B cells are precursors to the Ab-secreting plasma cell (Frøland and Natvig, 1973).

While the mammalian analogue of the bursa has not completely been elucidated, lymphocytes similar to bursa-derived cells have been found to develop from stem cells in mammalian fetal liver (Tyan and Herzenberg, 1968) and bone marrow (Mitchell and Miller, 1968). Using both *in vitro* and *in vivo* studies, Owen et al. (1975) have suggested that B cell maturation in mammals is a multifocal event, and is not strictly dependent upon gastrointestinal influence, as was once thought.

In summary, then, B cells, can be described as bursa-derived, or 'bursal equivalent'-derived, small lymphocytes (Roitt et al., 1969) which appear to be directly related with Ab synthesis.

(2) Thymus-dependent or "T" Lymphocytes

The second lymphocyte population this review will consider is that of the thymus-dependent "T" cell (Roitt et al, 1969). These again are morphologically defined as small lymphocytes. Functionally, however, they are quite different from the previously discussed B lymphocytes.

The thymus is considered to be derived from epithelial cells from the third and fourth pharyngeal pouches (Venzke, 1952), and becomes infiltrated with small lymphocytes, probably originating from circulatory stem cells which have become enmeshed in the thymic analogue (Moore and Owen, 1967; Owen and Ritter, 1969). These stem cells then appear to be "processed" within the thymus. They have been shown, not only to differentiate morphologically to small lymphocytes, but to also develop various surface markers not found on either stem cells or thymus-independent lymphocytes (Frøland and Natvig, 1973; Jondal et al., 1973; Raff, 1971).

A further stage of differentiation is involved in the maturation of thymic lymphocytes whereby a proportion of these cells are released into the peripheral lymphoid tissues (Davies, 1969; Miller, 1962; Weissman, 1967) and, in the process, once again exhibit a reorganization of surface antigens (Aoki et al., 1969; Greaves et al., 1975). It is this peripheral, thymus-derived small lymphocyte which has come to be termed the "T lymphocyte" or "T cell" (Roitt et al., 1969).

T cells have been demonstrated, in experiments where animals have been neonatally thymectomized (Miller, 1961; 1962), to control cell-mediated immunity and certain Ab responses. Furthermore, studies of immunological disorders, such as those found in congenitally athymic nude mice or Di George's syndrome patients (reviewed in Greaves et al., 1975), have revealed that a correlation exists between the lack of a thymus and the inability to develop cell-mediated immune responses. In most cases, these deficiencies can be overcome by reconstitution with T cells (Greaves et al., 1975). Conversely, in Bruton-type agammaglobulinemic patients, who exhibit a deficiency in immunoglobulin (Ig)-bearing cells, and in serum Ig levels, cell-mediated responses remain reasonably unimpaired

(Good et al., 1962).

This again exemplifies the humoral versus cellular dichotomy in the overall immune response. In general, it appears that T cells are largely responsible for cell-mediated responses although, as will be discussed below, they are also instrumental in controlling certain Ab responses. B cells, on the other hand, are primarily involved in antibody formation and are thereby important in the humoral immune response.

(3) Killer or "K" Cells

A third lymphoid cell population has been observed by Greenberg et al. (1975). These cells, which have been demonstrated to be capable of Ab-dependent, non-phagocytic cytotoxicity against Ab-coated target cells, have been termed "killer" or "K" cells. While they appear to be lymphoid on the basis of morphology, they fail to demonstrate the surface antigenic determinants of either B or T lymphocytes. Although functionally similar, lymphoid K cells are distinguishable from a myeloid population of K cell (Greenberg et al., 1973) since the former are not adherent to glass and, as mentioned, are morphologically different.

(4) Macrophages

A fourth population of cell involved in immune responses - one which appears to be of increasing importance as research progresses - is the macrophage. These large, wandering phagocytic reticuloendothelial cells are possibly the most primitive of those in the immune response (Metchnikoff, 1905). They are derived from bone marrow monocytes (reviewed in Van Furth, 1970) but are not necessarily end cells, since they can either multiply by fission or further differentiate to form epithelioid and "giant cells" (Weiss, 1972).

Functionally, they not only act in a phagocytic capacity (re-

viewed in Stossel, 1974 a; b), but also appear to be required, at least *in vitro* (Erb and Feldmann, 1975a, 1975b, 1975c; Treves *et al.*, 1976; Paraskevas, unpublished data), for the development of humoral Ab responses. This requirement may be due to the release of macrophage products required for culture maintenance. Possibly, too, the macrophage may play a role in antigen (Ag) presentation for immune response triggering. These aspects will be discussed in detail in following pages.

In accord with present knowledge, then, macrophages, T cells, B cells and K cells constitute the basic populations of immunocompetent cells. The means of differentiating these cells and their interactions in immune responses will now be briefly discussed.

II. Surface Markers on Immunocyte Populations

(1) B Cells

In view of the fact that B lymphocytes are directly involved in the humoral immune response, it is not surprising that they exhibit significant amounts of membrane-bound Ig (SMIg) which can be detected by a variety of methods (reviewed in Auti *et al.*, 1974 and Warner, 1974). These methods include:

1) cell visualization techniques using either labelled anti-Ig Ab's or an intermediary Ig layer in a "sandwich" type of reaction,
2) activation of biological processes such as lymphocyte transformation or specific complement-dependent cytolysis.
3) physical methods such as electrophoresis or removal by anti-Ig-coated matrices, and
4) quantitative methods such as hemagglutination inhibition, radioimmunoassay or cell surface iodination followed by specific precipitation of the labelled Ig.

One technique which has been used in work presented in this thesis is the "reverse immune cytoadherence" (RICA) rosetting method developed by Paraskevas _et al_. (Paraskevas, Lee, Orr and Israels, 1971; Paraskevas, Lee and Israels, 1971). In this method, an $F(ab')_2$ hybrid Ab with specificity for mouse gamma globulin at one combining site and a protein coated onto sheep erythrocytes (SRBC) of the other is used to form an intermediate bridge through which a rosette can be formed around the Ig^+ cell by the coated SRBC.

It is now generally accepted that B cells display a higher density of SMIg than T cells. Even antigen-activated T cells, which exhibit 10^2 to 10^3 molecules of SMIg per cell, are much more sparsely covered than B cells, which bind an average of 10^5 molecules per cell (Warner, 1974).

A surface Ag on murine B cells which has been used for differentiation is the mouse B lymphocyte antigen (MBLA)(reviewed in Greaves _et al_., 1975; Owen _et al_., 1975). This Ag has been shown to serve as a marker both for resting B lymphocytes and for their Ab-secreting progeny (Greaves _et al_., 1975). It has been a valuable probe in experiments where attempts have been made to establish the ontogeny of the B lymphocyte (Owen _et al_., 1975).

Two other surface components found on B cells are receptors for the Fc portion of Ig (FcR's) and for the third component of complement (C3R's) (reviewed in Aiuti _et al_., 1974 and Shevach _et al_., 1973). These markers aid in distinguishing between B and T lymphocytes although, as will be mentioned, it is possible that a subpopulation of peripheral T cells may also display FcR's. T cells, however, are not $C3R^+$. (Greaves _et al_., 1975).

(2) T Cells

It has been previously suggested that, upon differentiation from stem cell to lymphocyte and finally to mature T cell, surface markers of

thymocytes are acquired and, subsequently, altered. Perhaps the most commonly utilized of these markers is the murine theta (θ) or Thy 1 antigen (Vitetta et al., 1974), first described by Reif and Allen (1963). Raff (1969) has shown that this antigen is present on the surface of peripheral T cells as well as thymocytes. In fact, maturation of T cells involves differentiation from non θ-bearing stem cells to densely θ-positive (θ^+) thymic lymphocytes which undergo a slight loss of the antigen to yield a moderately θ^+ T cell (Aoki et al., 1969).

Another series of antigenic markers which has been defined on mouse thymus and thymus-derived cells is the Ly series (Boyse et al., 1968; Cantor and Boyse, 1975(a)(b); Cantor et al., 1976; Feldmann et al., 1977). It is this group of antigens which, in recent years, has been used to differentiate between functional subclasses of T cells. Lymphocytes bearing the Ly 1 Ag (Ly 1^+ cells) have been shown to act either as "helper T cells" (Cantor et al., 1976; Feldmann et al., 1977) in enhancing the Ab response against thymus-dependent Ag's such as SRBC, or as precursors of these cells. Cells that do not exhibit Ly 1, but bear the Ly 2 and Ly 3 markers (Ly 2^+3^+ cells) are considered to act as killer T cells in graft rejection (Cantor and Boyse, 1975b) and as "suppressor T cells" in the regulation of an anti-SRBC Ab response (Cantor et al., 1976; Feldmann et al., 1977). Whether or not these latter two functions are characteristic of the same or different T cell subpopulations has not as yet been conclusively established (Cantor et al., 1976). At any rate, the Ly antigen series is proving to be a valuable tool in the study of T cell subpopulations.

Another T cell marker is the thymic leukemia or TL antigen, of which there are four specificities (reviewed in Greaves et al., 1975). This Ag is present only on thymocytes and, upon differentiation of these cells to peripheral T cell, becomes undetectable. Although this antigen

was used to ascertain T cell origins and to demonstrate a reorganization of surface Ag's during thymocyte maturation (Owen and Raff, 1970), it remains relatively uninvolved with peripheral T cell studies. Consequently, for the purposes of this review, it will not be discussed further.

However, the problem of the presence or absence of Ig on T cell surfaces lends itself to somewhat greater consideration. In most studies (reviewed in Warner, 1974), Ig has not been detected on thymus cells. Studies using peripheral T cells, on the other hand, yield conflicting results. Hammerling and Rajewsky (1971) noted that all peripheral T cells carry surface IgM but in far smaller quantities than that carried by peripheral B cells. On the other hand, others (Nossal et al., 1972; Grey et al., 1972) have found no significant increase in surface Ig on peripheral T cells when compared to thymocytes.

T cells which have been stimulated or activated by specific Ag may exhibit SMIg (Goldscheider and Cogan, 1973). In fact, work by Paraskevas et al. (1972,1976) and Lee and Paraskevas (1972) indicates that Ig may be detected on T cell surfaces within six hours after antigenic stimulation. While some authors have reported this newly demonstrable Ig to be synthesized by the T cell (Goldschneider and Cogan, 1973), this has not been directly proven. It may well be that the SMIg is cytophilically acquired from extraneous sources and is not produced by the T cell itself (Hudson et al., 1974; Hunt and Williams, 1974).

In conjunction with this ability of T cells to acquire cytophilic Ig, it has been demonstrated recently that a subpopulation of these lymphocytes exhibit FcR's (Basten et al., 1975; Paraskevas and Lee, 1976; Stauf and Herzenberg, 1975). These studies have demonstrated that approximately 24 per cent of splenic T cells display FcR's. It appears then that where T lymphocytes were generally considered to be FcR^- (reviewed in

Greaves et al., 1975; Shevach et al., 1973), they now can be shown to possess a subpopulation which is FcR^+.

Lastly, it should be briefly mentioned that primate T cells exhibit an addition differentiative surface marker in the form of a receptor for certain heterologous erythrocytes. These cells are thereby capable of rosetting the RBC to form "E-rosettes" (reviewed in Greaves et al., 1975, and Aiuti et al., 1975) which can subsequently be distinguished visually and/or separated physically from non-rosetting cells.

(3) Macrophages

As with B cells, macrophages also exhibit FcR's and C3R's (Stassel, 1974(a). The FcR's appear to react, at least for purposes of opsonization, with IgG. However, receptors for the Fc of IgM have been reported on monocytes (Shevach et al., 1973). C3a, which acts as an important chemotactic factor in the phagocytic response, also reacts with the macrophage plasma membrane (Stossel, 1974 a) via membrane-bound receptors (Shevach et al., 1973).

A less immunologically specific property of macrophages also distinguishes them from B and T cells. Macrophages can be differentiated from lymphocytes by their innate ability to adhere to glass surfaces (Mosier, 1967). This characteristic allows their relatively easy isolation from mixed cell populations.

(4) Membrane Redistribution: The Capping Phenomenon

It has been demonstrated that the addition of anti-Ig to lymphoid cells exhibiting high concentrations of SMIg, in the absence of metabolic inhibitors, can trigger polarization of the surface Ig molecules which are subsequently pinocytosed (Taylor et al., 1971). This phenomenon is not confined to Ig^+ lymphocytes. Ashman and Raff (1973) have shown that receptors on T cells for SRBC can indeed polarize or "cap," and other anti-

bodies, antigens or mitogens have been shown to cap their receptors (reviewed in Greaves _et al._, 1975). This phenomenon, or similar effects, may provide a trigger mechanism for the activation of lymphocytes (Greaves _et al._, 1975), and may explain some preliminary results from work presented in this thesis.

Before turning to these experiments, however, a general discussion of Ag recognition procedures and the cellular interactions involved therein is presented below.

III. Antigen Recognition and Cellular Interactions in the Immune Response

(1) T-dependent and T-independent Antigens

It has been demonstrated (reviewed in Greaves _et al._, 1975) that while both T and B cell stimulation is required in the responses against many antigens, other immunogens can trigger a T-independent response. A response is said to be thymus-independent if "the response of neonatally thymectomized, lethally irradiated and bone marrow or foetal liver-reconstituted animals, or of congenitally hypothymic nude mice, is not significantly different from appropriate control animals over a wide dose range of Ag, or if anti-θ serum plus complement treatment of cells does not interfere with their ability to respond" (Greaves _et al._, 1975).

