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SUPPRESSOR CELLS AND SUPPRESSOR FACTOR(S) IN THE IMMUNE RESPONSE IN THE ADULT OUTBRED RABBIT

By

EYAL TALOR

Thesis submitted to the School of Graduate Studies, University of Ottawa, as partial fulfillment of the requirements for the degree of Doctor of Philosophy in Microbiology and Immunology.

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UNIVERSITÉ D'OTTAWA
UNIVERSITY OF OTTAWA

To my son

J O N A T H A N

ABSTRACT

Antibody-forming cells (AFC) actively secreting antibodies can be detected in large numbers in the spleen over a period of several weeks and in lesser numbers and only transiently in the bone marrow and the circulation following either primary or secondary immunization IV with sheep erythrocytes (SRBC) or horse erythrocytes (HRBC). AFC are not detected at all in the thymus nor in any of the other lymphoid organs. Memory cells, capable of being stimulated by the specific antigen in in vitro culture to transform into overt AFC, are detected only in the spleen during the first 3 months post-primary immunization. However, they are present in increasing numbers in the thymus beginning 4 to 6 months post-primary immunization as well as in the spleen. These results suggest(ed) that suppressor cells activated in the thymus may inhibit any AFC which may be activated during the primary or secondary immune responses in the thymus. In this investigation, it was demonstrated that suppressor cells are indeed activated in the thymus by the fifth day post-primary or secondary immunization capable of inhibiting the secretion of antibodies from autologous or allogeneic immune splenic AFC thereby preventing the generation of hemolytic plaques in the plaque-forming cell assay. These suppressor cells, referred to as immune thymus suppressor cells or ITSC, secrete a soluble factor in short term (4 hr) incubation in vitro which is capable by itself of inhibiting antibody secretion from autologous or allogeneic immune splenic AFC in vitro. This factor is referred to as immune thymus suppressor factor or ITSF. ITSF acts in an antigen-specific manner and its activity is not MHC restricted. ITSC and ITSF totally inhibit the secondary immune response induced in in vitro culture of splenic memory MNC and

the antigen, and the primary immune response in vivo when injected IV into rabbits on five consecutive days beginning with the injection of the antigen. ITSF added to in vitro cultures of immune splenic mononuclear cells suppresses only the secretion of specific antibodies; the cells are not impaired in their ability to synthesize and secrete nonspecific immunoglobulins. Thymocytes obtained from unimmunized rabbits and supernatants of cultures of normal rabbit thymocytes did not exhibit any suppressor activity in any of the situations in which ITSC and ITSF were found to be suppressive. Isoelectric focusing of ITSF revealed 12 to 15 protein bands, thus indicating the highly heterogeneous nature of ITSF. The potential use of ITSF, once purified, as an immunosuppressive agent in conjunction with other immunosuppressive agents such as Cyclosporin-A and Immuran in patients with autoimmune diseases and in allograft recipients is discussed.

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LIST OF ABBREVIATIONS

α_1AT	α_1-antitrypsin
α_2M	α_2-macroglobulin
Ab	Antibody
ADCC	Antibody-dependent cell-mediated cytolysis
AFC	Antibody forming cell(s)
AFP	Alpha fetoprotein
AM	Alveolar macrophages
APP	Appendix
ARC	Antigen receptor bearing cell(s)
BCDF	B cell differentiation factor
BCG	Bacillus Calmette-Guerin
BCGF	B cell growth factor
BM	Bone marrow
BSF	Bone marrow suppressive factor
C'	Complement
C'3	Third component of complement
C'1-C'9	First to ninth components of complement
CEA	Carcinoembryonic antigen
CF	Cytotoxic factor
FMI	Cell mediated immunity
Con A	Concanavalin A
CRP	C-reactive protein
CTL	Cytotoxic T lymphocyte(s)
DHSR	Delayed hypersensitivity skin reaction
DNP_{50}-BGG	Dinitrophenyl bovine gamma globulin
DSF	Diffusable suppressor factor
E	Erythrocytes - red blood cells (RBC)

E rosette	Formation of rosettes by T cells - SRBC
EA	Erythrocyte complexed with antibodies
EAC	indicator erythrocytes - C' and IgM complexed OxRBC
EAG	indicator erythrocytes - IgG complexed OxRBC
EAM	indicator erythrocytes - IgM complex OxRBC
EGME	Ethylene glycol monoethyl ether
ELISA	Enzyme linked immunosorbant assay
EPF	Early pregnancy factor
FCS	Fetal calf serum (heat inactivated at $56^{\circ}C$, 30 min)
FITC	Fluorescein isothiocyanate
Fc	Crystallizable fragment of Ig
FcG	Fc of IgG
FcGR	Fc receptor for IgG
FcM	Fc of IgM
FcMR	Fc receptor for IgM
G	Gravity
GAT	L-glutamic acid^{60}-L-alanine^{30}-L-tyrosine^{10}
GISF	GAT induced antigen specific T suppressor factor
GT	L-glutaminc acid^{50}-L-tyrosine^{50}
GVH	Graft versus host
HARBMS	Horse anti rabbit bone marrow serum
HARTS	Horse anti rabbit thymocyte serum
HBM	Human bone marrow
HBSS	Hank's Balanced Salt Solution
HCG	Human chorionic gonadotropin
HDL	High density lipoprotein
HI	Humoral immunity
HLA	Human Leucocyte antigen

hrs	Hours
HRBC	Horse erythrocytes
HSF	Histamine suppressor factor
HSSF	HRBC stroma sonicate filtrate
HuRBC	Human erythrocytes
IBF	Immunoglobulin binding factor
IDL	Intermediate density lipoproteins
IDS	Inhibitor of DNA synthesis
IF	Immunofluorescent
IFN	Interferon
IL-1	Interleukin 1
IL-2	Interleukin 2
Ig	Immunoglobulin
IgG	Immunoglobulin G
IgG-BF	IgG-binding factor
IgM	Immunoglobulin M
1^{O}IM	Primary immune response
1^{O}IMM	Primary immunization
2^{O}IM	Secondary immune response
2^{O}IMM	Secondary immunization
3^{O}IMM	Tertiary immunization
IRA	Immunoregulatory-α-globulin
IRSF	Immune response suppressive factor
ITSC	Immune thymus suppressor cell
ITSF	Immune thymus suppressor factor
IV	Intravenous
IU	International units

K cell	Killer cell
LAF	Lymphocyte activating factor
LCF	Lymphocyte chemotactic factor
LDL	Low density lipoprotein
LPDP	Lipoprotein deficient plasma
LPS	Lipopolysaccharide
LSS	Lipid suppressor substance
LT	Lymphotoxin
MØ	Macrophage
mAB	Monoclonal antibody
MAF	Macrophage activating factor
MCF	Macrophage chemotactic factor
MDSS	Marrow derived suppressive supernatant
MF	Mitogenic factor
MHC	Major histocompatibility complex
MIF	Migration inhibitory factor
MLR	Mixed leucocyte reaction
MNC	Mononuclear cell(s)
MNC-B	MNC depleted of B cells
$MNC\text{-}M\emptyset_1$	MNC depleted of monocytes or macrophages once
$MNC\text{-}M\emptyset_2$	MNC depleted of monocytes or macrophages twice
MNC-T	MNC depleted of T cells or non-T cells
MNC_m	Mitomycin-A treated MNC
MO	Monocytes
MW	Molecular weight
NIP	Normal immune suppressive protein
NK cell	Natural killer cell

NOCC	Naturally-occurring cell-mediated cytolysis
NSE	Non-specific esterase staining
NTC	Normal thymus cell
NTS	Normal thymus supernatant
OFA	Oncofetal antigens
OxRBC	Ox erythrocytes
PAG	Purified α_2-glycoprotein
PAPPS	Pregnancy-associated plasma proteins
PB	Peripheral blood (circulating MNC)
PBS	Phosphate buffer solution
PFC	Plaque forming cell(s)
PHA	Phytohaemmaglutinin
PLN	Popliteal lymph node
PP	Peyer's patches
PPD	Purified protein derivative
PWM	Pokeweed mitogen
RBC	Erythrocytes (red blood cells)
RRBC	Rabbit erythrocytes
SAA	Serum amyloid A
SAF	Suppressor activating factor
SBF	B cell derived suppressor factor
SC	Subcutaneous
SD	Standard deviation
SDIP	Spleen derived immunosuppressive protein
SG	Specific gravity
SIF	Soluble inhibitory factor
SIRS	Soluble immune response suppressor