In thymus-dependent responses, IgG is the predominant class of Ab. However, in T-independent responses, the response matures little beyond IgM production (Greaves _et al._, 1975).

Examples of T-independent antigens are type III pneumococcal polysaccharide (SIII) (Howard _et al._, 1972), polymerized flagellin (Feldmann and Basten, 1971), _E. coli_ lipopolysaccharide (LPS) and polyvinylpyrrolidone (PVP) (Andersson and Blomgren, 1971). A common characteristic

of these compounds is the existence of repeating identical epitopes or antigenic determinants within the molecule. Consequently, it appears that this polymeric nature may circumvent a need for presentation or focussing of the antigen to the B cell by some other lymphocyte, possibly the T cell (Greaves et al., 1975). However, as Shiozawa et al. (1977) have pointed out, no conclusive evidence correlating the molecular structure of the Ag with the degree of T cell help required has been documented. Furthermore, these antigens are mitogenic in nature, which may also be important in their T independency (Bretscher, 1975; Shiozawa et al., 1977).

(2) Antigen Receptors on Lymphoid Cells

It appears that the receptor for Ag on B cells is SMIg (reviewed in Greaves et al., 1975, and Warner, 1974). In unstimulated cells, the predominant class of SMIg is IgM, while certain IgM^+ cells also demonstrate IgG and IgA. Following antigenic stimulation, the density of SMIg decreases and a shift to predominantly IgG occurs (Warner, 1974). However, Vitetta and Uhr (1975) have proposed that IgD may, in fact, be the predominant cell surface receptor. Regardless of the particular Ig class involved, the fact that the B cell SMIg acts as the AgR is well defined.

The situation, however, appears to be somewhat more complex in thymus-derived cells. Since the majority of normal T cells are FcR^- and Ig^-, an alternate method of Ag recognition is required.

Supporting evidence for a macrophage requirement in Ag recognition by T cells is quite strong. Erb and Feldmann (1975 a;b;c) have shown that in vitro responses to both soluble and particulate antigens require the presence of syngeneic macrophages. In these experiments, cell to cell contact was not required, indicating that stimulation of the T helper function was triggered by, or at least required, a soluble macrophage product.

Shevach et al. (Rosenthal and Shevach, 1973; Shevach and Rosenthal, 1973; Shevach, 1973; Thomas and Shevach, 1977) have, to some degree,

substantiated this evidence in guinea pigs. They submit that the actual moiety which is recognized by the T cell is an antigen-modified product of the I region of the macrophages major histocompatibility complex (MHC). In other words, the I region-associated, or "Ia" product may be involved in direct presentation of the immunogen to the T cell.

Cohn and Paul (1974) have shown that, in a response to dinitrophenylated guinea pig albumin (DNP-GPA), the macrophages controlled the degree of responsiveness to various concentrations of Ag. Furthermore, previously immune macrophages could induce an increased response in immunologically virgin T cells upon challenge with DNP-GPA. Their experiments indicate that cytophilic Ab adherent to the activated macrophage could bind more Ag to the macrophage surface, thereby creating a more efficient T cell stimulator.

Although these studies indicate that Ag is presented to the T cell by the macrophage, results showing that T cells can bind Ag directly cannot be ruled out. Ag suicide techniques, which involve inactivation of Ag-binding cells upon binding to a radiolabelled Ag of a high specific activity have demonstrated that T cells can, in fact, bind Ag (Basten *et al.*, 1971) and, in so doing, direct a specific immune response. Furthermore, the ability of T cells to bind SRBC has been previously discussed, which indicates that direct physical contact between T cell and Ag likely occurs in this case. In addition, T cells have been shown to form rosettes with SRBC coated with T-dependent antigens such as bovine gamma globulin (BGG), bovine serum albumin (BSA) and keyhole lympet hemocyanin (KLH) (reviewed in Greaves *et al.*, 1975).

The nature of the receptor for these antigens is thought to be Ig, since this would most easily correlate with the B cell model (Greaves

et al., 1975). However, Greaves (1975) has described recent evidence indicating that the AgR on T cells may actually be a receptor for Ia products. This receptor may possess some qualities resembling the idiotypic determinant and Ag-binding site of Ig.

To summarize so far, the author wishes to compromise to some extent and suggests that, from available data, Ag recognition by T cells may, in some cases, occur through direct interaction with the Ag and a complementary receptor, which may be Ig. However, another much more complex mechanism, with possible involvement of the macrophage in presentation of the Ag to the T cell, is likely important in many reactions. The receptor in this case could possibly be directed toward an Ag-modified macrophage product.

(3) Cellular Interactions

In several instances, this review has alluded to the high degree of cooperation between T and B lymphocytes. It is now appropriate to briefly review the areas in which this arises.

As mentioned, there are certain antigens which require T cell mediation to stimulate a mature IgG-type of response. Experiments have demonstrated that, with hapten-carrier conjugates where there is little repetition of identical epitopes on the same molecule, the T cell is responsible for recognition of the carrier portion. The Ab response, on the other hand, which is mediated by the B cells, is directed against the haptenic determinant (Katz et al., 1970; Mitchison et al., 1970). Furthermore, it appears that, in order for a secondary anti-hapten response to occur in cells primed with the specific carrier, the hapten and carrier for secondary challenge must be physically linked (Mitchison et al., 1970). This indicates that there may be some form of physical presentation, or focusing, of the hapten to the B cell by the T cell (reviewed in Greaves

et al., 1975). Exactly how this occurs has yet to be determined.

Non-antigen-specific cooperation between T and B cells has been established in the so-called "allogeneic effect" (Katz et al., 1971), whereby an ongoing GVH response can eliminate the carrier specificity of a secondary anti-hapten response. Consequently, even when a heterologous and/or non-immunogenic carrier is used in the boosting hapten-carrier conjugate, a substantial secondary anti-hapten response can be obtained. This effect has been demonstrated in guinea pigs (Katz et al., 1971), mice (Kreth and Williamson, 1971), rats (McCullagh, 1970) and murine tissue culture (reviewed in Greaves et al., 1975).

Not only can T cells exhibit a helper function by enhancing immune responses, but recent studies in many systems have shown that they can exert a suppressive or regulatory function. Reviews of the vast amount of literature regarding this phenomenon have been excellently compiled by Möller (1975). However, in the scope of this thesis, it is the author's intention to simply draw attention to the fact that T cells, or a subpopulation thereof (Cantor et al., 1976; Feldmann et al., 1977), may act in a regulatory manner, possibly invoking a state of homeostatic balance in the immune system.

Two somewhat contradictory theories, known as the "one signal" (Coutinho and Möller, 1974) and "two signal" (Bretscher, 1975; Bretscher and Cohn, 1968) hypotheses have been proposed in attempts to explain the control of tolerance and immunity. The one signal theory postulates that the signal for immune B cell triggering develops from T helper cells, for T-dependent antigens, or from the mitogenic properties of T-independent antigens. Tolerance arises simply from the presence of a suboptimal concentration of Ag.

The two signal theory, on the other hand, proposes that a first signal is produced upon binding of the haptenic portion of an Ag to an

Ig-type surface receptor on the B cell. This signal alone is tolerogenic. However, a second signal, presumably arising from the interaction of a T cell-derived helper factor with its appropriate B cell surface receptor and the already bound Ag, can stimulate the cell toward immunity. Consequently, the ratio between the number of signals one to two is thought to discriminate between immunity and tolerance. As with the one signal hypothesis, the immunogenicity of T-independent antigens is believed to be due largely to their mitogenic properties.

In view of the functional diversification of T lymphocytes, it is evident that, as well as interactions between T and B cells, T-T cooperation also occurs. For example, Cantor and Asofsky (1972) have shown that in the GVH response, nonrecirculating thymus-derived cells directed the specificity of the response, thereby acting as precursors to the effector cells. On the other hand, the recirculating T cells acted as amplifiers of the response.

Pilarski et al. (1977) have recently reported that a helper subpopulation of T cells is required in the concanavalin A (Con A) stimulation of cytotoxic effector T cells. Furthermore, in delayed-type hypersensitivity (DTH) reactions, Bullock et al. (1975) propose that development of effector T cell function may involve participation of at least two distinct precursor cells.

Feldmann et al. (1975), in studying the response against oxidized ferrodoxin (OFd), a polypeptide which possesses both carboxy (C)-terminal and amino (N)-terminal determinant groups, observed that the best Ab responses were obtained only when T cells primed with the C-terminal group were mixed with T cells primed with the N-terminal determinant. Cells primed with either determinant, when cultured in the absence of

cells primed to the other, would not exert a helper function.

Paraskevas and Lee (1976a) have observed that a subpopulation of T cells which can be shown to carry SMIg within six hours after immunization can provide a significant helper function in Ab responses to T-dependent Ag's. It was also demonstrated that this effect, in addition to lymphocyte-macrophage collaborations constitutes a complex immunoregulatory network. The observations by many investigators in recent years that soluble products from these cells can, in fact, facilitate this intercellular communication has opened up a new discipline of "Factorology" within studies of the immune response. An overview of these mediators will be presented in the following chapter.

IV. Soluble Mediators in the Immune Reponse

Soluble mediators have been observed in a number of systems. They appear to consist of a wide variety of chemical compounds which range from nucleic acids, to enzymes, to other proteins and glycoproteins of uncertain composition. In fact, it is very likely that some of these factors may be identical, since certain activities are not found in the absence of others.

A number of these factors have been heroically reviewed by other authors (Friedman, 1975a; Waksman and Namba, 1976), although even these compilations can by no means be considered complete. In view of the large number of soluble mediators described in the literature, then, this endeavor will, for the most part, limit its discussion to some of those involved in the humoral immune response.

(1) Immunosuppressive Factors

One of the earliest documented soluble mediators was an alpha-2-glycoprotein isolated from rat, rabbit, human and bovine serum by Mowbray

et al. (1963). This material was originally shown to suppress homograft rejection although it was later found to also suppress the hemagglutination titre of a murine anti-SRBC response if administered to the animal 10 to 20 hours before the Ag. The suppressive activity was found to be inseparable from RNAse activity in the serum fraction (Mowbray, 1967). A similar compound was also found in increased amounts in transplant recipients (Phillips *et al*., 1975). Furthermore, an immunoregulatory alpha globulin (IRA) which interferes with Ag recognition has been isolated from human plasma by other authors (Cooperband *et al*., 1972), indicating that this may be an important naturally occurring suppressor factor.

Feldmann (Feldmann, 1972; Feldmann and Basten, 1972) has proposed the existence of a murine suppressor factor which is produced *in vitro* by Ly $2^{+}3^{+}$ suppressor T cells. Upon incubation with Ag for 16 to 24 hours, a mediator is produced which is Ag specific, carries Ia specificity and decreases the 7S and 19S Ab responses both *in vivo* and *in vitro*.

Soluble suppressor factors have also been demonstrated to regulate IgE production. Tada *et al*. (1973; 1975) have shown that extracts from T cells primed with an extract of *Ascaris suum* (Asc) or a dinitrophenylated derivative of Asc (DNP-Asc) in Freund's complete adjuvant (FCA) markedly decrease the IgE response *in vivo* for up to 40 days after inoculation of the extract. Furthermore, this activity was due to a protein with a molecular weight of 35,000 to 65,000 Daltons, which migrated with a beta to alpha mobility upon electrophoresis (Okumura and Tada, 1974; Tada *et al*., 1975).

It was also shown that, in the mouse, a factor obtained by the same system, but by priming with a carrier protein different than Asc, could suppress the IgG response to DNP conjugated to either KLH or BGG

by specificity toward the carrier protein (Tada et al., 1975). This suppression was genetically controlled since the factor was not effective across an H-2 barrier. In addition, from in vitro experiments, it was observed that egg albumin (EA)-specific suppressor factor was only effective if T helper cells for EA were present. Similarly, no suppressive effect for KLH-specific suppressor factor was found in the absence of KLH-specific helper T cells. From these results, the authors implied that the specific suppressor factor likely does not act directly on the B cell (Tada et al., 1975).

In addition to Tada's IgE suppressor factor, Carter (1976) has demonstrated a suppressing mediator obtained in the sera of B6D2 F_1 hybrid mice 8 hours after the injection of 10 μg EA in FCA. If this serum is injected into syngeneic recipients one hour before administration of 1 μg EA in $Al(OH)_3$, an immunization that usually elicits a strong IgE anti-EA response, this response can be drastically reduced. The factor was shown to be carrier specific, have a short half life, and likely act on the early stages of the response, since no effect was detected if it was administered after Ag. Also, SRBC-specific suppressor serum, produced under similar conditions, has been demonstrated to inhibit to some degree the 7S and 19S anti-SRBC responses.