SIRS-ox	Oxidized SIRS
SPL	Spleen
SR	Sacculus rotundus
SRBC	Sheep erythrocytes
SRS-A	Slow reacting substance of anaphylaxis
SSF	Soluble suppressive factor
SSSF	SRBC stroma sonicate filtrate
T-ARC	Antigen reacting T cells
TCGF	T cell growth factor
TF	Transfer factor
T_G	T cells with receptor for FcG
T_H	T helper cell(s)
THY	Thymus
T_M	T cells with receptor for FcM
T_S	T suppressor cell(s)
Tse	Suppressor effector T cell(s)
TSF	T cell suppressor factor
Tsi	Suppressor inducer T cell(s)
TsiF	T cell suppressor inducer factor
Tsp	Suppressor precursor T cell(s)
TRSA	T cell released suppressor activity
Tμ	T cells with an FcM receptor
Tσ	T cells with an FcG receptor
TT	Tetanus toxoid
VLDL	Very low density lipoproteins

1. GENERAL INTRODUCTION

1.1. Early concepts of the immune system

The concept of immunity is derived primarily from the study of resistance to infection. It was known for centuries before the discovery of the germ theory of infectious disease that recovery from illness was accompanied by the ability to resist reinfection i.e. reappearance of the same disease (Bulloch, 1938; Burnet, 1969; Roitt, 1974). Chinese physicians in the eleventh century observed that the inhalation of smallpox crusts ground into a powdery snuff prevented the subsequent occurrence of smallpox. Later, the technique of variolation, the intradermal application of powdered smallpox scabs, was used in the middle East (Richter, 1982). This primitive form of immunization was brought to England in the eighteenth century by Pylariyni and Timoni and was later popularized by Lady Mary Wortley Montagu. The future of modern immunobiology was assured with the discovery by Edward Jenner in 1798 that the injection of cowpox crusts protected humans from smallpox. Louis Pasteur in 1881 developed standardized killed and attenuated vaccines. The term "vaccine" (from vacca: L, Cow) was coined by Pasteur in honor of Jenner's contribution to the prevention of smallpox. Robert Koch discovered the tubercle bacillus in 1880 and laid down the rules for systematic clinical and pathological investigation (the Koch Principles) while studying the mode of the tubercle bacillus infection. His attempts to develop a vaccine for tuberculosis led to the observation of the phenomenon known today as delayed hypersensitivity.

In 1885, Roux and Yersin isolated a soluble potent exotoxin secreted by Corynebacterium diphtheriae. Von Behring and Kitasato in 1890 innoculated animals with this exotoxin and observed that the sera of these animals possessed a toxin-neutralizing substance which they termed antitoxin. The

capacity to neutralize the toxin could be transferred by the serum to uninoculated animals, a process called passive immunization. This work paved the way to modern immunotherapy. Buchner in 1889 discovered Complement and termed it "alexine". Pfeiffer in 1895 and Bordet in 1899 extended the work on complement and found it to be a serum constituent distinct from antibody which also participates in the killing and elimination of invading bacteria.

The turn of the century was witness to one of the major events in the field of immunology, namely the emergence of two distinct theories governing the mechanism of resistance to infectious microorganisms; (i) the humoral theory which emphasized the protective role of chemical products (antibodies) elaborated by cells, and (ii) the cellular theory which emphasized the protective effects of certain cells in the host. Paul Ehrlich was the main protagonist of the humoral or antibody theory of resistance and Elie Metchnikoff, Ehrlich's major antagonist, proposed the cellular theory of immunity. In his view, the phagocytes or the scavanger cells of the body constituted the primary mechanism for the elimination of the foreign invader. This conflict was resolved by the discovery of opsonins by Sir Almroth Wright in 1904. Wright demonstrated that normal serum contains factors capable of coating the infections microorganisms, thus facilitating their engulfment, phagocytosis and intracellular degradation by the phagocytic cells. The opsonins were later shown to be, in great part, naturally-occurring antibodies present in low concentration in the circulation of normal healthy subjects.

1.2.Contemporary concepts of the immune system

A contemporary definition of immunity would be "all those physiological mechanisms that endow the animal with the capacity to recognize materials as foreign (i.e. different from self) and to mobilize

the appropriate mechanisms to enable it to neutralize, eliminate, or metabolize them with little or no injury to self".

Resistance to infection results from the mutual co-operation of two functionally distinct systems - nonspecific (or innate) immunity and specific (or acquired) immunity. The nonspecific immune system operates continually in the normal individual to provide it with a limited degree of resistance to infection by pathogenic microorganisms. It functions primarily to prevent invasion by the pathogens. This system consists of (i) mechanical barriers (skin and mucosal membranes), (ii) non-specific antibacterial secretions such as sebum, sweat, tears; saliva and genital secretions, (iii) enzymes in secretions (i.e. lysozyme), (iv) the alternate pathway of Complement (C) fixation (C'3, C'5-C'9), (v) phagocytic cells, and (vi) ADCC lysis of the invading organism by FcG bearing lymphocytes (Barret, 1983). The FcG and C'3b receptor-bearing macrophages, monocytes and neutrophils kill microorganisms by phagocytosis and intracellular degradation via the release of lysozymes and other enzymes into the intracellular vacuoles. Phagocytosis takes place most efficiently in the presence of opsonins (Stossel, 1975; Klebanoff and Clark, 1978). Opsonins consist of (i) IgG antibodies present in subthreshold (below the level of detection using conventional immunoassays but detected by radioimmunoassay) concentration and (ii) C'3b which adheres to the antigen (bacteria) following C' fixation via the alternate pathway (Herman et al, 1979, Newman and Johnson, 1979).

The initial step in the phagocytosis of the invading microorganism (bacteria, rickettsia, fungal spores, protozoa) is the interaction of the phagocytic cell, via its receptors, with the IgG antibody and/or C'3b attached to the antigen. The FcG receptor-bearing lymphocytes can also

lyse the invading microorganisms extracellularly following their interaction with the FcG of the opsonizing antibodies (Segal and Hurwitz, 1977). This mechanism by which target cell lysis occurs is referred to as antibody-dependent cell-mediated cytolysis (ADCC). The effector cell is referred to as the killer (K) cell. The ADCC reaction constitutes a very important and effective immune mechanism as it can function in the presence of antibodies which are non-C' fixing irrespective of their concentration or are C' fixing but are present in too low a concentration to bring about complement-mediated lysis of the target (Ziegler and Henney, 1977).

An additional component of the non-specific immune system is the naturally-occurring (C' and antibody-independent) cell-mediated cytotoxic (NOCC) cell. The effector or cytotoxic cell is referred to as the natural killer (NK) cell. The NK cells are lymphocytes or monocytes which may have, but need not be devoid of, the conventional surface membrane receptors for SRBC, FcG, FcM and C'3b. These NK cells are considered to constitute a major defense against malignant and transformed cells referred to as immune surveillance (Quan et al, 1982). Although it does not confer resistance toward infectious organisms, it is nevertheless considered by the discipline to constitute a component of the non-specific immune system (Kaufmann et al, 1981).

The specific or acquired immune system consists of antibodies (humoral immunity or HI) and sensitized lymphocytes (cell mediated immunity or CMI) as well as phagocytic cells and the classical pathway of complement fixation (C'1 to C'9). Although the latter two are "non-specific" and are not induced by the antigen, they function in an accessory capacity to facilitate the elimination of the invading organism (antigen) by antibodies and sensitized cells (Boyle and Boros, 1980; Mayer et al, 1981). Cell-mediated

immunity is not infrequently associated with tissue destruction as occurs in tuberculosis, allograft rejection and autoimmune responses (Waksman, 1971). The effector cells involved in these CMI reactions in vivo are sensitized T lymphocytes which are activated by the sensitizing antigen(s). Subsequent interaction of these actively-sensitized T lymphocytes with the specific antigen results in the release of mediators referred to as lymphokines (Cohen et al, 1979). The relevant lymphokines have been identified as migration inhibitory factor (MIF), macrophage chemotactic factor (MCF), macrophage activating factor (MAF), lymphocyte chemotactic factor (LCF), mitogenic factor (MF), transfer factor (TF), cytotoxic factor (CF) and gamma interferon. These lymphokines are responsible for the tissue damage and lesions (Cohen et al, 1979). The classical or prototype lesion of the CMI reaction is the delayed hypersensitivity skin reaction (DHSR) following the intradermal challenge of a tuberculin-sensitized individual with tuberculin or PPD (Bloom, 1967; Landsteiner and Chase, 1942). The reaction evolves and progresses over a period of 24-72 hours. The induration usually attains maximum intensity by 72 hours post-challenge. Upon contact with the antigen, the sensitized lymphocytes release MIF and MCF which are specific for monocytes. The monocytes which are chemotactically attracted to the challenge site transform, upon interaction with MAF, into "angry" macrophages activated to a high level of phagocytic activity. The number of specifically sensitized lymphocytes may be increased by the action of TF which confers upon a previously uncommitted cell the properties of an actively sensitized cell. The sensitized lymphocytes are induced to proliferate by MF. Both TF and MF may be considered to be amplification factors in the generation of sensitized lymphocytes. The death of the invading organism and injured

cells in the reaction site is attributed to the action of CF (Kops et al, 1984; Van Loveran and Askenase, 1984).