Still another mediator has been studied by Pierce and his colleagues (Jandinski et al., 1976; Peavy and Pierce, 1976; Pierce et al., 1975; Tadakuma and Pierce, 1976). Soluble immune response suppressor (SIRS) is produced in the supernates of murine spleen cells treated with ConA in vitro and incubated for 48 hours. SIRS has been demonstrated to decrease the number of plaque-forming cells (PFC) against heterologous RBC in vitro. The action appears to be mediated via the macrophage, but

the authors suggest that this is likely not the only mode of action, since the suppression seems to be greater than if macrophages alone were suppressed (Tadakuma and Pierce, 1976). In view of this, SIRS has been demonstrated to decrease DNA synthesis of spleen cells 24 to 48 hours before a decrease in PFC was noticeable (Pierce et al., 1975; Tadakuma and Pierce, 1976).

This factor apparently is produced by T cells of the Ly $2^{+}3^{+}$ phenotype (Jandinski et al., 1976) and, at least to some degree, is strain-dependent (Peavy and Pierce, 1976). It has also been reported that SIRS activity cannot be demonstrated in the absence of migration inhibition factor (MIF), a T cell product important in the mediation of cellular immunity (Waksman and Namba, 1976). This gives rise to speculation that the two may be the same.

Since 1974, an hypothesis has been presented in a series of papers by Fridman et al. whereby an immunoglobulin binding factor (IBF), presumably consisting of released Fc receptor, may be involved in suppression of the immune response (Fridman and Goldstein, 1974; Fridman et al., 1974; Gisler and Fridman, 1975; Gisler and Fridman, 1976; Guimenzanes et al., 1976; Neuport-Sautes et al., 1975). The factor was first found to be produced in culture supernates from rat lymph node cells sensitized to guinea pig encephalitogenic protein (GEP) in vivo and, subsequently, rechallenged in vitro for 24 hours with GEP (Fridman et al., 1974). It has since been observed in the supernates of allogeneically stimulated, cortisone-resistant normal thymocytes and activated T cells (ATC) (Fridman and Goldstein, 1974). IBF is an actively synthesized protein, as shown by its inability to be produced by cycloheximide and puromycin treated cells (Fridman and Goldstein, 1974; Neuport-Sautes et al., 1975). It has a

molecular weight of approximately 150,000 Daltons and has been demonstrated to bind complexed IgG but not IgM or IgG monomers (Fridman et al., 1974).

Specificity for the Fc portion of IgG was established by inhibition of complement-dependent hemolysis (Fridman and Goldstein, 1974). In addition, IBF expressed the ability to suppress, in vitro, the 19S and 7S Ab responses to SRBC, a thymus-dependent Ag, and DNP-aminoethyl dextran, a thymus independent Ag. This suppression was maximal if added 72 hours after culture initiation, thereby implicating the terminal differentiation of antibody-forming cell precursors as the process most affected (Gisler and Fridman, 1975; 1976). IBF activity in these experiments was also found not to be antigen specific.

Using direct immunofluorescence and rosetting techniques, this group of investigators has shown that during incubation of murine ATC, the number of cells exhibiting FcR's decreases, while the amount of IBF released into the medium increases proportionately (Neuport-Sautes et al., 1975). Both of these phenomena are blocked by inhibition of protein synthesis. Furthermore, both IBF-producing and FcR^{+} cells can be removed by beads coated with IgG-anti-IgG complexes, implying that there may be a correlation between Fc receptors released from the cell surface and IBF release (Neuport-Sautes et al., 1975).

Other soluble humoral response suppressor factors that have been documented include an allogeneic suppressive factor (Yonkosky et al., 1976) which, after production by allogeneically stimulated spleen cells, such as in a GVH reaction, can be shown to decrease the response against heterologous RBC. In addition, suppressor factors have also been shown to be produced by B cells (Duwe and Singhal, 1976; Gorczynski, 1974).

Lee and Paraskevas have observed a suppressor factor which is found in optimal concentrations in the serum of mice 7 days after immuni-

zation with heterologous RBC or with soluble or semi-particulate antigens in FCA (Lee and Paraskevas, 1975; 1976 a ; 1976 b). Since the biological activity is retained in the filtrate after passage of the serum through a UM-10 Diaflow ultrafiltration membrane (Amicon Corp., Lexington, Mass.) and is semipurified in this manner, it is referred to as "UM-10-FIL." Using both fluorescent Ab studies and the RICA assay, it was discovered that UM-10-FIL could significantly decrease the number of Ig^{+} cells when incubated with normal murine spleen cells for 45 minutes at 37°C (Lee and Paraskevas, 1976b). Furthermore, it was established that when UM-10-FIL was injected intraperitoneally (i.p.) into normal mice and heterologous RBC were administered 45 minutes later, the 7S and 19S Ig responses were markedly suppressed (Lee and Paraskevas, 1976a). This suppression was demonstrated to be antigen specific in that serum from horse RBC (HoRBC)-inoculated animals did not suppress the response against SRBC.

(2) Immunostimulatory Factors

As is expected from the previous discussion, while some soluble mediators suppress humoral Ab responses, others are capable of enhancement. For example, Schimpl and Wecker have demonstrated a factor, present in the supernates of 24-hour cultures of allogeneically stimulated spleen cells, which can replace thymocyte function in both the 19S and 7S responses against heterologous RBC *in vitro* (Schimpl and Wecker, 1972; 1973; Wecker *et al.*, 1975). This factor is neither strain nor Ag specific and has a molecular weight of 45,000 to 60,000 Daltons. They have also demonstrated that a factor in 24 hour supernates from homologous spleen cell cultures can replenish a partially deprived T cell pool, but is not capable but is not capable of totally replacing thymic function (Schimpl and Wecker, 1972).

Watson (1973) has shown that three thymus replacing factors can be obtained in supernates from spleen cells which are stimulated with either SRBC or allogeneic cells. When these supernates were fractionated by gel filtration, one component with a molecular weight of approximately 150,000 Daltons was found to be heat, periodate and trypsin sensitive. This mediator, a T cell product, was not Ig and was shown to be slightly mitogenic for B cells. The other two factors were of smaller molecular weights and T cells were not required for their production.

Friedman (1975b), also using allogeneically stimulated spleen cell supernates, has demonstrated the ability of soluble factors therein to increase the anti-SRBC response in vitro. Again, it was found that this was due to enhanced stimulation of B cells.

Taussig et al. (1975) have observed mediators produced by T cells in response to synthetic polypeptide antigens such as poly (Tyr,Glu)-poly DL Ala--poly Lys [(T,G)-A--L] and poly (Phe, Glu)-poly DL Ala--poly Lys [(Phe,G)-A-L]. These mediators, which appear to be Ia products, can increase the PFC response against these antigens and can cooperate across allogeneic barriers. Using the allogeneic system, the authors demonstrated that the ability of a strain to respond to these Ag's lies not in the ability of the T cells to produce the factor but in the capacity of the B cells to respond to the mediator. It should be pointed out, however, that the validity of these results has come under recent question (Munro and Taussig, 1977).

Shiozawa et al. (1977) have described a carrier specific helper factor which is extracted from educated T cells. They propose that the helper activity arises when the factor is focussed on the B cell by the carrier portion of the bound Ag. This stimulates an Ab response as pro-

posed in the Bretscher-Cohn two signal hypothesis. This mediator has been demonstrated to be allogeneically restricted in that it cannot operate across an H-2 barrier.

It has also been shown that thymocytes can induce a factor which can restore Ig production to anti-thymocyte serum (ATS)-treated spleen cells _in vitro_ (Shinohara _et al._, 1976). Production of this factor was not inhibited by inhibitors of protein synthesis or DNA replication.

Still another enhancing mediator was discussed by Feldmann (1972) and Feldmann and Basten (1972). This compound, presumably a 7S IgM moiety, has been said to be released from the T cell surface membrane _in vitro_ and combines with Ag to invoke Ag-specific enhancement of PFC response. This enhancement apparently is the resultant of sensitization of macrophages which, when added to suspensions of B cells and Ag, can then direct a T cell-dependent response. It has been proposed that this effect may be similar to the (T,G)-A--L and (Phe,G)-A--L helper effect of Taussig _et al._ (1975).

Erb and Feldmann have also proposed that a genetically restricted factor (GRF), produced by macrophages, can also stimulate Ly $1^{+}2^{+}3^{+}$ T cells to respond as helper T cells (Erb and Feldmann, 1975a; b; c). This factor again appears to be an I-region gene product.

Other factors from macrophages have been shown to be mitogenic for B cells in the absence of Ag (Moller and Coutinho, 1975) and can replace macrophages in the induction of a primary anti-SRBC response. For example, β-mercaptoethanol has been demonstrated to fulfill, to some degree, such a function (Erb and Feldmann, 1975c).

In addition, RNA released from macrophages and peripheral lymphocytes can direct Ab synthesis toward the specificity of the RNA-producing

cell. This RNA consequently has been termed "informational RNA" or "i-RNA" (reviewed in Heller, 1974). Furthermore, RNA can combine with Ag to form a complex which, while not informational, can react with Ag-specific recipient cells to stimulate Ab production. RNA has also been observed to produce a non-specific adjuvant effect in immune responses.

Work by Paraskevas *et al*. demonstrated that a mediator was discovered in mouse serum 6 hours after i.p. injection of either Ag in FCA or FCA alone which could increase the number of SMIg-bearing spleen cells upon addition of Ag, as shown by RICA (Orr, 1974; Orr and Paraskevas, 1974; Paraskevas, Lee and Orr, 1976). In efforts to duplicate this activity *in vitro*, it was observed that thymocytes prestimulated with BCG, the *Mycobacterium* component of FCA which is required to facilitate this response, could produce, in their 3 hour culture supernates, a similar factor. This mediator, when added to normal spleen cell suspensions in the presence of Ag and 7S Ig, could increase the number of $SMIg^+$ cells therein, supposedly by facilitating Ig uptake by a subpopulation of T cells (Orr, 1974; Paraskevas, Lee and Orr, 1976). Since this activity was eluted in the 4S peak after gel filtration, while the activity fr[illegible] hour immune serum was found in the 7S fraction and required only the addition of Ag, it was assumed that the two mediators were likely id[illegible]cal but that the serum factor was already bound to 7S Ig.

The 4S activity was attributed to an "immunoglobulin-ant[illegible] complexing factor" (IACF) and was found to be produced by cortison[illegible] resistant, nylon wool nonadherent, long-lived T cells in spleen, [illegible] lymph node cell populations (Orr, 1974; Paraskevas, Lee and Orr, 1[illegible] Paraskevas, unpublished data). Furthermore, glycolysis and DNA-de[illegible] RNA synthesis were shown not to be required for IACF production.

active protein synthesis was necessary, since cycloheximide-treated thymocytes did not produce the factor (Orr, 1974).

IACF is trypsin-sensitive, RNAse-resistant and heat-resistant at 56°C for 30 minutes. It is believed to consist of subunits of less than 10,000 molecular weight which can be dissociated at pH 2.5 and, upon neutralization, still maintain their original activity (Orr, 1974).

When incubated with Ag and Ig, IACF does not only increase the number of $SMIg^+$ spleen cells; it has also been shown to increase the 7S Ab response against heterologous RBC when injected with Ag into nude or ATxXB Balb/c mice. The 19S response, however, does not appear to be affected to any degree (Orr, 1974; Paraskevas, Lee and Orr, 1976).

At the time of these experiments, it was also observed that thymocytes, prestimulated with 6 hour immune serum and subsequently cultured at 37°C for 3 hours, produced supernates which could increase the number of RFC by RICA when incubated with normal spleen cells and NMS in the absence of Ag (Paraskevas, Lee and Orr, unpublished data). This activity was attributed to a third mediator, immunoglobulin cytophilicity-enhancing factor, or ICEF. It is the purpose of this thesis to further establish the existence of such a factor and to examine its possible biological significance.

MATERIALS AND METHODS

I. Animals

Unless otherwise stated, all animals used in these experiments were 6 to 8-week-old Balb/c male mice (Jackson Laboratories, Bar Harbor, Maine; Canadian Breeders, St. Constant, P.Q.).

II. Preparation of 6 Hour Immune Serum and Normal Serum

Mice were injected intraperitoneally (i.p.) with either 250 μg or 500 μg human fibrinogen (FIB, Canadian Red Cross, Winnipeg, Man.) in 0.2 ml of a 0.15M saline/FCA emulsion. Six hours later, they were anesthetized with chloroform and bled by cardiac puncture. The blood was clotted at room temperature for 30 minutes and the serum recovered. Since 250 μg FIB gave responses similar to those from 500 μg, the lesser amount was used in all but the initial experiments.

Normal Balb/c serum (NMS) was obtained from non-immunized animals in the above manner.

III. Preparation of ICEF Supernates

For each milliliter of ICEF supernate desired, 40×10^6 to 50×10^6 viable Balb/c thymocytes were incubated with 0.2 ml of "6 hr. serum" for 30 minutes at 37°C in a total volume of 1.0 ml Hanks' Balanced Salt Solution (HBSS, Gibco Canada Ltd., Calgary, Alta.). The cells were then washed twice by centrifugation at 100x g for 7 minutes followed by re-suspension in approximately 4 ml HBSS. After the final wash, the cells were resuspended in 1.0 ml HBSS and incubated 3 hr. at 37°C. At this time, they were centrifuged at 1000x g for 5 minutes and the supernate was collected. When aseptic conditions were required, the incubations were performed in sterile Eagle's Minimal Essential Medium (MEM) pH 7.4 (Gibco).

As a control, thymocytes were stimulated with NMS instead of 6 hr. serum and the above procedure was duplicated. The supernates obtained in these cases are designated as "normal serum-stimulated supernates" or "NSS."