In contrast, humoral immunity is seldom associated with tissue damage. The interaction of precommitted antigen receptor-bearing T lymphocytes (T-ARC) with the ARC-dependent antigen results in the stimulation of previously uncommitted virgin B lymphocytes. These precursors of antibody forming cells (AFC) transform into medium and large lymphoblasts which divide and dedifferentiate into antibody-forming and secreting lymphoblasts and plasmablasts (Ashman, 1982; Richter, 1982). The antibodies interact only with the original immunizing antigen (as compared with the lymphokines which act indiscriminately on cells in the microenvironment). The majority of the antibody-secreting B lymphoblasts and plasmablasts transform into endstage non-antibody synthesizing or secreting plasma cells with a half life of 3-5 days; these cells lose the capacity to secrete the antibodies before they lose the capacity to synthesize them and will therefore invariably contain antibodies in the cytoplasm (Ashman, 1982). A minority of the B lymphoblasts differentiate into small, mature, antigen-specific, long-lived (up to 30-40 years) memory lymphocytes. These memory lymphocytes are capable of being reactivated immediately to antibody synthesis upon re-exposure to the original immunizing antigen (Klinman, 1972; Teale et al, 1981). The requirement of only memory B lymphocytes in the secondary response, as opposed to clonally-selected T-ARC lymphocyte and uncommitted virgin B lymphocytes in the primary response, tends to explain the more explosive nature of the secondary as compared to the primary antibody response.

Antibodies, which may be considered to constitute the mediators of HI, interact with and kill the invaders only following interaction with

and activation of the complement system via the classical pathway (C'1 to C'9). Osmotic lysis of the invader occurs only following activation of C'9 (Boros and Rapp, 1965). The antibodies also neutralize toxins, prevent viral reinfection, and form immune complexes with the extrinsic antigens which are eliminated by phagocytosis and intracellular degradation (Metzger, 1974).

The majority of the naturally-occurring antigens require interaction with T-ARC lymphocytes prior to activation of the B lymphocytes and their transformation, proliferation and dedifferentiation into antibody-secreting lymphoblasts and plasmablasts. These antigens are referred to as T or ARC-dependent antigens. These antigens characteristically consist of a non-immunogenic component referred to as the carrier to which are attached multiple numbers of different antigenic determinants referred to as epitopes (Katz et al, 1970; Paul et al, 1970). A small number of antigens initiate antibody synthesis in the absence of T-ARC cells and they are referred to as T-independent antigens. These antigens have been characterized as large polymeric molecules with repeating identical antigenic determinants. Examples of such antigens are dextran, lipopolysaccharide and polymerized flagellin. The immune response to the T-dependent antigen is initially IgM antibody synthesis followed by IgG antibody synthesis whereas the response to the T-dependent antigen is initially IgM antibody synthesis followed by IgG antibody synthesis whereas the response to the T-independent antigen is primarily if not exclusively an IgM antibody response (Buck et al, 1979, Layton et al, 1981; Pike and Nossel, 1984).

It is generally agreed that two signals are required to initiate B lymphocyte activation by the antigen. In the case of the ARC-dependent

antigens, these signals are provided by the ARC cell and the antigen. In the case of the T-independent antigen, one signal is provided by the antigenic determinant on the antigen while the other is provided by the carrier constituent of the antigen (Pike and Nossal, 1984; Bradley-Mullen, 1982).

The discovery and subsequent characterization of the interleukins, which are soluble factors produced by monocytes/macrophages and T lymphocytes, has led to a better understanding of the mechanisms which sustain the survival and proliferation of the immunocompentent cells. The T cell activating factor or interleukin 1 (IL-1), secreted by the monocyte, stimulates the production by T lymphocytes of a T cell growth factor (TCGF) referred to as Interleukin 2 (IL-2) (Maizel et al, 1981; Mizel, 1982; Gery, 1982; Gillis and Mizel, 1981; Palacios, 1982). T cell proliferation is dependent upon the interaction of IL-2 and IL-2 receptors expressed on the activated T cell (Reska-Kuntz et al, 1984; Lipkowitz et al, 1984). The induction of B lymphocyte proliferation and differentiation involves at least two interleukins produced and secreted by the T lymphocyte in the presence of IL-1. These are referred to as B cell growth factor (BCGF) and B cell differentiation factor (BCDF) (Ford et al, 1981; Howard et al, 1982). The differentiation of activated B lymphocytes in the presence of BCGF induces B lymphocyte proliferation and the synthesis or unmasking of specific receptors for BCDF. The interaction of these receptors with BCDF promotes differentiation of the blasts into antibody-secreting cells. (Korsmeyer et al, 1983; Muraguchi et al, 1984).

Immunoregulatory mechanisms operate to limit the extent of the immune response. Up to the late 1960's the main question addressed was: what cellular mechanisms initiate the immune response? The question today would

appropriately be what terminates the immune response? Gershon (1970) was the first to describe antigen-specific suppressor cells and his findings have been confirmed by numerous investigators. The immunoregulatory mechanism consists of the T helper and T suppressor lymphocytes. The T helper lymphocytes augment or enhance antibody synthesis. They possess surface receptors for FcM and are referred to as T_M or T_μ lymphocytes. Suppression or inhibition of antibody synthesis is effected by the T suppressor lymphocytes. Suppressor cells have cell surface receptors for FcG and are referred to as T_G or T_γ cells (Moretta et al, 1975; Moretta et al, 1976; Moretta et al, 1977). With the emergence of monoclonal antibodies specific for subpopulations of T lymphocytes, the circulating suppressor T and helper T lymphocytes have been identified by their interaction with OKT8 and OKT4 monoclonal antibodies and are referred to as T8 and T4 lymphocytes, respectively (Reinherz et al, 1980; Thomas et al, 1981; Kung and Goldstein, 1980). The suppressor cell mechanism is relevant to humoral as well as cellular immunity. Both responses are terminated by suppressor T cells. A second mechanism operating to limit the HI response is feedback suppression by antibody molecules themselves. The IgM antibodies which appear first following immunization with the antigen stimulate the synthesis of IgG antibodies and the latter, as they increase in concentration, suppress further synthesis of IgM antibodies. In the absence of IgM antibodies, the stimulation to synthesize IgG antibodies is lacking and therefore increased concentration of IgG antibodies will cause feedback inhibition of IgG antibody synthesis (Herzenberg et al, 1981). The stimulating IgM antibodies and the suppressive IgG antibodies may act by stimulating the T_M and T_G lymphocytes, respectively.

At this point in time, it is generally accepted that the immune response is terminated by antigen-specific suppressor cells. The cellular origin of these suppressor cells and the mechanism by which they act to cause this suppression is still speculative and unresolved. The different mechanisms of suppression will be discussed in detail in Chapter 3.

2. RATIONALE, HYPOTHESIS AND OBJECTIVES

As discussed in the Introduction, the thymus has been shown to provide the host with ARC cells necessary for the induction of a primary antibody response to T or ARC-dependent antigens.