IV. Reverse Immune Cytoadherence (RICA)

(1) Preparation of Hybrid Ab

A 5S hybrid $F(ab')_2$ rabbit Ab, one site specific for BSA, the other directed against myeloma proteins of the mouse 7Sγ1 (γF) or 7Sγ2a (γG) subclasses, was prepared as outlined by Paraskevas, Lee and Israels (1971) and Paraskevas, Lee, Orr and Israels (1971). Briefly, rabbit anti-mouse γF and γG (anti-F,G) serum was prepared as outlined in Paraskevas, Lee, Orr and Israels (1971). Rabbit anti-BSA serum was prepared in a similar manner using 1.0 mg BSA (Miles Laboratories, Kankakee, Ill.) in 1.0 ml of an FCA-saline emulsion as the immunogen. The antibodies were then purified by passage of 5 ml immune serum over an immunosorbent column consisting of 50 mg of the appropriate Ag coupled to 1.0 g CNBr-activated Sepharose 4B (Pharmacia Canada Ltd., Montreal, P.Q.) equilibrated in 0.15M borate saline, pH 8.0. The columns were then washed with 100 ml 0.15M borate saline, pH 8.0. The Ab's were subsequently eluted in 5 ml 0.1M glycine-HCl buffer, pH 2.5 followed by 5 ml of borate saline, and neutralized to pH 7.0 with 1N NaOH.

The resultant Ab preparations were stored at -20°C and subsequently pepsin digested and reduced in 2-mercaptoethylamine/HCl as described by Paraskevas, Lee and Israels (1971). The reducing agent was removed at 4°C on an Ag 50 WX4 (mesh 100-200; Bio Rad Laboratories, Richmond, Calif.) cation exchange column (10 x 200 mm). The eluted protein was neutralized with 1N NaOH and reoxidized by stirring under O_2 for 2 hr at room temperature. This preparation was concentrated by high pressure ultrafiltration through a UM-10 Diaflow membrane (Amicon Corp., Lexington, Mass.) to approximately 2 ml and applied to a Sephadex G-200

column (27 x 1000 mn; Pharmacia Canada Ltd.) which was equilibrated with 0.15 M borate saline pH 8.0. The eluted 5S fraction was then concentrated to approximately 5 mg/ml by UM-10 filtration and stored at 4°C.

The anti-BSA-anti- F,G hybrid Ab and anti-BSA $F(ab')_2$ dimers were isolated by passage over the CNBr-activated Sepharose 4B-BSA immunosorbent previously mentioned. Here, the immunosorbent-antibody mixture was stirred and allowed to sit 2 hr. at 4°C. After extensive washing with 0.15M borate saline pH 8.0, the bound Ab was eluted slowly in 5 washes of 2.1M glycine-HCl buffer, pH 2.5, to give a total volume of 7.3 ml. Those washings showing significant concentrations of protein by absorbance measurements at 280 nm were combined and neutralized to pH 7.0 with 1N NaOH.

This eluate was then mixed overnight at 4°C with bis-diazotized benzidine (BDB)-γF,G aggregates prepared as described in Paraskevas, Lee and Israels (1971). The unbound anti-BSA $F(ab')_2$ recombinants were then removed by centrifugation at 10,000 rpm for 20 minutes at 4°C in a Lourdes Betafuge, Model A-2, centrifuge (Lourdes Instrument Corp., Old Bestpage, N.Y.). The pellet containing the immunosorbent-hybrid Ab complexes, was then washed in 0.15M borate saline, pH 8.0. Subsequently, the hybrid Ab was dissociated and collected in 0.1M glycine-HCl buffer, pH 2.5 and the pH neutralized.

The specificity of the anti-BSA-anti-F,G hybrid Ab was monitored by Ouchterlony diffusion. Only hybrid Ab which precipitated BSA, γF and γG, when these were present together, was used in the experiments presented herein.

(2) Coating of SRBC

SRBC were coated with BSA by the method described by Paraskevas, Lee and Israels (1971). The only difference is that 0.12 mg BSA/ml of 2% tanned SRBC was used in place of horse spleen ferritin as originally outlined.

(3) RICA Assay

In the experiments presented in this thesis, $1x10^6$ lymphocytes in 0.1 ml of either HBSS or MEM pH 7.4, were incubated overnight at 4°C with 0.3 ml hybrid Ab and 0.03 ml BSA-coated SRBC (BSA-SRBC). The rosette-forming cells were subsequently observed under phase contrast microscopy at 400x magnification. Ig^+ cells were identified as RFC around which a ring of at least 4 BSA-SRBC is bound.

V. ICEF Treatment of Normal Spleen Cells

$1x10^7$ normal spleen cells (NSC) were incubated for 30 minutes at 37°C with 0.2 ml NMS and 0.2 ml ICEF supernate in HBSS, pH 7.4, in a final volume of 1.0 ml. After this time they were washed twice and resuspended to a concentration of $1x10^7$ total cells/ml. Subsequently, 0.1 ml of this suspension was subjected to testing by RICA.

VI. Fraction of NMS Involved in Ig Uptake

NMS was passed on a Sephadex G-200 column (27 x 1000 mm; Pharmacia, Canada) which had been equilibrated with 0.15M borate saline, pH 8.0, and previously calibrated with Blue Dextran 200,000 (molecular weight $2x10^6$, Pharmacia, Canada) and the 19S, 7S and 4S fractions were collected. These were concentrated to the original serum volume (2ml) using a UM-10 membrane (Amicon). The fractions were then substituted for NMS in the treatment of normal spleen cells by ICEF in order to establish which component(s) of NMS was responsible for supplying the cytophilic Ig.

VII. Preparation of Anti-Theta (anti-θ) Serum

AKR female mice 6 to 8 weeks old (Jackson) were injected i.p. with $1x10^7$ thymocytes from 4 to 6-week old C3H female mice (Jackson) weekly for 6 weeks. Ten days after the last injection, the mice were anesthetized with chloroform and the blood was collected by cardiac puncture. The serum was separated by overnight incubation at 4°C followed by centrifugation of the clotted material at 1000x g for 5 minutes. It was then decomplemented at 56°C for 30 minutes and absorbed for 1 hr at 40°C with C3H and Balb/c RBC.

Following titration against thymocytes, spleen cells and bone marrow cells, the anti-θ serum was aliquoted and stored at -20°C until use. The optimal concentration for use was designated as that which killed approximately 95% of thymocytes.

VIII. ICEF Treatment of Anti-θ Resistant Spleen Cells

In order to study which cell population may be involved in Ig uptake, $1x10^7$ viable Balb/c spleen cells were incubated for 45 minutes at 37°C with 0.2 ml anti-θ serum (1:10 final dilution) and 0.2 ml guinea pig complement (GP-C; Miles; final dilution 1:10). After this time, the number of viable cells was counted and compared to that obtained from NSC treated with GP-C in the absence of anti-θ serum. This treatment has been shown to produce a cell population enriched for B cells and macrophages, but deficient in T cells (Raff, 1969).

The remaining cells were washed twice in HBSS, resuspended to $1x10^7$ total cells/ml, treated with the appropriate volumes of ICEF supernate and NMS, and subjected to RICA analysis. Controls included untreated anti-θ resistant cells, anti-θ-resistant cells treated with NSS in either the presence or absence of NMS, and anti-θ-resistant cells treated with ICEF in the absence of NMS.

IX. T Cell Enrichment by Nylon Wool Columns

Spleen cell populations highly enriched for T cells were obtained by passing NSC over nylon wool columns in a modification of the methods of Schwartz *et al*. (1975; 1976) and Julius *et al*. (1973). Approximately 35g of nylon fiber (FT-242, Fenwal Laboratories, Morton Grove, Ill.) were boiled for 30 minutes in 0.2N HCl and subsequently washed in 10 volumes of distilled, deionized water (DDW). It was then soaked for 5 days at 37°C in DDW, which was changed daily. The wool was dried at 37°C for 3 days and approximately 1g portions were placed in a 10 cc volume in a 10 cc Plastipak disposable plastic syringe (Beckton, Dickenson and Co. Ltd., Mississauga, Ont.) and autoclaved at 121°C for 15 minutes.

Before use, the columns were flushed with 85 ml 0.15M phosphate buffered saline, pH 7.0, followed by 100 ml RPMI 1640 with 10% heat-inactivated (30 minutes at 56°C) fetal calf serum (FCS; Gibco). This medium was drained to the top of the nylon matrix and the column was incubated 1 hr. at 37°C. After this time, it was flushed with 20 ml Dulbecco's PBS (DSS; Dulbecco and Vogt, 1954), pH 7.2, containing 10% FCS (DSS-10 FCS).

Spleens were teased with forceps into RPMI plus 10% FCS and the resultant cell suspension was washed once in this medium then resuspended in DSS-10 FCS. Approximately 200×10^6 viable nucleated cells, as examined by 0.1% trypan blue exclusion, were loaded onto the column in 2.0 ml. These were passed just into the nylon matrix and incubated for 45 minutes at 37°C. At 15 minute intervals, the cells were slowly passed further into the matrix by the addition of 1 to 2 ml DSS-10 FCS. After the incubation period, the cells were eluted slowly from the column in DSS-10 FCS and the first 25 ml were collected.

Nylon wool nonadherent (NNA) cells obtained in the above manner were

centrifuged at 100x g for 10 minutes and washed twice in MEM. Viable cells were counted, as determined by trypan blue exclusion, and the effectiveness of T cell enrichment was determined by assaying for the presence of $SMIg^+$ cells by RICA.

X. Effect of ICEF on Uptake of Ig by NNA Cells

To study the ability of certain T lymphocytes to bind cytophilic Ig in the presence of ICEF, 5×10^6 washed NNA cells were incubated with 0.1 ml ICEF supernate plus 0.1 ml NMS in a final volume of 0.5 ml for 30 minutes at 37°C. At this time, they were washed twice with MEM, resuspended to approximately 10×10^6 cells/ml, and 0.1 ml of this suspension was subjected to analysis by RICA. Controls included the incorporation of NSS in lieu of ICEF and the substitution of MEM for ICEF or NMS in certain suspensions. In addition, the activity of each ICEF preparation was monitored on NSC.

XI. Effect of ICEF-Treated NNA Cells on PFC Responses *In Vivo*

Since ICEF appeared to increase the cytophilicity of Ig for T cells (see Results, Section IV-2), the possible effect of these Ig^+ T lymphocytes on a thymus-dependent Ab response was studied. Again, NNA cells were obtained and washed as above. In these experiments, only sterile media and aseptic conditions were used in order to avoid infection of the recipient animals.

Viable NNA cells were counted and the entire population thereof was divided into two. These aliquots were treated with the appropriate volumes of ICEF plus NMS or NSS plus NMS. After treatment, the cells were washed twice with sterile MEM, resuspended to 50×10^6 viable cells/ml and 0.2 ml of this suspension (10×10^6 cells) were injected intravenously (i.v.) into adult thymectomized, lethally-irradiated (800 R; Eldorado A ^{60}Co. therapy

machine, Atomic Energy of Canada Ltd.) Balb/c female mice which had been reconstituted with $20x10^6$ syngeneic bone marrow cells three to four weeks previously (ATxXB mice). The mice were then immunized i.p. with $5x10^8$ SRBC (National Biological Laboratories Ltd., Dugald, Man.) which had been stored at 4°C in Alsever's solution and washed at least twice in 0.15M PBS pH 7.0. Seven days later, the recipients were anesthetized with chloroform, checked macroscopically for possible remnants of thymic tissue and the spleens were harvested. The PFC assay, according to the method of Cunningham and Szenberg (1968) was then performed and the numbers of 19S and 7S Ab-secreting cells per spleen were calculated.

In this assay, the cells were washed once in a 1:1 solution of MEM:HBSS, pH 7.4 and resuspended to 2.0 ml in this medium. Thirty µl of cell suspension was then combined with 30 µl of undiluted GP-C and 30 µl of a $2.5x10^9$ washed SRBC/ml suspension. For indirect PFC assays, 30 µl of a 1:10 dilution of rabbit anti-mouse Ig (RAMIg), prepared as for the anti- F,G mentioned previously, was also included. The resultant suspension was then transferred to slide chambers prepared as described in Cunningham and Szenberg (1968), sealed with molten paraffin and incubated at 37°C for 45 minutes. The slides were observed under 100x magnification and the number of PFC/spleen were calculated. Counts for 7S PFC were obtained by subtracting the direct count (19S PFC) from the corresponding indirect count (19S + 7S PFC).

XII. Effect of ICEF on PFC Production in Normal Mice

In order to establish any possible influence of ICEF on Ab responses of normal cells *in situ*, $5x10^8$ SRBC, stored and washed as above, were injected i.p., along with 0.3 ml of either ICEF or NSS supernate, into normal 6 to 8-week-old Balb/c mice. Seven days later, the mice were sacrificed by cervical dislocation, the spleens were harvested, and the PFC

assay, as outlined, was performed.