Recent investigations by Richter et al (1986), Barron et al (1986) and Berry et al (1986) in the normal adult outbred rabbit in this laboratory have shown that the thymus has a second immunologic function, as an organ in which memory B cells may survive for prolonged periods of time. These memory cells which are not detected in the thymus until 4 to 5 months post-primary intravenous (IV) immunization, can subsequently be reactivated to antibody forming cells (AFC), following reexposure to the specific immunizing antigen in vitro. However, these investigators were consistently unable to demonstrate overt AFC in the thymus following in vivo exposure to the antigen irrespective whether the thymus cells were assessed for AFC (by the PFC assay) following primary or secondary in vivo immunization. This consistent failure to detect AFC within the thymus suggests that (i) the thymus is not conducive for the survival of AFC; (ii) there exists a blood-thymus barrier barring the infiltration of circulating AFC; (iii) the circulating AFC are dying AFC and cannot survive within the lymphoid organs they infiltrate other than the bone marrow; or (iv) there exist suppressor mechanisms within the thymus which mitigate the detection of infiltrating or endogenously (parenchymal)-generated or activated AFC.

The finding of memory cells in the thymus beginning 4 months post-primary intravenous immunization (Richter et al, 1986) suggests that suppressor cells might be present in the thymus during the phase of active antibody synthesis and during the early post-primary immunization period, capable of inhibiting the synthesis of antibodies by the infiltrating AFC.

In accordance with these observations, and in light of past findings, it was felt that the role(s) of the thymus in the primary and secondary immune responses merited renewed investigation in order to elucidate whether or not suppressor cells could be found in the thymus following primary and secondary in vivo immunization.

The HYPOTHESIS which was addressed is that antigen-specific suppressor cells appear in the thymus of the rabbit shortly post-primary immunization and that they are capable of inhibiting the synthesis and/or secretion of antibodies by the AFC.

The OBJECTIVES of this investigation were as follows: (i) to determine whether antigen-specific suppressor cells are detected in the thymus post-primary and post-secondary IV immunization, (ii) to identify and characterize the suppressor cells, (iii) to elucidate the mechanism by which the suppressor cells exert their suppressive effects (i.e. whether they secrete a soluble suppressor factor or suppress via cell-cell contact), and (iv) to define a physiological role for the suppressor cells and the suppressor factor(s) in vivo.

3. HISTORICAL REVIEW

3.1. INTRODUCTION

In order to abide by the regulations of the department of microbiology and immunology, University of Ottawa, concerning the length of a Ph.D. thesis, the scope of the discussion of the historical review will be limited to a discussion of the specific and non-specific suppressor mechanisms governing specific antibody synthesis and non-specific immunoglobulin (Ig) synthesis, respectively. Other topics which could conceivably be subjects for discussion such as the regulatory mechanisms governing the cellular mediated immunity, and the relationship of the suppressor mechanism with immune tolerance, will be omitted for the sake of brevity.

The concept of suppressor cells and/or suppressor factors as the primary down regulators of the immune response did not take hold prior to 1970 and yet it seems rather obvious today. This state of affairs may be attributed to the fact that, for decades, the primary concerns of immunologists revolved around understanding the mechanisms of activation of the immune system and enhancement of the immune response to infectious agents on the assumption that a greater response means greater protection. One may indeed wonder, in amazement, why immunologists and immunology textbooks did not address the matter of down-regulation of the immune response. If one examines the lymph node of an animal immunized subcutaneously within the area of drainage of the node 4 to 6 days previously, one will observe a marked and seemingly unregulated proliferation of cells and greatly enlarged follicles (2 to 4 times their normal size). The lymph nodes undergo marked hypertrophy due to cellular proliferation and not edema. There is also a degree of anaplasia and

pleomorphism which would suggest a malignant process to the wary eye of the pathologist would he not be aware of the circumstances which led to the lymph node hypertrophy. However, if one looks at the lymph node 10 to 15 days following immunization, the microscopic architecture will have returned to normal and there is no sign to suggest the apparently disorderly and chaotic changes that had occurred in the lymph node only 6 to 9 days previously. Since lymphocyte proliferation ceases within 7 to 10 days following immunization and antibody synthesis and secretion ceases within 10 to 15 days following immunization, there must be a mechanism(s) which terminates the proliferative activity and antibody synthesis. Mechanisms must be activated to restore the immune system to homeostasis following its "pathologic" activation by antigenic stimulation. However, it was not until the late 1960's when Gershon and his associates demonstrated the existence of lymphocytes capable of suppressing the immune response, which they referred to as suppressor cells, that investigations of the mechanisms which regulate the immune response were initiated in earnest.

In discussing immunoregulatory mechanisms in 1986 it is important to recognize that there appear to exist two, not one distinct, of these mechanisms. The more obvious and the more dramatic of the two is the one which regulates specific antibody (Ab) synthesis by the B cells following immunization. The second and less apparent of the two is the one which regulates the synthesis of non-specific Ig's by the B cells. This latter mechanism is often not considered by investigators concerned with the suppression of Ab's.

Under normal circumstances the concentration of the circulating Ig's will be maintained at an almost constant level between the advent of

puberty and extreme old age. This constancy of circulating Ig represents the balance between synthesis and degradation of Ig and therefore infers the existence of mechanisms which regulate Ig synthesis by the B lymphocytes. Since there is a wide variation in the concentration of circulating Ig in different individuals (the range is between 1200 mg percent to 2000 mg percent; which takes into account 2 SD from the mean) it is obvious that the two arms of this regulatory mechanism - enhancement of Ig synthesis and suppression of Ig synthesis operate at different levels in different individuals.

The specific immune system is composed of lymphocytes of two distinct lineages, the T and B lymphocytes as well as the obligatory participation of macrophages/monocytes, probably in an accessory capacity. The T and B lymphocytes cannot be distinguished from each other by light microscopy in the non-immune animal. Following immunization, the small mature B lymphocytes undergo activation, transformation, proliferation, and dedifferentiation into the antibody forming cells, the lymphoblasts and plasmablasts, which synthesize and secrete the antibodies. The T cells consist of two classes of cells which appear to act antagonistically with respect to the immune response - the helper (T_H) cells stimulate the B cells to generate antibodies and the suppressor (T_S) cells act to terminate the immune response by mechanisms not yet fully understood. One of these mechanisms involves its interaction with the helper cell, preventing it from further stimulating antibody synthesis by the AFC B cell. A second regulatory mechanism involves the inhibition of the proliferative response of the B cells, thus inhibiting the clonal expansion of these cells.

The specific suppressor mechanism in the normal individual is not

normally detectable since it is in a dormant state. It can only be detected following immunization with antigen. Once activated, the antigen-specific suppressor cells function to down regulate the specific immune response following which they must either enter a dormant state or die since they can no longer be detected in the animal 1-2 months following immunization with the specific antigen. It is important to emphasize that specific suppressor cells only affect the synthesis and secretion of specific antibody directed against the specific immunizing antigen and that they have no effect on Ig synthesis in general.

Although the majority of investigators have identified the suppressor cell as a T cell, one cannot disregard the not insignificant number of reports in the literature which have presented evidence in favor of macrophages and B cells as suppressor cells (these will be discussed in Chapters 3.3. and 3.4., respectively).

In addition to the demonstration of cells capable of suppressing the immune response a large number of investigators have disclosed that the mechanism by which these cells act involves the secretion of soluble suppressor factors which by themselves are capable of suppressing the immune response. As can be anticipated from the above discussion of specific and non-specific suppressor cells, both specific and non-specific suppressor factors have been identified (these will be discussed in chapter 3.5.). Furthermore, it has been shown that antibodies themselves may function to regulate their own synthesis, this feedback inhibitory mechanism will be discussed in Chapter 3.5.9.3.

3.2 T SUPPRESSOR CELLS

3.2.1. Introduction

Prior to the discovery of suppressor T cells by Gershon and co-workers in 1970, little or no concern was given to the fact that suppressor cells are involved in the termination of the immune response.

By far the largest number of investigations concerned with the suppression of the immune response have disclosed that suppressor T cells are involved in the down-regulations and/or the termination of the immune response. These suppressor T cells may be of two main types, one is the antigen-specific T suppressor cell which suppresses the specific immune response toward the immunizing antigen, the other is the antigen-nonspecific T suppressor cell which suppresses the immune response to unrelated antigens in vitro and may in fact represent the regulatory arm of the immune system concerned with the maintenance of a constant amount of circulating Ig, also referred to as homeostasis.

In humans suppressor T cells, both specific and nonspecific, have been described in the circulation, tonsilar and splenic tissue. In the animal most of the reports in the literature have sited suppressor T cells in the spleen, circulation and lymph node. Although much work has been done in the past 15 years characterizing and isolating these suppressor T cells, little or no effort was made to correlate their in vitro action with their role in vivo.