XIII. Effect of ICEF on PFC Production in T Cell-Deficient Mice

In an attempt to observe whether or not ICEF could act as a thymus-replacing factor, much in the same way as IACF was shown to function (Orr, 1974; Paraskevas *et al.*, 1976), the effect of ICEF on T cell-deficient mice was studied. ATxXB Balb/c females were injected i.p. with 0.3 ml ICEF or NSS plus $5x10^8$ washed SRBC. Five days later, the animals were anesthetized with chloroform, examined macroscopically for thymic tissue and their spleens were harvested. The PFC assay, as outlined above, was then carried out except that the volume of MEM:HBSS used for resuspension was only 1.0 ml.

XIV. Production of ICEF by IACF-Treated Cells

Since ICEF is produced from thymocytes stimulated with Ig and six hour immune serum which presumably contains IACF and Ag (Orr, 1974; Paraskevas *et al.*, 1976), it was decided to study the ability of thymocytes which had, instead, been stimulated with IACF culture supernate in conjunction with Ag and NMS, to produce ICEF, as had been indicated previously (K.B. Orr and S.T. Lee, unpublished data). Each ml of IACF was produced by treating $10x10^6$ viable thymocytes with 100 µg BCG (Difco Laboratories, Detroit, Mich.) at 37°C for 30 minutes. The cells were subsequently washed twice in HBSS, pH 7.4, resuspended to 1.0 ml, incubated 3 hr. at 37°C and their supernate collected after centrifugation at 1000x g for 5 minutes.

ICEF was produced by pre-incubation of 300 µl "IACF produced *in vitro*" with 300 µl NMS and 100 µl FIB which was at a concentration of 2.5 mg per ml. This incubation was done at 37°C for 30 minutes, after which $40x10^6$ to $50x10^6$ viable thymocytes in 300 µl HBSS, pH 7.4, were

added. The resultant suspension was incubated for 30 minutes at 37°C, washed twice in HBSS, pH 7.4, resuspended to 1.0 ml and further incubated for 3 hr. at 37°C. The supernate was then collected after centrifugation at 1000x g for 5 minutes.

Two hundred µl of the supernate plus 200 µl NMS were then added to $10x10^6$ normal spleen cells in HBSS, pH 7.4, and incubated 30 minutes at 37°C. The cells were washed twice with HBSS, resuspended to $10x10^6$/ml and 100 µl of this suspension was subjected to RICA analysis.

Controls included the substitution of HBSS for either IACF, NMS or FIB in the 30 minutes "stimulation" toward ICEF production. Furthermore, the activity of the stimulating BCG culture supernate was monitored by treating NSC with the supernate plus NMS and FIB in the appropriate manner, and subjecting the treated cells to RICA analysis.

XV. Production of IACF by ICEF-treated Thymocytes

Preliminary experiments (K.B. Orr and S.T. Lee, unpublished results) had demonstrated that when ICEF, which had been produced from IACF-stimulated thymocytes was used with NMS to stimulate normal thymocytes, the resultant 3 hr. culture supernate exhibited characteristics by RICA similar to IACF. These results, consequently, indicated the possible existence of a cyclical mechanism of recurring stimulation. It was decided to attempt to duplicate these experiments with appropriate controls.

One ml ICEF was incubated with 250 µl NMS for 30 minutes at 37°C. At this time, $30x10^6$ viable thymocytes in 30 µl were added and the resultant suspension was incubated for a further 30 minutes at 37°C. The cells were then washed 3 times in MEM, pH 7.4, resuspended to 1.0 ml and incubated at 37°C for 3 hr. The supernates were collected after centrifugation at 1000x g for 5 minutes.

Two hundred µl of the supernate, 100 µl NMS and 100 µl FIB at 2.5 mg/ml were incubated for 30 minutes at 37°C, at which time 6×10^6 normal spleen cells in 100 µl were added. This suspension was incubated for 30 minutes at 37°C, washed twice with MEM, resuspended to 10×10^6 cells/ml, and a 100 µl aliquot was subjected to analysis by RICA.

Controls for the stimulation procedure involved substitution of NSS for ICEF and of MEM for NMS. Controls used to establish the RICA characteristics of the "secondary" supernate involved the substitution of MEM for FIB or NMS.

XVI. Production of ICEF at 4°C

This experiment was designed to determine whether or not ICEF could be produced and/or released from cells at a temperature which would inhibit most metabolic activity. In triplicate experiments, thymocytes were stimulated with IACF, FIB and NMS as described earlier. These cells were then washed twice in HBSS, pH 7.4, resuspended to 1.0 ml and incubated 3 hr. at 4°C. At this time, the supernates were collected and used, with NMS, to treat NSC which were subsequently studied by RICA. Details of these procedures have already been presented. In one additional experiment, the stimulation by IACF, NMS and FIB was performed at 4°C as well.

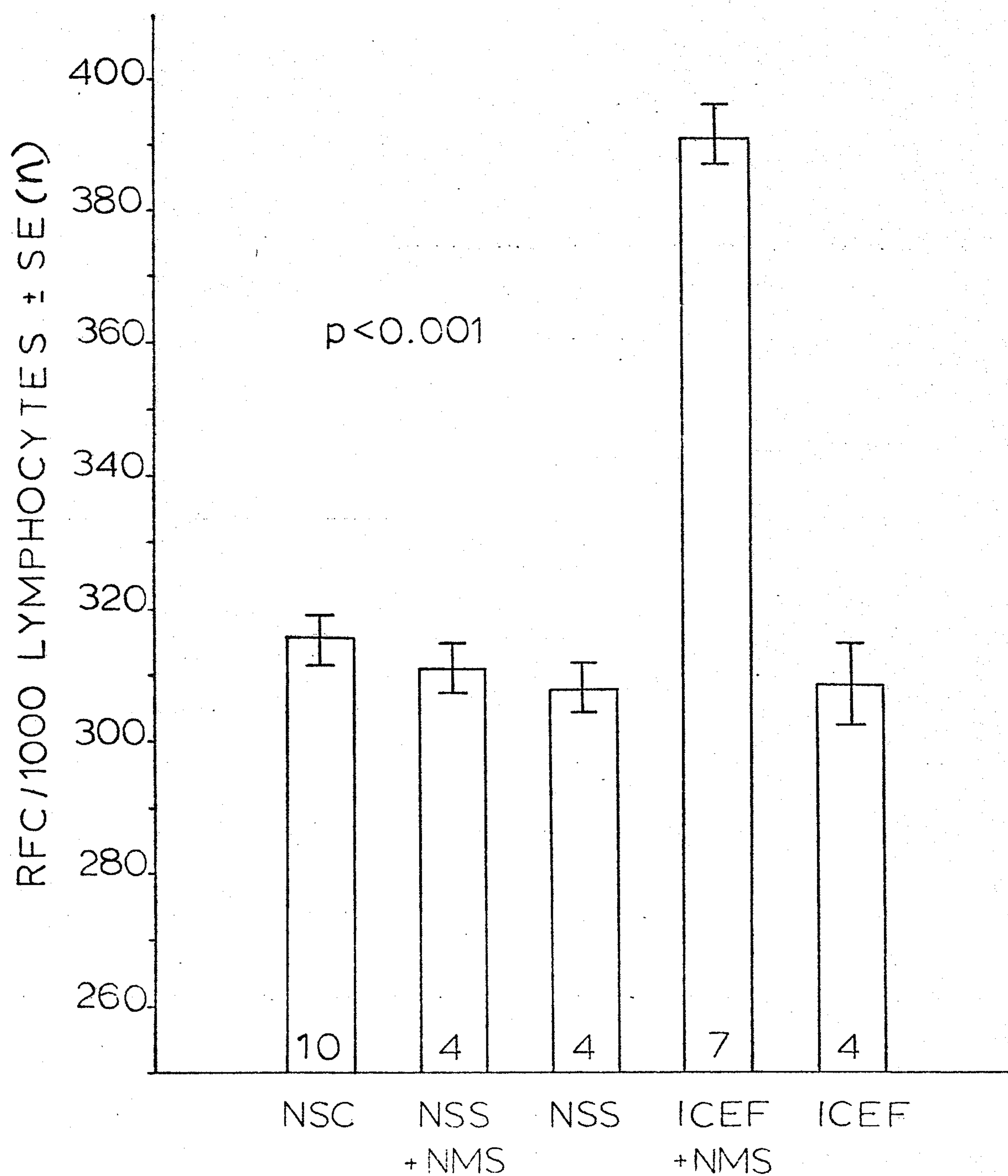
Controls include treatment of NSC with ICEF produced at 37°C and NSS produced at 4°C. The activity of the stimulating IACF was also monitored by RICA on NSC.

RESULTS

I. ICEF Treatment of Normal Spleen Cells

As illustrated in Figure 1, the treatment of NSC with ICEF and NMS significantly increases the number of RFC observed ($p < 0.001$). Normal and control counts are between 300 to 320 RFC/1000 lymphocytes. Cells treated

FIGURE 1: EFFECT OF ICEF ON RFC FROM NORMAL BALB/c SPLEEN CELLS



with ICEF plus NMS, on the other hand, produce a mean count of 390.7 RFC/ 1000 lymphocytes $\pm$ a standard error of 4.4. It should also be pointed out that both ICEF and NMS are required to facilitate this increase.

II. Active Serum Fraction of NMS in Ig Uptake

Figure 2a shows the fractionation pattern, as measured by optical density at 280 nm (OD_{280}), of the NMS after passage on Sephadex G-200. For further separation of the 19S and 7S peaks, the respective fractions from two passages of serum were pooled and passed again through Sephadex G-200 columns. The OD_{280} scans of these eluates are given in Figure 2b and 2c respectively. These fractions, in addition to the 4S fraction, which appeared to be reasonably well separated from the 7S fraction in Figure 2a and, therefore, was not purified further, were concentrated and tested for their ability to supply cytophilic Ig.

Table 1 shows that it is the 7S fraction of NMS which appears to be required by ICEF to facilitate an increase in $SMIg^+$ cells. With this fraction plus ICEF, a mean count of 361.5 $\pm$ a standard error of 3.9 was obtained. Again, both ICEF and the 7S fraction were required for the increase to occur.

When the results were compared by Student's Two-Tailed t Test, it was found that ICEF + 7S-treated cells showed significantly more $SMIg^+$ cells than ICEF + 19S-treated cells ($p < 0.01$), ICEF + 4S-treated cells ($p < 0.02$), NSS + 7S-treated cells ($p < 0.01$) and NSC ($p < 0.001$). The fact that the IECF + 7S counts are slightly lower than ICEF + NMS-treated cells can be explained by the degree of manipulation which was required to obtain the 7S fraction. Consequently, some activity may have been lost.