This chapter will introduce the reader to antigen-specific as well as antigen-nonspecific suppressor T cells, the mechanism of their induction and their mechanism of action.

3.2.2. Methods utilized to detect suppressor cells

Suppressor cells suppress the immune response. Therefore, the methods used for their detection measure their ability to suppress an immunological response which would otherwise occur in their absence. Three assays which have been carried out in the vast majority of investigations are: (i) the inhibition of the immune response in vivo following the transfer of suppressor cells induced in vitro or in a syngeneic host, (ii) the inhibition, by suppressor cells, of the immune response generated in vitro by lymphoid cells of an immunized animal, and (iii) the inhibition, by suppressor cells, of the phytomitogen-induced blastogenic response by normal autologous or allogeneic lymphoid cells.

3.2.3. Mechanism of induction of T suppressor cells

3.2.3.1. Introductory statement

A proper understanding of the mechanism of T suppressor cell activation, generation or induction is complicated by the fact that two different, distinct terminologies exist to define suppressor cells. One is defined on the basis of differential rosetting of the T cells with EAG and EAM indicator cells; the other is based upon the reactivity of the T cells with specific monoclonal antibodies directed toward two particular T cell surface constituents referred to as T4 and T8. The (Ortho) antisera are referred to as OKT4 and OKT8, respectively. A second complicating feature is the often repeated failure of investigators to recognize two distinct classes of suppressor cells - one functioning to regulate the synthesis of non-specific immunoglobulins (Ig) and the other responsible for the regulation of the synthesis of specific antibodies.

The evidence up to 1980 strongly indicated that these two suppressor mechanisms are functionally distinct. However, recent evidence suggests that, in fact, the cellular interactions underlying the activation of

suppressor cells for both specific antibody synthesis and non-specific Ig synthesis are similar, if not identical. Therefore, they will be discussed together rather than separately.

To date, little progress has been made in our understanding of the initial reaction which must take place between the antigen and the cell or cells involved in the induction of overt suppressor cells. It is not clear whether only a single cell is involved from the activation to the effector stage or whether multiple cells of identical or distinct lineages must interact sequentially to facilitate the generation of overt suppressor cells. Recent investigations have shed some light as to the cellular interactions leading to the induction of suppressor cells and their identification, as is discussed below.

3.2.3.2. The mechanism of induction of human T suppressor effector cells (overt suppressor cells)

Moretta and his associates (Moretta et al, 1975; Moretta et al, 1976; Moretta et al, 1977; Moretta et al, 1979) investigated the regulatory mechanisms governing Ig synthesis by human B cells stimulated by PWM in vitro. They rosetted circulating T cells with EAG and EAM indicator erythrocytes, and thereby isolated sub-classes of helper and suppressor T cells. These cells were identified by their ability to facilitate and suppress Ig synthesis by autologous B cells in vitro, respectively. The helper T cells rosetted with the EAM indicator erythrocytes whereas the suppressor T cells rosetted with the EAG indicator erythrocytes. These cells were referred to as T_M or T_μ cells and T_G or T_γ cells, respectively, in view of the fact that the T_M cells possess surface membrane receptors for the Fc region of IgM (the μ heavy chain) whereas the T_G cells possess surface membrane receptors for the Fc region of IgG (the γ heavy chain). Moretta et al (1977) claimed that the T_G cells were activated into

functional suppressor cells as a result of their interaction with the FcG on the EAG indicator cells, that is, as a result of their rosetting with the EAG cells (Moretta et al, 1977, 1979).

Ballieux and his associates investigated the regulatory mechanisms governing the synthesis of specific antibodies by human B cells in vitro. They demonstrated that the helper and suppressor cells were the T_M and T_G cells, respectively. The T_G cells had to be preincubated with the antigen in order to be activated to transform into suppressor effector cells. These investigators also demonstrated the existence of a cell within the $T_M(T_\mu)$ helper cell population which could activate the $T_G(T_\gamma)$ suppressor effector cell following their incubation with the antigen. These cells were referred to as suppressor inducer T_M cells (Ballieux et al, 1982) in contradistinction to the helper inducer T_M cell (Ballieux et al, 1982). It was demonstrated that the suppressor inducer T cells (T_{si}) act on the suppressor precursor T cell (T_{sp}) which then transform into the T suppressor effector cells (T_{se}). The T_{sp} cells were shown to be autologous rosetting (ar^+) cells; they were capable of rosetting with autologous erythrocytes. However, the T_{si} and T_{se} cells were shown to be incapable of rosetting with autologous erythrocytes (i.e. they are ar^- cells). On the basis of differential rosetting and staining with specific monoclonal antibodies by the immunofluorescent technique, the T_{si}, T_{sp} and T_{se} cells were identified as $T_{\mu\ \gamma}^{+\ -}\ 4^+\ 8^-\ ar^-$, $T_{\mu\ \gamma}^{-\ -}\ 4^-\ 8^+\ ar^+$, and $T_{\mu\ \gamma}^{-\ +}\ 4^-\ 8^+\ ar^-$, respectively. Similar findings were reported by Thomas et al (1981) and Davidsen and Kristensen (1986). These results have since been confirmed in our laboratory by Richter and Jodouin (to be submitted for publication).

The suppressor inducer cell may interact with the suppressor precursor cell to induce it to transform into a suppressor effector cell by (i) cell-

cell contact, which entails cell surface recognition sites or receptors (as discussed above), or (ii) secreting a soluble suppressor factor which conveys the message "go" to the suppressor precursor cell to transform into the suppressor effector cell. At this point in time both mechanisms are considered to be equally credible. The latter mechanism is supported by the work of Lau et al (1984, 1985). They described a suppressor activating factor (SAF) secreted by a human T cell line (CEM cell) in culture, which activates the T suppressor effector cells to secrete a potent suppressor factor (T cell released suppressor activity or TRSA). The SAF was only slightly suppressive by itself. On the other hand, the TRSA was 100 to 500 times more suppressive than the SAF.

In 1980, Reinherz, Schlossman and their colleagues (Reinherz and Schlossman, 1980 ; Reinherz et al, 1980) introduced the use of monoclonal antibodies (mAb) for the separation of functional T cell sub-populations which govern the synthesis of Ig by B cells in culture stimulated by PWM. They utilized two mouse monoclonal antisera, referred to as OKT4 and OKT8, directed towards specific surface configurations present on normal circulating human T cells. The T cells which react with the OKT4 mAb were referred to as T4 cells and were demonstrated to be T helper cells, whereas the T cells which reacted with the OKT8 mAb were referred to as T8 cells and were demonstrated to be T suppressor cells.

The relationship between the T_M helper and T_G suppressor cells and the T4 helper cells and T8 suppressor cells remained a matter of contention and controversy until 1982 when it was resolved by Heijnen, Ballieux and their colleagues (Heijnen et al, 1982a; Heijnen and Ballieux, 1983). These investigators demonstrated that the overt T helper cell is a $T_{G\ M}^{-\ +\ -}8$ T cell whereas T suppressor effector cell is a $T_{M\ G}^{-\ +\ -\ +}4\ 8$T cell.

Most recently, Morimoto et al (1985a,b) have succeeded in raising monoclonal antibodies in immune mouse spleen MNC hybridoma cultures capable of selectively lysing the human T4 helper inducer and the T4 suppressor inducer cells in the presence of complement. One antiserum, referred to as anti-4B4, is capable of lysing the T4 helper inducer cells whereas the second antiserum, referred to as anti-2H4, is capable of lysing the T4 suppressor inducer cells. Thus, by using these two antisera it is possible to separate the helper cells from the suppressor inducer cells, both of which comprise the T_M helper cells.

3.2.3.3. Mechanism of induction of T suppressor cells in the experimental animal

3.2.3.3.1. Introductory statement

To date, the only experimental animal in which immunoregulatory mechanisms have been extensively investigated is the mouse. Two major facts about T lymphocytes have become apparent over the past 15 years: the first, as demonstrated by the early work of Claman and Chaperon (Claman and Chaperon, 1966), Davies (Davies, 1969) and Miller and Mitchell (Miller and Mitchell, 1969), is the helper function carried out by a cell with a cell surface phenotype Lyl^+, and by Gershon (Gershon, 1974) the suppressor function carried out by a cell with a cell surface phenotype $Ly2^+$ of the T lymphocyte pool which participates in the humoral immune response; the second, as initially described by Cantor, Boyse and associates (Cantor and Boyse, 1977; Cantor and Weissman, 1976; Cantor and Gershon, 1979; and Cantor et al, 1978), is that the circulating T cell pool is very heterogeneous consisting of a large number of subsets, each with distinct differentiation surface membrane markers and immunologic function.

Investigations of the regulatory phenomenon of specific immune suppression (Araneo et al, 1979; Eardley et al, 1978; Feldmann et al, 1977;

Germain et al, 1978; McDougal et al, 1979; Perry et al, 1978; Sy et al, 1980; Tada and Okumura, 1980; Waltenbaugh et al, 1977; and Weinberger et al, 1980) have disclosed the importance of multiple interactions among these T cell subsets in the induction of overt antigen-specific suppressor cells. Several distinct model systems of the different interactions between the various T cell subsets and soluble suppressor factors (soluble suppressor factors will be discussed in Chapter 3.5.) secreted by these different T cell phenotypes involving both humoral (Cantor et al, 1978; Eardley et al, 1978, 1979a,b; Waltenbaugh et al, 1977) and cell-mediated immunity (Germain et al, 1978; Perry et al, 1978; Greene et al, 1979) have emerged (This thesis is concerned only with the suppression of the humoral immune response and no further reference will be made to the induction of suppressor cells in the CMI response).

These data raise the important question as to whether there are many different pathways of T cell-mediated suppression, or whether the described pathways, in the different experimental systems, are fragments of a large single sequence. Recent findings have suggested the possibility of integrating the great majority of the results into a single overall scheme of cellular interaction leading to the induction of overt suppressor T cells in the mouse.

It is not possible to include in this analysis all the reports on T-cell mediated suppression in the mouse. Rather, I will concentrate on the three major models, for the induction of T suppressor cells, for which a large body of data is available. For greater detail, see reviews in references (Benacerraf et al 1977; Cantor and Gershon, 1979; Claman et al, 1980; Germain, 1980; Germain and Benacerraf, 1980; Greene and Benacerraf, 1980; Pierce and Kapp, 1976; and Tada and Okumura, 1980).

3.2.3.3.2. The mechanism of induction of T suppressor effector cells in the mouse (overt suppressor cells)

A suppressor pathway that is activated concomitantly with normal immunization has been described by Eardley, Gershon, Cantor and associates. Exposure of purified splenic T cells, with a surface marker Lyl^+ (helper cells) to SRBC in vitro activates an inducer suppressor T cell, within this helper cell population, with a cell surface phenotype Lyl^+, $I\text{-}J^+$, Qal^+ (I-J and Qal being surface configuration common to T suppressor inducer/precursor cells) (Cantor et al, 1978; Eardley et al, 1978, 1979a,b; Gershon, 1974). This cell in turn acts, via a factor secreted by the activated cell (Yamauchi et al, 1981), on a non-primed suppressor precursor T cell of the phenotype Lyl^+, 2^+, 3^+, $I\text{-}J^+$, Qal^+ which transforms into or activates a suppressor effector T cell with an Lyl^+, 3^+ phenotype, which can be detected only in its (suppressor precursor) mature state, and which serves as the actual suppressor effector cell. This suppressor effector T cell acts in an MHC-restricted manner to inhibit Lyl^+ helper cell activity.

The second pathway for the induction of suppressor T cells in the mouse is one of the best studied, and earliest described, and involves the generation of specific suppressor T cells in responder mice by the administration of large antigen doses over an extended (4-week) period (Okumura et al, 1977; Tada, 1977; Tada et al, 1976; Tada et al, 1979; Tada et al, 1975; Takemori and Tada, 1975; Taniguchi et al, 1976a,b; Taniguchi and Tokuhisa, 1980). The T suppressor cells recovered from such treated animals in vivo are $Ly2^+$, $I\text{-}J^+$ which bind to the specific antigen used for their induction. This cell, the suppressor inducer, acts on an antigen-primed, Lyl^+, 2^+, 3^+, $I\text{-}J^+$, radiation-sensitive, T cell via the secretion of an antigen-specific soluble suppressor factor,

to permit the production of an activated $Ly2^+$, 3^+, $I\text{-}J^+$ phenotype, suppressor effector T cell (Tada, 1977; Tada et al, 1979; Taniguchi and Tokuhisa, 1980). The authors suggested that this step may involve two distinct cells, rather than a change in the surface phenotype of the $Ly1^+$, 2^+, 3^+ cell. The suppressor effector cell ($Ly2^+$, 3^+, $I\text{-}J^+$) once induced secretes a suppressor molecule that acts in an MHC non-restricted manner to inhibit $Ly1^+$ helper T cell function.

The third model for the induction of suppressor T cells involves the suppression of the humoral responses to synthetic polypeptide antigens, L-glutamic acid^{60}-L-alanine^{30}-L-tyrosine^{10} (or GAT) and L-glutamic acid^{50}-L-tyrosine^{50} (or GT). These systems have been studied by Germain and Benacerraf and colleagues (Germain and Benacerraf, 1978; Germain et al, 1978a,b, 1979, 1980) and by Kapp and Pierce and associates (Kapp, 1978; Kapp et al, 1974, 1976, 1977; Théze et al, 1977a,b; Waltenbaugh et al, 1977a,b; Araneo and Kapp, 1980; Turck et al, 1985; Sorensen and Pierce, 1986). Immunization of nonresponder mice with GAT results in the activation of an antigen-specific T suppressor inducer cell, or T_{S1} cell (Kapp et al, 1974). The Ly phenotype of these cells has not been determined in the nonresponder mice but shown to be $Ly2^-$, $I\text{-}J^+$ in (responder X responder) F_1 mice (Lake et al, 1986). This suppressor inducer cell once activated secretes a suppressor factor, GAT-T_SF_1 (Kapp et al, 1976) which is antigen-specific. Its function is the induction, together with the antigen, of a second T suppressor (T_S) cell T_{S2}, from resting T cells (Germain and Benacerraf, 1978; Germain et al, 1978b). The precursor of the T_{S2} is a $Ly1^+$, 2^+, 3^+ cell and its activation by the GAT-T_SF_1 yields an activated $Ly2^+$, 3^+ which acts as the suppressor effector T cell (T_{S3}) in nonresponder mice (Lopez et al, 1986) and has the $Ly2^+$

phenotype in the (responder X responder) F_1 mice (Lake et al, 1986), both of which act via the secretion of a nonspecific mediator on Lyl^+ T cells as well as B cells.

GT-specific suppression differs from GAT suppression mainly in the existence of non-suppressor nonresponder strains in mice and the effect of two gene products which control the ability of these mice to affect suppression to this antigen (Debre et al, 1975a,b. 1976; Germain et al, 1978; Théze et al, 1977a; Waltenbough et al, 1977a,b; Kapp et al, 1983). These genes appear, on the one hand, to affect the ability of T_{S1} to be activated and produce GT-T_SF_1 and, on the other, to permit T_SF_1 to evoke actual suppression via T_{S2} induction (Germain et al, 1980). Both GAT and GT-induce suppressor effector cells and act on T or B cells in the presence of MØs (Araneo and Kapp, 1980; Lopez et al, 1986).

A sufficient number of similarities exist among the systems described to attempt a synthesis of this data. It is possible to suggest a single major pathway of T suppressor cell activation which incorporates the key features of each of the described models. (i) The initiation of the pathway by an Lyl^+, antigen specific T cell, which intern produces a T_SF (antigen-specific) that acts in an MHC non-restricted manner. (ii) In a second stage, the triggering of T_{S2} whose T_SF_2 acts in an MHC restricted manner to (iii) activate an antigen primed T_{S3} which acts as the suppressor effector cell. It should be noted that either T_{S2} themselves, or the T_{S3} cells, may act, not only via the production of a nonspecific mediator, but directly on a target cell via cell surface receptors or antigen-bridging mechanisms.

It is clear, however, that several major issues remain unresolved at present; (i) How does the antigen trigger the T_{S1} cell? (ii) What

determines whether T_{S2} are of the antigen-specific (antigen-primed) or normal resting T cell type? (iii) How are $T_S F$ signals transmitted? and (iv) How does the actual suppression of the target cell occur? These and other questions concerning the induction of T_S cells should be answered in the near future.