FIGURE 2: OD_{280} OF ELUTED FRACTIONS OF NMS FROM SEPHADEX G-200

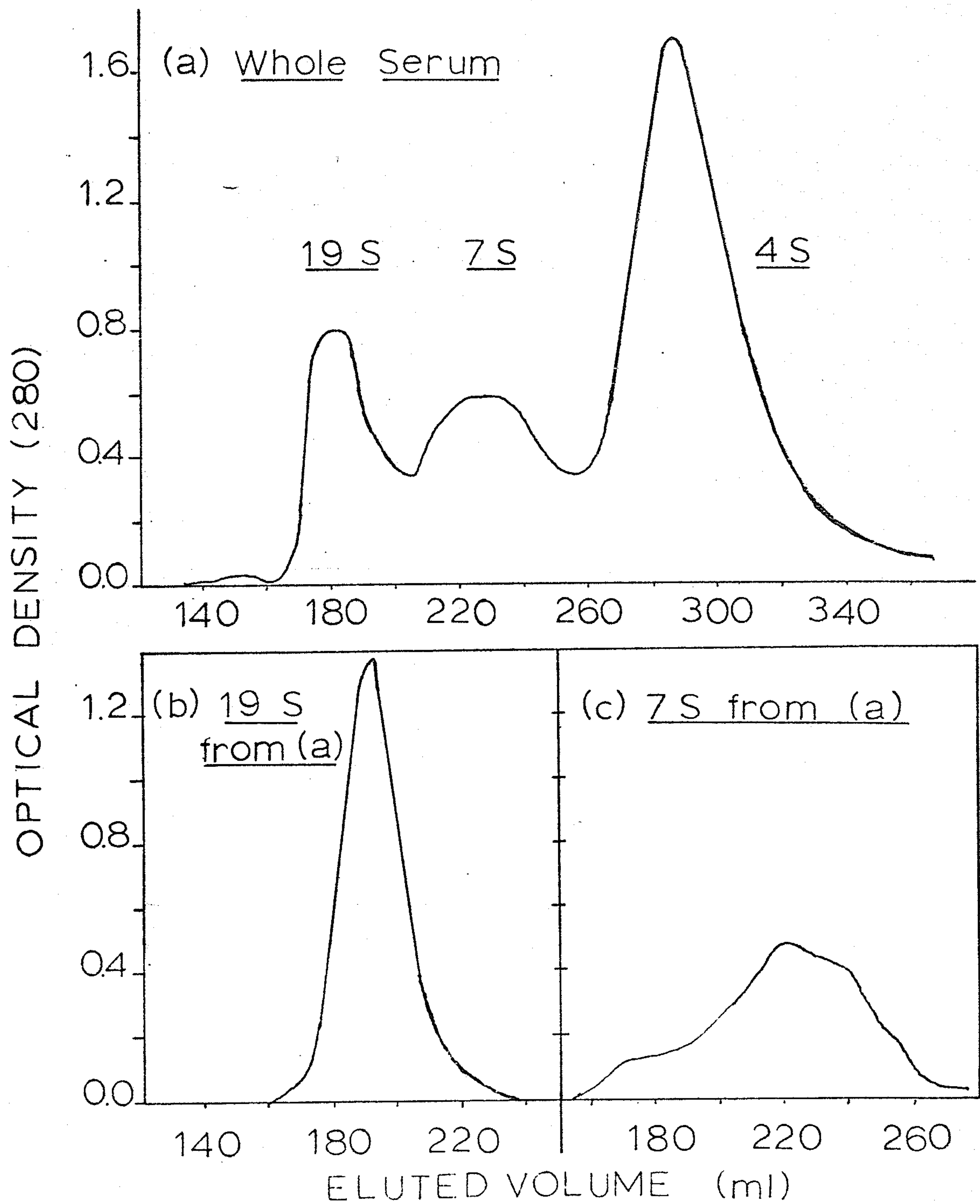


TABLE 1: EFFECTIVE NMS FRACTION IN ICEF ENHANCEMENT OF Ig CYTOPHILICITY

TREATMENT							RFC/10^3 LC
NSC	NSS	ICEF	NMS	4 S	7S	19S	$\pm$ SE (n)
+	-	-	-	-	-	-	318.6 $\pm$ 4.5(9)
+	+	-	-	-	-	-	307.8 $\pm$ 3.8(4)
+	+	-	+	-	-	-	314.6 $\pm$ 4.5(5)
+	-	+	-	-	-	-	308.0 $\pm$ 6.1(4)
+	-	+	+	-	-	-	396.0 $\pm$ 12.2(5)
+	+	-	-	+	-	-	316.5 $\pm$ 12.5(2)
+	-	+	-	+	-	-	307.0 $\pm$ 21.0(2)
+	+	-	-	-	+	-	305.8 $\pm$ 12.6(4)
+	-	+	-	-	+	-	361.5 $\pm$ 3.9(4)
+	+	-	-	-	-	+	287.0 $\pm$ 10.0(4)
+	-	+	-	-	-	+	308.0 $\pm$ 10.8(4)

LEGEND: + ≡ added to treatment
- ≡ absent from treatment
LC ≡ lymphocytes

III. RICA Results of ICEF Treatment of Anti-θ + GP-C-Resistant Spleen Cells

Experiments with IACF (Orr, 1974) indicated that the increase in RFC in normal spleen cells treated with Ag, Ig and IACF was due to Ig uptake by T cells since no increase was observed in anti-θ plus GP-C-treated spleen cell populations. Because of the pattern observed when NSC were treated with ICEF, similar results were expected when this factor was used to treat T cell-depleted spleen cells. Consequently, it was surprising to find that when T cells were removed, treatment with ICEF + NMS produced a consistently significant loss of surface Ig ($p < 0.001$), as shown in Table 2.

RFC counts for the control anti-θ-treated populations approximate 410 RFC per 1000 lymphocytes. This is expected since the percentage of Ig^+ cells should increase upon T cell removal. However, when these θ- cells were treated with ICEF + NMS, the RFC count decreased to a mean of 293.6 RFC/1000 lymphocytes. Again, both NMS and ICEF were required to elicit this decrease.

It appears then that, in the absence of T cells, the combination of these two components mediates the demonstrable loss of SMIg from spleen cells. The exact mechanism and significance of this phenomenon has not been further studied.

IV. Effects of ICEF on T Cell Enriched Spleen Cell Populations

(1) Effectiveness of T Cell Enrichment

Table 3 shows that an average of only 3.6% of NNA cells were $SMIg^+$ by RICA. In experiments where nonspecific RFC were controlled by the absence of hybrid Ab in the RICA assay, this figure dropped to 1.67%. Since RFC counts on untreated NNA cells were generally used to simply monitor the effectiveness of T cell enrichment for other experiments, background counts were not included in several instances.

TABLE 2: EFFECT OF ICEF ON ANTI-Θ RESISTANT SPLEEN CELLS

TREATMENT				$RFC / 10^3$ LC
$\alpha\Theta^R$SC	NSS	ICEF	NMS	±SE (n)
+	-	-	-	420.4 ± 7.6 (5)
+	+	-	-	416.8 ± 13.5 (4)
+	+	-	+	418.0 ± 6.4 (4)
+	-	+	-	408.3 ± 10.4 (5)
+	-	+	+	293.6 ± 12.3 (5)

LEGEND: $\alpha\Theta^R$SC ≡ anti-Θ+GP-C resistant spleen cells

\+ ≡ added to treatment

\- ≡ absent from treatment

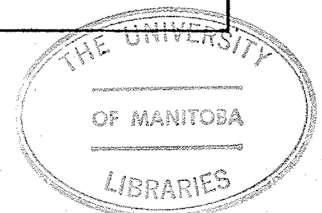

THE UNIVERSITY OF MANITOBA LIBRARIES

TABLE 3: RICA ANALYSIS OF NNA CELLS

EXPT	RFC /10^3 LYMPHOCYTES			
	NSC	NNA	BG-NNA	NNA-BG_{NNA}
1	300	39	20	19
2	324	55	35	20
3	320	38	27	11
4	326	23	ND	ND
5	285	38	ND	ND
6	308	40	ND	ND
7	326	20	ND	ND
MEAN $\pm$ SE	312.7 $\pm$1.5	36.0 $\pm$4.4	27.0 $\pm$4.3	16.7 $\pm$2.8

LEGEND: BG_{NNA} ≡ background RFC (no hybrid Ab) for NNA cells

ND ≡ test not performed

(2) Effect of ICEF on Ig Uptake by NNA Cells

As can be seen in Table 4, treatment of NNA cells with ICEF + NMS resulted in a substantial increase in RFC. Because nylon wool adherence is a nonspecific enrichment method which has been shown to remove a large percentage of FcR^+ cells such as B cells and macrophages (Schwartz and Paul, 1976), nonadherent cell populations would be expected to vary in their concentrations of cells capable of binding cytophilic Ig. This, in fact, appears to be the case since there is some degree of variation among experiments in the ability of NNA cells to bind Ig in the presence of ICEF. Consequently, the data presented in Table 4 are expressed as the percent increase in RFC/1000 lymphocytes by ICEF-treated cells over NSS-treated cells. To calculate this parameter, the equation

$$\%\text{ increase over controls} = \frac{[(\text{ICEF+NMS})\ \text{RFC}/10^3\ \text{lymphocytes}]\ [(\text{NSS NMS})\ \text{RFC}/10^3\ \text{lymphocytes}]}{[(\text{NSS+NMS})\ \text{RFC}/10^3\ \text{lymphocytes}]} \times 100\%$$

was employed. In 3 of 4 experiments, substantial increases varying from 50.0% to 103.2% were observed. In a fourth experiment, the data for which is not shown, only a 13% increase was observed. This observation, however, could be statistically rejected on the basis of the Quesenberry and David (1961) test for outlying results. The results then show an approximate 80% increase in the number of RFC formed following ICEF treatment.

(3) Effect of ICEF-treated NNA Cells on PFC *In Vivo*

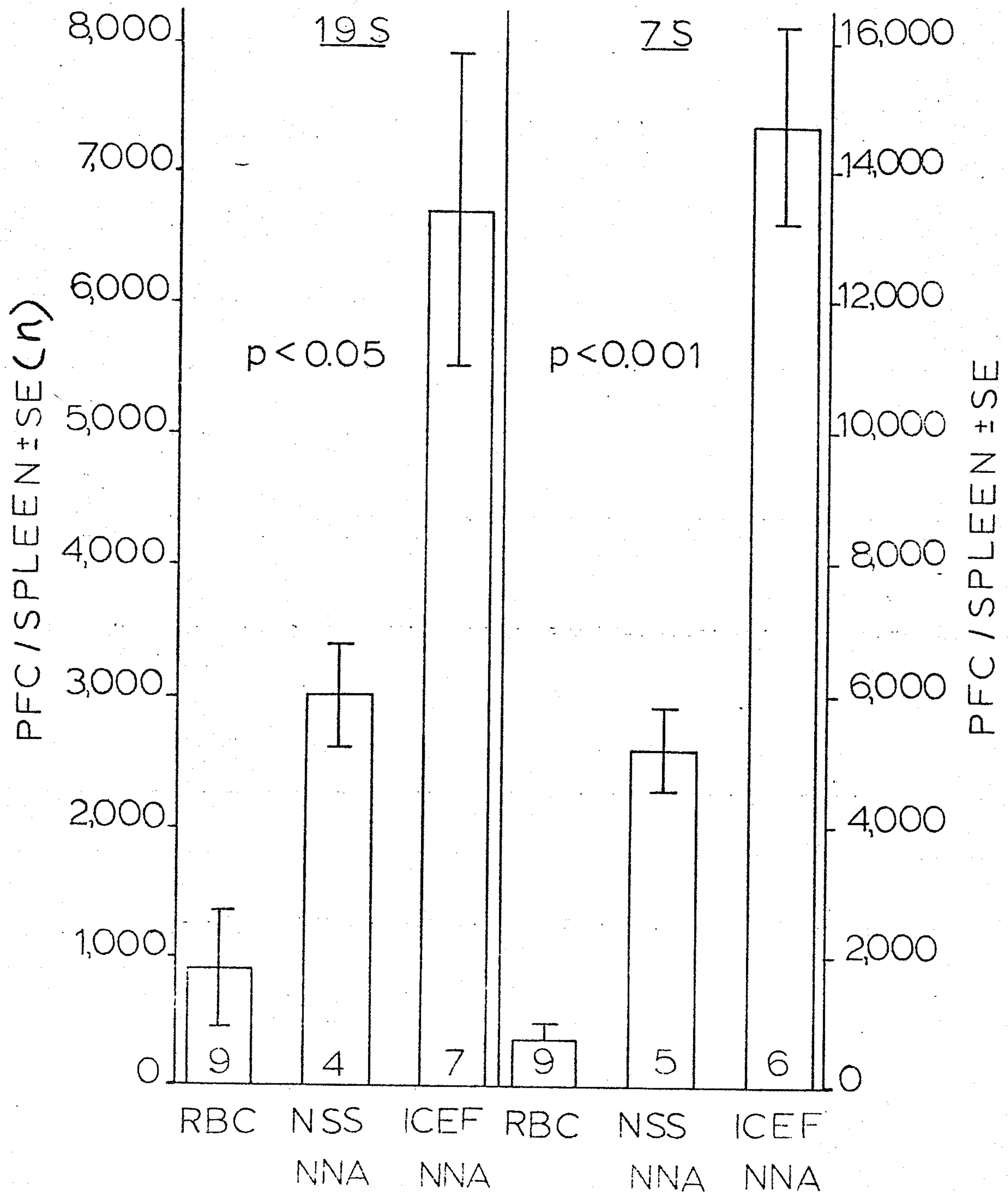
Data from three separate experiments show that when NNA cells which had been treated with ICEF and NMS were used to reconstitute ATxXB mice, a consistenly significant enhancement of the PFC response was obtained, as illustrated in Figure 3. The most significant enhancement occurred in the 7S response ($p < 0.001$) where the number of PFC, at 14,712/spleen, was almost triple that of control animals. An approximate doubling

TABLE 4: RICA ANALYSIS OF ICEF-TREATED NNA CELLS

EXP'T.	$RFC/10^3$ LYMPHOCYTES				% INCREASE
	NSS+NSC	ICEF+NSC	NSS+NNA	ICEF+NNA	
1	290	363	106	196	84.9
2	308	408	62	126	103.2
3	326	364	40	60	50.0
				MEAN % INCREASE ± SE	79.4 ± 15.6

NOTE: all NSS and ICEF treatments included NMS

FIGURE 3: EFFECT OF ICEF+NMS-TREATED NNA CELLS ON ANTI-SRBC PFC IN ATxXB MICE



of the 19S response was facilitated by ICEF-treated cells.

V. Enhancement of PFC in Normal Mice by ICEF

The data presented in Figure 4 show that when ICEF was simply injected into normal mice, a substantial increase in both 19S and 7S PRC responses against SRBC was generated. Animals which were injected with NSS instead of ICEF showed no striking differences in the responses when compared to those which received no supernate.

It should be stressed that these results were obtained following only a single dose of supernate administered simultaneously with Ag. Several parameters, such as dosage, route of administration, time-course of supernate injection and the number of supernate injections, could have been examined, which may have led to an even more significant enhancement. These experiments, however, were not performed in this study.

VI. PFC Results in ICEF-Treated ATxXB Mice

As shown in Table 5, the PFC results obtained from ATxXB mice which had been injected with ICEF and SRBC are somewhat nebulous. While the means for the 19S and 7S anti-SRBC responses indicate an approximate doubling in the ICEF-treated animals, the variation obtained in both cases is so extensive that these differences cannot be considered significant ($p < 0.1$ for 19S; $p < 0.2$ for 7S). Increasing the sample size did not alleviate this problem.