3.2.4. The mechanism of action of T suppressor effector cells

3.2.4.1. Introductory statement

The mechanism of action of suppressor cells, that is the manner whereby suppressor cells inhibit, abort or down regulate the immune response is not well understood at the present time. Obviously, there are limited possibilities by which suppressor cells can exert their effect on the immune system. These include: (i) direct interaction of the suppressor cells with the antibody forming cells, inhibiting their proliferation and/or synthesis and secretion of antibodies, (ii) the direct interaction of the suppressor cell with the helper cell, resulting in neutralization or inhibition of helper cell activity, leading to cessation of antibody synthesis by the B cells, or (iii) secretion of a suppressor factor by the suppressor cell, which may then act on either the helper cell or the B cell, or both, resulting in the termination of antibody synthesis and the immune response.

3.2.4.2. The mechanism of action of T suppressor effector cells in humans and animals

Evidence to date supports postulated mechanisms (ii) and (iii) listed above. Moretta and his associates (Moretta et al, 1979) demonstrated that the human suppressor T cell must interact with the helper T cell and not the B cell in order to suppress Ig synthesis by the B cell in vitro. This effect was demonstrated to be mediated by a suppressor factor secreted by the suppressor cells (suppressor factors will be discussed in Chapter 3.5).

The investigations by Ballieux and colleagues (Uytdehaag et al, 1979a; Ballieux and Heijnen, 1983; Heijnen et al, 1981; Uytdehaag et al, 1981; Heijnen et al, 1979) support the results of Moretta et al (1979). Using an antigen-specific human cell-culture system, they demonstrated that suppression of the specific immune response in vitro is not the result of the interaction of suppressor T cells with the antibody forming cell or the helper cell, but is probably due to the actions of an antigen-specific suppressor factor secreted by the suppressor effector cell (discussed in Chapter 3.5.10.4.).

Smith et al (1984) showed that the plaque-forming cell (PFC) response to SRBC is suppressed when Con A is administered IV on several occasions to mice within a 24 hour period prior to or immediately after immunization. The magnitude and the duration of the suppression depended upon the concentration of the antigen used for immunization. Repeated immunization with the antigen abrogated the suppression induced by Con A if Con A was injected twice (4 and 24 hours) after primary immunization. Although, profound suppression of the anti-SRBC PFC response was observed in mice pretreated with repeated Con A injections (4 to 24 hours) prior to immunization with the antigen (SRBC), spleen cells from these mice did not exhibit suppressive activity when transferred into normal immunized syngeneic recipients or when co-transferred with normal syngeneic spleen cells into irradiated immunized recipients. The authors suggest the need for cell-cell contact or cellular co-inhabitation for suppression to occur. However, it is also possible that no suppressor cells were generated by Con A in vitro. Rather, Con A disrupted the architecture in the lymphoid organs and destroyed lymphoid cells, resulting in the abolition of the immune response.

Moller and associates (Ektedt et al, 1977) demonstrated that mouse spleen cells activated by Con A in vivo inhibited the anti-SRBC (T-dependent) response of normal syngeneic spleen cells in cultures, but not to a T-independent antigen (3,5-dinitro-4-hydroxy-phenacetyl-conjugated lipopolysaccharide) nor did they inhibit the activation of normal cells by phytomitogen (PWM) a polyclonal T-and B-cell activator. The suppression occurred only if the Con A-activated suppressor cells were added to the cultures at the initiation of the cultures. The addition of these suppressor cells 24 hours after the initiation of the culture did not cause suppression of the anti-SRBC response. This suggests that the Con A-induced suppressor cells do not act directly on B cells but rather on helper cells (T cells or macrophages) at a very early stage of the immune response to T-dependent antigens. In a recent communication (Moller, 1985), in vitro Con A-activated spleen cells were shown to be suppressor cells when co-cultured with normal syngeneic spleen cells and antigen (SRBC), but were found to act as helper cells in vivo in the anti-SRBC response when transferred to syngeneic untreated immunized mice. These cells were true helper cells as they enabled nude mice to respond to SRBC following their IV injection into the mice. This suggests that Con A activates both suppressor and helper T cells in vitro, as was reported earlier by Dutton and colleagues (Scavulli and Dutton, 1975), and that suppressor T-cells act on helper T cells in in vitro cultures to bring about the termination of the immune response. However, in vivo the Con A-activated helper cells gain the upper hand, be it due to their better survival in the injected syngeneic immunized animal or due to the fact that the helper cells outnumber the injected suppressor cells due to the presence of host helper as well as the injected helper cells.

Positively selected T8 human suppressor cells require much less IFN for activation in vitro and are more effective suppressors in smaller numbers than unfractionated IFN_{α}-activated circulating MNC (Schnaper et al, 1983). These cells suppressed the blastogenic response by normal autologous circulating MNC in the PHA-driven blastogenic response. The authors suggested that the suppression was due to absorption of IL-2 by the suppressor cells thus depriving the responder T cells of the IL-2 required for a blastogenic response.

Papermaster et al (1983) have reported that human circulating MNC cultured for 4-6 days in the presence of Con A and FCS suppress the production of human IFN by allogeneic human circulating MNC in culture in response to staphyloccal extoxin A. This suppression could be abrogated by the addition of IL-2. These findings suggest that the suppressor cells inhibit IFN production by sequestering IL-2 that is required for IFN production. Similar findings regarding the ability of suppressor cells to absorb IL-2 (TCGF) were made by Palacios and Moller (1981) and Moller (1985).

Lomnitzer et al (1984) have studied the suppressive activity of Con A-induced human circulating suppressor cells by comparing H^3-thymidine incorporation by autologous or allogeneic responder MNC alone and co-cultures of the Con A-pretreated MNC and the responder cells. The Con A treated MNC suppressed H^3-thymidine incorporation by the responder MNC. The authors attributed this suppression to inhibition of IL-2 production by the responder cells. Similar findings with ovine circulating and lymph node MNC were reported by Ellis and Demartini (1985). These cells suppressed the phytomitogen (Con A, PHA and PWM)-induced proliferative response of untreated autologous or allogeneic responder

cells by 50 to 70 percent as compared to controls. The addition of IL-2 early in the culture period abrogated the suppression by the Con A-activated suppressor cells. These Con A-induced suppressor cells were found to absorb IL-2 from the medium, thereby decreasing the availability of IL-2 to the responder cells. Here again, suppressor T cells seem to act on, or indirectly affect the activity of, helper T cells rather than acting directly on the antibody forming B cells.

In the past decade, many investigators have reported the secretion of antigen-specific (Heijnen et al, 1982; Utdehaag et al, 1981; Ballieux and Heijnen, 1982; Uytdehaag et al, 1979; and Heijnen et al, 1982) as well as antigen nonspecific (Rich and Pierce, 1974; Tadakuma and Pierce, 1978; Aune and Pierce, 1982; and Irons et al, 1984) suppressor factors by suppressor T cells, both in vitro and in vivo, which can suppress the immune response to both autologous and heterologous antigens in vivo and in vitro. The targets of these factors in vitro include T helper (Heijnen, Ballieux and colleagues) cells, B cells, and Fc of IgG (Fridman et al, 1981; Lowy et al, 1983; Fridman et al, 1984). Chapter 3.5. will be devoted to the different suppressor factors and their origin, characteristics and mode of action.

In the light of the data presented above, it would appear that the T suppressor effector cells exert their activity with respect to both nonspecific Ig synthesis and specific Ab synthesis by the B cells in an analogous manner. The terminal event involves the secretion of a soluble suppressor factor which will be discussed in Chapter 3.5.10.

3.3. MACROPHAGE SUPPRESSOR CELLS

3.3.1. Introductory statement

It has been a century since Elie Metchnikoff noted that during the inflammatory response leucocytes engulf microorganisms by phagocytosis, he coined the term macrophage to denote these cells. There are two main types of phagocytic cells: the polymorphonuclear leucocytes, which are circulating cells and which can migrate from the blood vessels into sites of inflammation; and the mononuclear phagocytes, which can be found in the circulation as well as fixed in tissues, the latter can also accumulate in inflammation sites. Both are capable of phagocytosis and pinocytosis. However, mononuclear phagocytes show a greater diversity in function and response. This diversity is attributed to the progressive maturation of these cells from the bone marrow precursors, and their experiences with endocytosis and the interaction with T lymphocytes.

The role of macrophages as secretory cells is important in their interaction with the extracellular milieu including their interactions with B and T lymphocytes. When macrophage activation follows interaction with lymphocytes, lymphokines, immunoglobulins or complement this is most likely to be mediated by binding to specific receptor molecules.