It is possible that the variability may be due to differences in the extent of T cell depletion in the ATxXB mice. It is demonstrated in this study that the amplification ability of IECF is probably due to its interaction with thymus-derived cells. Consequently, the presence of even small amounts of thymus tissue which were not effectively removed by thymectomy, and went undetected by macroscopic examination of the thorax upon sacrifice of the animal, could possibly account for the several elevated

FIGURE 4: EFFECT OF ICEF ON ANTI-SRBC PFC IN NORMAL MICE

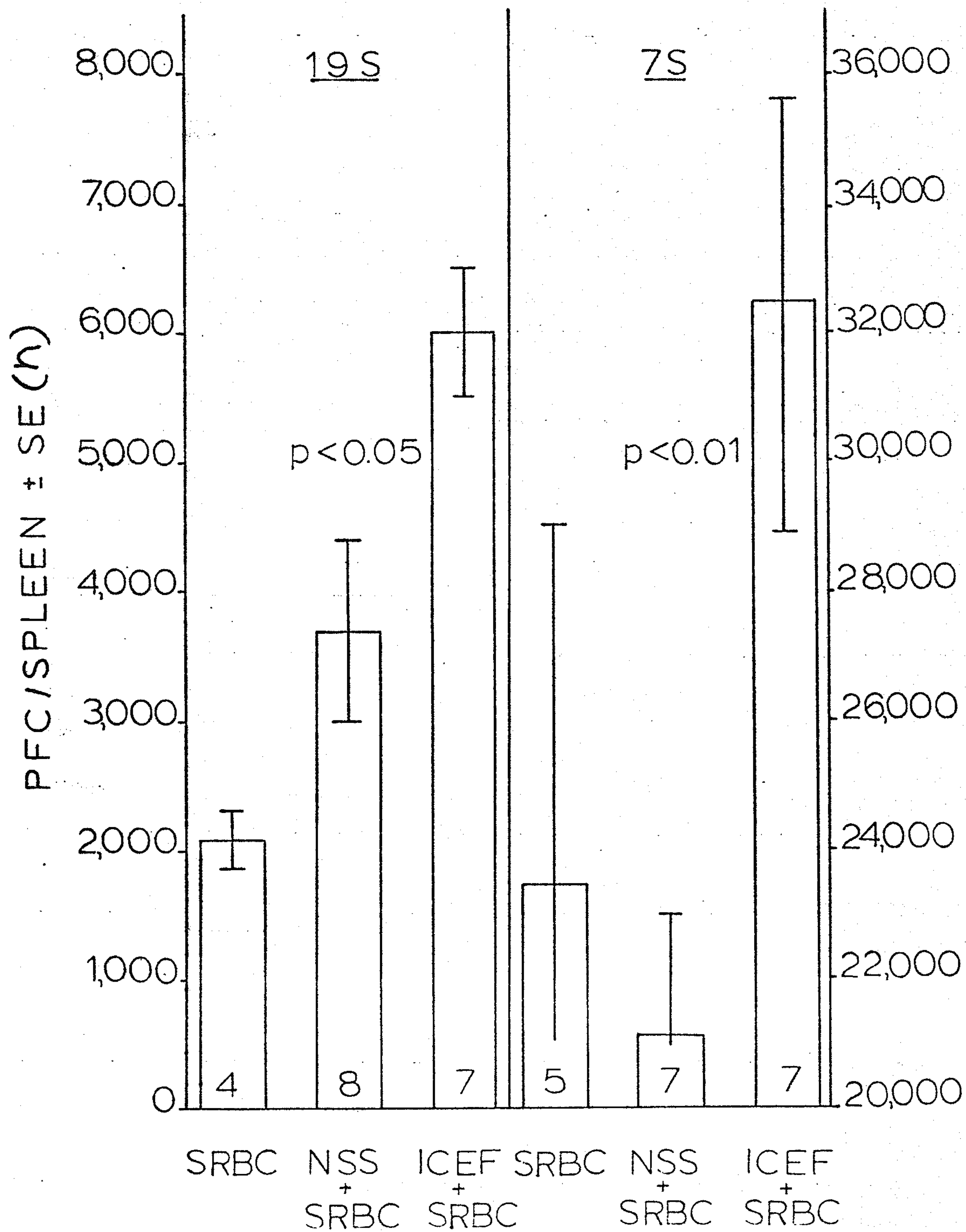


TABLE 5: EFFECT OF ICEF ON PFC IN ATxXB MICE

ASSAY NO.	RBC		NSS + RBC		ICEF + RBC	
	19S	7S	19S	7S	19S	7S
1	3,067	5,467	1,067	1,600	4,267	933
2	2,261	0	4,000	0	9,600	0
3	4,655	3,059	1,067	1,466	3,467	1,600
4	655	266	6,118	3,990	3,733	267
5	ND	ND	2,527	665	9,177	4,655
6	ND	ND	266	532	133	798
7	ND	ND	200	700	7,000	8,400
8	ND	ND	3,600	2,000	6,000	5,900
9	ND	ND	4,900	6,500	2,800	300
10	ND	ND	ND	ND	200	500
11	ND	ND	ND	ND	9,800	12,600
MEAN ± SE	2,659.5 ± 832.9	2,698.3 ±1,290.7	2,638.3 ±712.3	1,939.2 ±689.7	5,107.0 ±1,065.6	4,177.5 ±1,447.0

LEGEND: ND ≡ test not performed
all counts expressed as PFC / spleen

counts observed.

At any rate, these data provide no evidence that ICEF can act as an effective T cell-replacing factor in the anti-SRBC response.

VII. ICEF Production by IACF-treated Thymocytes

The data illustrated in Figure 5 demonstrate that a 3 hr culture supernate with characteristics by RICA which are similar to ICEF can be obtained from thymocytes stimulated with IACF plus Ag and NMS. Ag was not required for an increase in Ig uptake by spleen cells in the presence of this supernate. Furthermore, only the supernates from cells stimulated with all three of IACF, NMS and FIB could produce the increase when incubated with NSC and NMS. Control supernates from cells which had been preincubated with treatments lacking any one of these components failed to do so. Consequently, at least on the basis of RICA evidence, ICEF can be produced from thymocytes which have been stimulated with IACF plus Ag and NMS.

VIII. IACF Production by ICEF-treated Thymocytes

Not only can ICEF be produced by IACF-treated thymocytes but, on the basis of RICA, the converse is also true. A supernate exhibiting the RICA characteristics of IACF can be released from thymocytes pretreated with ICEF + NMS, as shown in Table 6. This supernate, when reacted with NSC in the presence of FIB and NMS, can elevate the numbers of $SMIg^+$ cells from control levels of approximately 300 RFC/1000 lymphocytes to 386 ($p < 0.02$).

It can be seen from Table 5 that both FIB and NMS are required for this increase. If one of these components is absent, Ig uptake will not occur. These results, as well as those presented in Section VII further substantiate previous results of Lee and Orr which alluded towards a cyclical pattern of co-stimulation.

FIGURE 5: RICA ANALYSIS OF SUPERNATES FROM IACF-TREATED THYMOCYTES

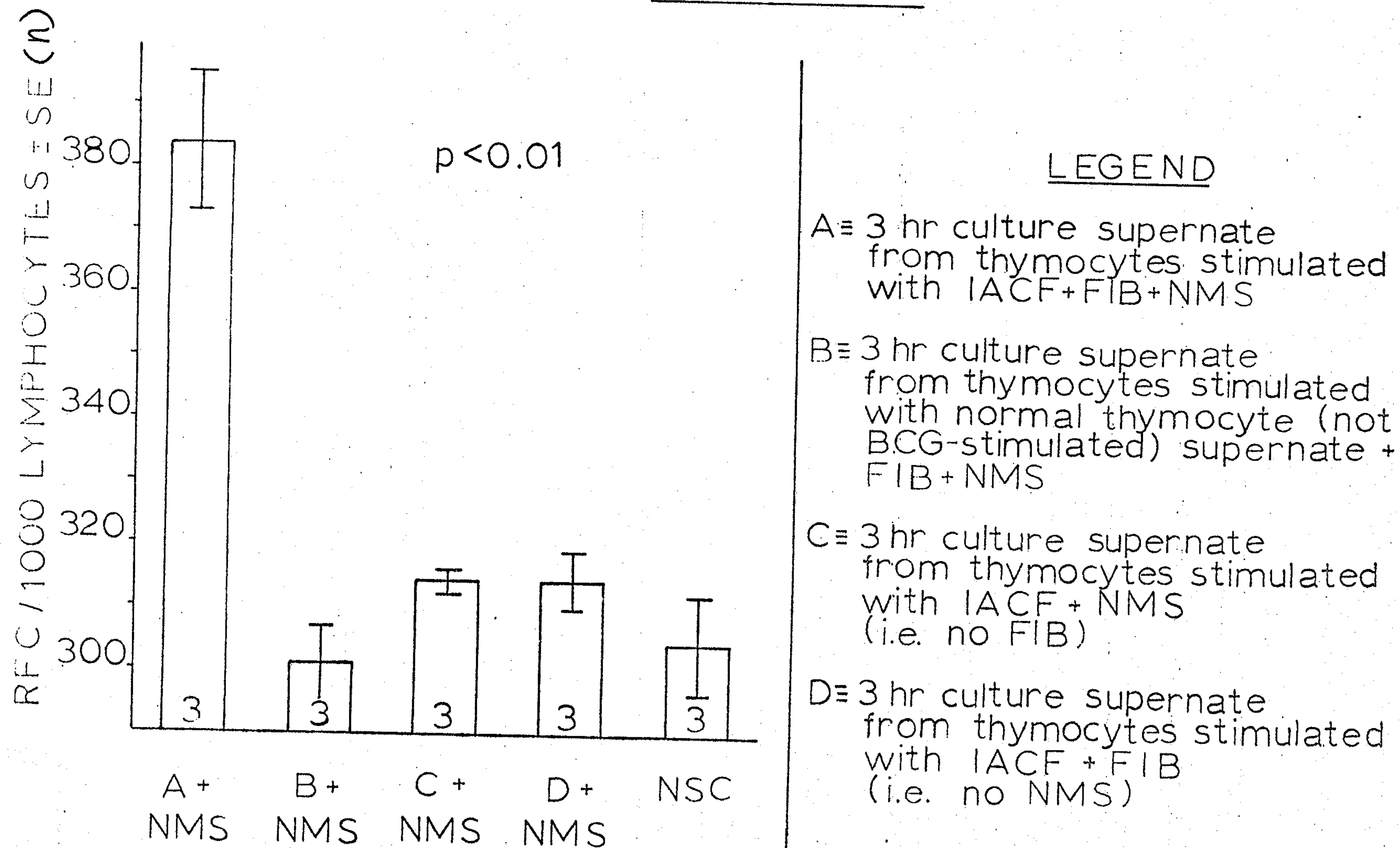


TABLE 6: RICA ANALYSIS OF SUPERNATES FROM ICEF-TREATED THYMOCYTES

SPLEEN CELL TREATMENT						RFC/10^3 LC ±SE (n)
A	B	FIB	NMS	ICEF	NSS	
+	-	+	+	-	-	386.0 ± 22.6 (3)
+	-	+	-	-	-	299.0 ± 24.5 (3)
+	-	-	+	-	-	297.0 ± 19.5 (3)
-	+	+	+	-	-	302.3 ± 8.4 (3)
-	-	-	+	+	-	393.0 ± 3.8 (3)
-	-	-	+	-	+	316.7 ± 3.2 (3)

LEGEND: A ≡ 3 hr culture supernate from cells stimulated with ICEF + NMS

B ≡ 3 hr culture supernate from cells stimulated with NSS + NMS

\+ ≡ added to treatment

\- ≡ absent from treatment

LC ≡ lymphocytes

TABLE 7: RICA ANALYSIS OF SUPERNATES PRODUCED AT 4°C

TREATMENT	RFC/10^3 LYMPHOCYTES			
	EXPT 1	EXPT 2	EXPT 3	MEAN±SE
IACF+FIB+NMS	372	367	370	369.7± 1.5
NTS+FIB+NMS	ND	307	305	306.0± 1.0
A +NMS	400	389	363	384.0±11.0
B +NMS	302	291	311	301.3±5.8
C +NMS	ND	ND	364	364.0
D +NMS	381	396	361	379.3±10.1
NSC	300	294	320	304.7±7.9

LEGEND:
- A ≡ ICEF produced at 37°C
- B ≡ NSS produced at 37°C
- C ≡ 3 hr culture supernate from thymocytes stimulated and cultured at 4°C
- D ≡ 3 hr culture supernate from thymocytes stimulated at 37°C and cultured at 4°C
- NTS ≡ 3 hr culture supernate from normal thymocytes incubated at 37°C
- ND ≡ test not performed

IX. Production of ICEF at 4°C

In a preliminary study into the requirement of active metabolism for ICEF production, the effect of low temperature (4°C) was examined. Table 7 shows that when the 3 hr incubation for ICEF production was performed at 4°C, a supernate resembling ICEF, which increased the number of RFC/1000 lymphocytes to 379 by RICA (supernate "D") was obtained. In the one experiment where both the 30 minute and 3 hr incubations were performed at 4°C (supernate "C"), the resultant RFC count of 364/1000 lymphocytes was consistent with the other increased counts by ICEF for that particular day. It would appear then that reducing the temperature at which the cells are stimulated and/or further cultured does not alter their ability to release ICEF.

DISCUSSION

From the preceding results, it appears that ICEF, in conjunction with NMS, can facilitate an increase in SMIg-bearing cells in a normal spleen cell population. The active NMS fraction which is involved in the Ig uptake is that which elutes in the 7S peak from a Sephadex G-200 column. This indicates that the increase in Ig^+ cells after treatment with ICEF is probably due to the cytophilic uptake of 7S Ig.

Experiments with NNA cells have demonstrated that the cells responsible for the uptake of this Ig are probably T lymphocytes. RICA results show that there is an approximate 80% increase in NNA RFC over control values after treatment with ICEF+NMS, while such treatment yields an approximate 25% increase in NSC RFC. Results from cell populations devoid of T. cells, however, do not demonstrate an increase in RFC under similar conditions. In fact, a surprising decrease of SMIg+ cells is observed in this case.

This removal of SMIg in the absence of T cells generates several questions. Firstly, is this phenomenon due to an internalization of the

Ig, possibly by capping and pinocytosis, or is it brought about by a displacement or shedding of the SMIg into the surrounding medium? Also, why are both NMS and ICEF required to facilitate this action? Perhaps, if ICEF does bind Ig, as has been postulated in our laboratory, it may produce a cross-linking of surface Ig and thereby induce capping and pinocytosis (Taylor et al., 1971).

Furthermore, what is the role of the T cell through which it appears to prevent the loss of B cell SMIg? It may provide some means of enzymatic degradation of ICEF, although this is somewhat unlikely and so far untested, explanation would not seem to be beneficial for the T cell. On the other hand, it may simply absorb ICEF and Ig to increase T cell SMIg while concomitantly reducing the amount of factor available for the removal of SMIg from T cells.

Another hypothesis worth considering is that the loss of B cell SMIg may also occur in the presence of T cells. This would imply that the approximately 25% observed increase in the normal system may, in fact, be greater. In other words, the difference between the observed increase and the actual increase would be masked by compensation for the decrease in B cell SMIg.

Studies using purified T cell populations may elucidate the validity of this hypothesis. From RICA results on NNA cells (Table 4), it can be seen that the largest percent increase in RFC counts for ICEF-treated cells is found in Experiment 2. However, even in this experiment, the percentage of NNA lymphocytes which bind Ig in the presence of ICEF is only 6.4% greater than the NSS-treated controls. This low percentage would not seem capable of overcoming a deficit in RFC as great as that found when SMIg is removed from B cells in the presence of ICEF. However, it should again be pointed out that NNA cells are not representative of the entire splenic T cell popu-

lation (Shiozawa et al., 1977; Tanaguchi et al., 1976) and may contain varying proportions of FcR^+ cells, as has been mentioned. In this respect, experiments incorporating them cannot unequivocally test this hypothesis.

Other authors (Basten et al., 1975; Stout and Herzenberg, 1975) have reported that 25% of splenic T cells may be FcR^+. Consequently, if the cytophilic Ig was to be bound solely through the FcR on the T cell, the ability to alleviate the large RFC deficit due to B cell SMIg loss would again be logistically improbable.

It is entirely possible, however, that the Ig may bind to the T cell via a receptor for ICEF, possibly in addition to the FcR. If such is the case, a sufficient number of T cells may bind Ig to overcome the loss of B cell RFC and still demonstrate a 25% increase in overall RFC.

In view of the complexity of this hypothesis and the unavailability of supporting data, the author does not strongly support it. Instead, he believes, if only on the basis of simplicity, that the RFC counts in normal spleen cells treated with ICEF + NMS may arise as a result of the direct adsorption of the factor onto T cells, thereby sequestering it from reactivity with B cells. More experimentation is obviously required in this area but, unfortunately, has not been performed here.

Studies using the PFC assay in vivo have further substantiated the effect of ICEF on T cells. NNA cells treated with ICEF + NMS, and subsequently used to reconstitute ATxXB mice, produced a significant enhancement of both the 19S and 7S Ab responses against SRBC. Similarly, when 0.3 ml of the factor were given i.p. with SRBC into normal adult mice, the resultant anti-SRBC responses were again significantly increased.

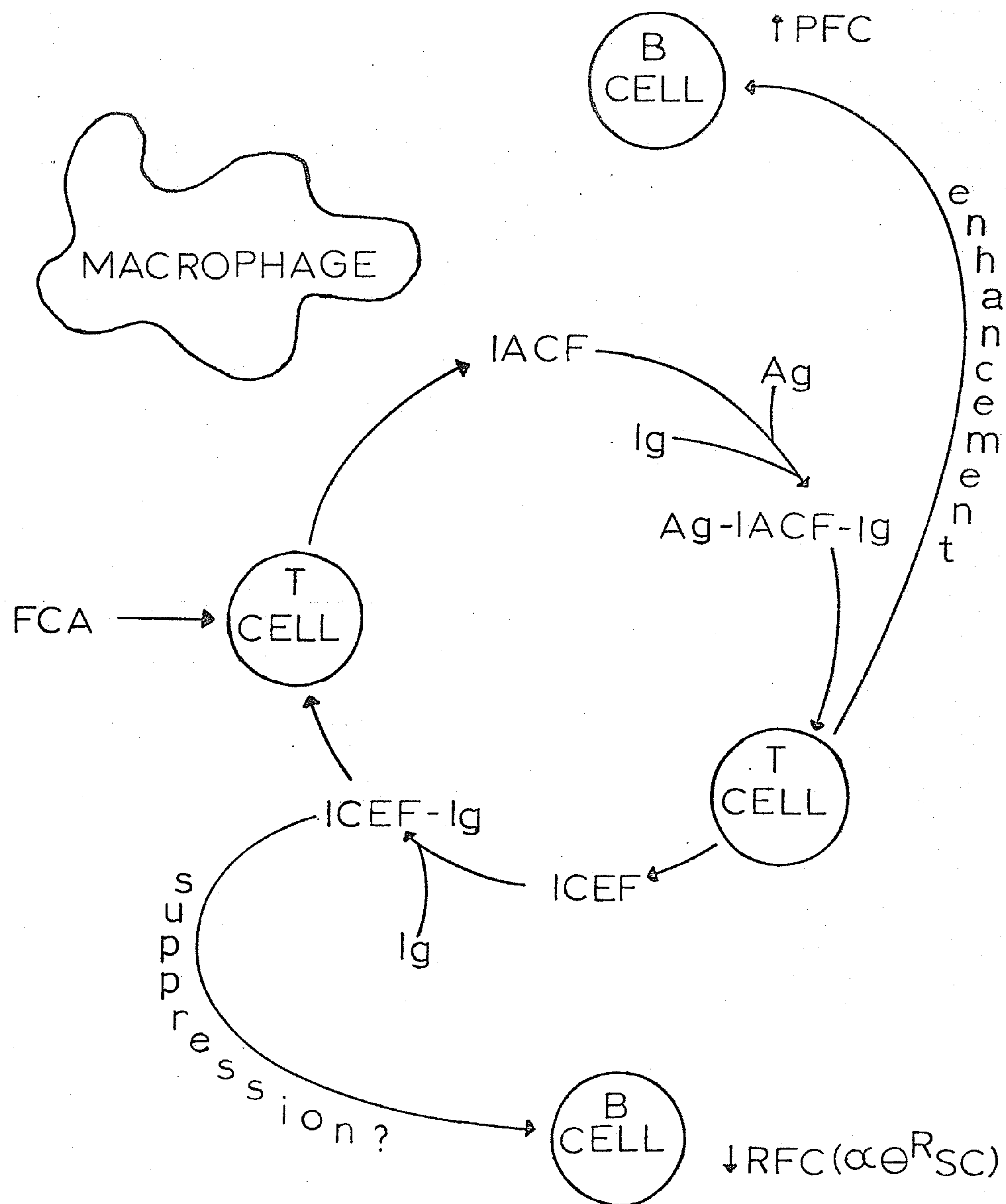
However, when ICEF was administered i.p. with SRBC into ATxXB mice, variable results were obtained. While the group mean showed a slight in-

crease in the ICEF-treated animals, the variability of results rendered this difference insignificant. It has been suggested that this variability may have arisen from the presence, in some animals, of remnant immunocompetent thymic tissue following thymectomy and irradiation. The ability of these residual T cells to react with ICEF and serum Ig could direct an increase in the anti-SRBC response in these mice, while the animals which were effectively thymectomized would demonstrate a much lower response. Therefore, while the results from this particular exercise are admittedly inconclusive, the problems encountered can be explained by other observations that ICEF is capable of stimulating T cells to enhance an anti-SRBC PFC response.

Another facet of this mediator appears to involve a certain reciprocity between ICEF and IACF. Since ICEF was first observed in culture supernates from thymocytes pretreated with serum collected six hrs. after immunization, which is believed to contain some form of IACF (Orr, 1974; Paraskevas et al., 1976), it is not surprising that it should also be detected in supernates from thymocytes pretreated with IACF + NMS + FIB. As presented in Figure 5, this is in fact the case. Furthermore, IACF, or at least a mediator exhibiting similar RICA properties, can be produced from cells pretreated with ICEF + NMS.

This observation lends itself to the hypothesis of a revolving or circular amplification process which is illustrated in Figure 6. In this model, FCA can stimulate T cells to produce a mediator, IACF, which can, upon reaction with Ag and Ig, increase the ability of Ig to bind to T cell surfaces. Apparently, a small population of macrophages (4%) is required for the production of this factor (Paraskevas, unpublished data), which is consistent with work reported by other authors (Erb and Feldmann, 1975a; b; c).

FIGURE 6: CELLULAR INTERACTIONS IN IACF-ICEF REGULATION OF IMMUNE RESPONSES



The SMIg^{+} T cells then can facilitate the production of both an enhanced Ab response and a second mediator, ICEF. This mediator requires only Ig to further stimulate the production of additional IACF. It can be seen how such a system could perform an amplification function similar to that described previously by Paraskevas and Lee (1976a; b; c) where synergistic interaction between Ig^{+} and Ig^{-} T cells early in the immune response can enhance the helper nature of these cells.

The observation that ICEF is capable of removing SMIg from B cell surfaces may implicate this factor in a regulatory role as well. Milton and Mowbray (1972) and Lee and Paraskevas (1976b) have demonstrated that the loss of SMIg from lymphocytes correlated well with the suppressive effects of IRA and UM-10-FIL, respectively. Consequently, it is not unlikely that the same situation arises here.

In fact, such a function would support the hypothesis that the extent of B cell SMIg loss is dependent upon the amount of ICEF not sequestered by the T cells. Therefore, once all T cell receptors for ICEF are filled and the amplification process reaches its peak, unbound ICEF could act upon B cells in a regulatory function. While the prospect is very interesting, PFC studies to date can neither confirm nor refute this theory. However, on the basis of the RICA results obtained, this hypothesis is incorporated into the model in Figure 6.

As far as the physicochemical properties of ICEF are concerned, little has been studied to date. At the outset of this project, it was decided that establishment of the factor's biological role should be of a higher priority than any detailed examination of its physicochemical characteristics. It has been found, however, that ICEF can be released from thymo-

cytes at 4°C, indicating that active metabolism may not be required for its production. These results correlate well with preliminary studies by others working with the author which show that the factor can also be released by thymocytes treated with cycloheximide (Paraskevas, unpublished data). Therefore, ICEF production appears to be independent of ongoing protein synthesis. These results lead to speculation that the factor may simply dissociate from the cell membrane during the 3 hr incubation. Further studies on this aspect, however, have not been undertaken here.

Although comparison of ICEF to other reported mediators would be somewhat premature until additional purification and physicochemical analysis is performed, this mediator may be somewhat similar to the IBF of Fridman and his colleages which was reviewed earlier in this presentation. Both mediators are produced by "educated" T cells. IBF is a product of allogeneically or GEP-primed cells while those producing ICEF are "educated" or stimulated by early immune serum factors. Both appear to bind Ig, but it must be noted that 7S Ig is required for ICEF while IBF binds only complexed IgG. The ability of ICEF to bind complexed 7S Ig has not been examined.

Functionally, IBF produces a nonspecific suppression of the anti-SRBC response *in vitro*. ICEF, while on one hand increases Ab responses *in vivo*, may suppress the response via a feedback type of regulation. Since ICEF produced from cells treated with FIB/FCA-six hr. immune serum can enhance an anti-SRBC response, and since no Ag is required to demonstrate ICEF activity by RICA, this mediator, too, may act in a non-antigen-specific manner, although this has not been further tested.

ICEF shares the same characteristics as the factor described by Shinohara *et al*. (1976) in that its release is not inhibited by the absence

of protein synthesis. However, the mediator proposed by these authors appears to act in a T cell-replacing capacity. ICEF, on the other hand, demonstrates a requirement for T cells in order to enhance an Ab response.

Areas of future studies on ICEF should include methods for isolation and purification of the factor from the supernate, physicochemical analysis of the active compound(s), elucidation of the subpopulation of cells responsible for its production and establishment of the significance of the factor's ability to generate a decrease of SMIg in B cell populations.

In conclusion, then, the data presented in this thesis demonstrate the existence of an immunoglobulin cytophilicity-enhancing factor (ICEF). Additional evidence, also documented herein, has shown that this mediator can enhance an *in vivo* thymus-dependent immune response in the presence of T cells. It also implicates ICEF with another factor, IACF, in a cyclical amplification process within a T cell-dependent response. In addition, the author proposes that ICEF may serve to regulate these responses by a feedback type of inhibition. Both the stimulatory and regulatory roles probably involve changes in SMIg on T and B lymphocytes, respectively.

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