Many studies have been conducted on macrophages from various organs, including; blood, peritoneal excudate, and spleen - implicating them in the suppression of the immune response. One of the more comprehensive studies was conducted on alveolar macrophages.

The fluid layer lining epithelial surfaces in the respiratory tract contains substantial numbers of immune cells. The capacity of alveolar macrophages (AM) to support T and B cell activation in response to antigenic and polyclonal stimulation has been studied in in vitro models.

3.3.2. Macrophage suppressor cells in the human

Reports on human AM function in vitro have been relatively consistent. With the exception of two earlier studies (Daniele et al, 1977; and Laughter et al, 1977), more recent publications on their activity in lymphocyte cultures indicate that human AM exhibit reduced accessory cell function(s) relative to macrophages from other sources. This disparity has been attributed by several authors to the use of indicator lymphocyte populations in the original studies which were not adequately depleted of endogenous accessory cells (Schuyler and Todd, 1981; Ettenson and Roberts, 1983). The main features of recent findings are as follows: (a) while small numbers of human AM are capable of partially reconstituting pokeweed mitogen (PWM)-induced immunoglobulins (Ig) secretion when added to cultures of adherent-cell depleted lymphocytes, (b) higher numbers are suppressive (McMannon et al, 1984; Lawrence et al, 1982).

Human AM appear capable of reconstituting polyclonal T cell phytomitogen responses of autologous T cells (Yeager et al, 1982; De Shazo et al, 1983; Toews et al, 1984) but are less effective than blood monocytes in parallel tests (Ettensohn and Roberts, 1983; Toews et al, 1984), moreover, they become suppressive in the same cultures if they are present above threshold numbers (Yeager et al, 1982; De Shazo et al, 1983; Ettensohn and Roberts, 1983).

The model of AM downregulation of the immune response predicts that alteration of AM population, resulting in enhanced accessory cell activity comparable to that found in circulating monocytes, would predispose the individual toward disease(s) associated with immunological hypersensitivity in the lung tissue. In this regard it has been suggested that AM from allergic asthmatic patients show decreased suppressor activity

(Aubas et al, 1984). There is even more compelling evidence for alteration of AM population in patients with pulmonary sarcoidosis. Enhanced cellular immune reactivity in the lower respiratory tracts of suffers (Crystal et al, 1984) which is accompanied by increased IL-1 secretion by their AM as compared with AM from normal individuals (Hunninghake, 1984; Brambilla et al, 1986) and is concomitant with increase in antigen presenting capacity (Venet et al, 1985) and phagocytosis (Grant et al, 1986). The analysis of the distribution of surface markers on AM from sarcoid patients indicates the presence of large numbers of monocytes recently recruited from the peripheral blood (Hance et al, 1985). It has also been recognized that a proportion of AM migrate into the interstitium, and eventually reach the draining lymph nodes where they may further exert their suppressive action (Corry et al, 1984).

3.3.3. Macrophage suppressor cells in the experimental animal

Unequivocal evidence of active suppression of polyclonal T and B cell activation (Holt, 1978, 1979; Holt et al, 1981, Shellito and Kallreider, 1984), of failure to present protein antigens to T cells (Holt and Batty, 1980) and of suppression of antigen-driven T cell proliferation (Holt et al, 1981) have been consistently reported in studies on rat AM. The suppressive activity of rat AM was noted at AM/T cell ratios as low as 1:100 (Holt, 1979a).

Canine AM appear similar to those of rats, and actively suppress polyclonally-induced T and B cell proliferation in vitro (Ansfield et al, 1979). A single study on monkeys concluded that phytohaemagglutin-reactive cells could not be demonstrated in endobronchial lavage in vitro unless AM were first removed from these cells collected by that method (Kazmierowski et al, 1976), suggesting that monkey AM can suppress this response.

The activity of rabbit AM in vitro is related to their relative density in culture. At low AM/T cell ratios, they serve as accessory cells for mitogen (Con A)-induced T cell proliferation and support both antigen (SRBC)-induced plaque forming cell responses and lymphocyte proliferation in vitro. However, in increasing numbers and ratios approaching 1:1 they perform increasingly poorly in the capacities mentioned above and may become suppressive (Pennline et al, 1979; Pennline and Herscowitz. 1981; Schuyler and Todd, 1981).

Mouse AM appear permissive to in vitro T cell proliferation when tested immediately after removal from donors (Holt, 1980), they become suppressive following culture in vitro for 24 hours (Sestini et al, 1984).

Cell mixing experiments performed in several laboratories have reported the phenonemon of active suppression by AM of autologous T cells from man, rat, rabbit and dog (Holt et al, 1981; Ettensohn and Roberts, 1983; Toews et al, 1984). T cell activation driven by specific antigen appears to be more sensitive to this form of suppression than that driven by polyclonal mitogens, and appears to be more sensitive than B cell activation (McCombs et al, 1982). It has also been suggested that AM suppression may vary between T cell subpopulations (Warner et al, 1981; McCombs et al, 1982) and does not show MHC restriction (Holt, 1979b).

The notion that AM downregulate immune processes in the lower lung by suppressing T cell function was first advanced by Mackaness (1971), as an explanation for the delayed kinetics of expression of anti-listerial immunity in the lungs of reinfected mice. The in vitro evidence discussed above is consistent with the operation of such a control mechanism and the wide range of lymphocyte activities shown to be susceptible to AM signals suggests that these cells may potentially regulate and inhibit T cell

responses to irrelevant environmental antigens in the lower lung which are continuously deposited on the alveolar surface. A less substantial, but significant in vivo supporting evidence is also available. The induction of an inflammatory response in the lungs of rats which triggers the influx of blood monocytes into the alveoli, has been shown to abrogate the lymphocytostatic activity of the AM population in treated animals (Holt, 1978) and the use of identical agents in the rabbit overcomes barriers which normally limit the development of cell mediated immunity to inhaled antigen (Peterson et al, 1977).

It should be noted that the regulatory activity of AM does not appear restricted to lymphocytes, and apparently extends to regulate effector functions of natural killer (NK) cells (Robinson et al, 1984; Farrel et al, 1985) and possibly neutrophils (Pennington et al, 1983). The underlying mechanism(s) by which AM mediate suppression have not been completely defined, but prostaglandin secretion by AM is indicated to play a significant role (Farrel et al, 1985).

Nonspecific suppression by macrophages of the immune response to various antigenic stimulations is not by any means the exclusive domain of AM. Other macrophages, both circulating and tissue fixed macrophages could be induced in vitro to become suppressor macrophages by various agents; BCG-induces suppression by splenic macrophage in C57Bl/6 (B6) mice (Schrier et al, 1980), induction of suppressor splenic macrophages by Fusarenon-X (a mycotoxin) in BALB/mice (Masuda et al, 1982) and macrophages suppression of anti-sheep erythrocyte (SRBC) and anti-Toxoplasma antibody responses after in vivo priming with Toxoplasma (Susuki and Kobayashi, 1983). Yoshikai et al (1983) and Kettman et al (1986) described nonspecific suppression by peritoneal

exudate macrophages from Corynebacterium parvum primed mice, that were able to suppress antibody formation to SRBC in vitro as well as in vivo upon transfer to syngeneic mice. Nakano and Nakano (1985) reported macrophage-like cell nonspecific suppression of contact sensitivity induced by intravenous injection of oxazolone-conjugated syngeneic spleen cells. Rat splenic MNC precultured in vitro for 5 days, in the absence of any exogenous stimulation, exhibited marked suppressive activity on the secondary cytotoxic T lymphocyte (CTL) response. The suppressor activity was produced by macrophages rather than by lymphocytes (Veit, 1982). Katayama et al (1982) reported in vivo suppression by macrophages of the delayed hypersensitivity to dinitrophenyl bovine gamma globulin (DNP_{50}-BGG) in the guinea-pig. Macrophages as well as T cells were shown to suppress the in vitro induced human autologous mixed lymphocyte reaction (Smolen et al, 1981; Fernandez and Macsween, 1981). Jadus and Peck (1986) reported the suppression of the immune responses both in vitro and in vivo to T-dependent and T-independent antigens by immature monocytes obtained from spleens of newborn mice.

Macrophages act in many ways to augment the immune response: they process and present antigen to T-lymphocytes, they interact with antibody via FcG receptors to eliminate foreign invaders, they get out of the circulation into areas in the tissue where they may encounter sensitized lymphocytes in the cell mediated arm of the immune response, enhance the inflammatory response and mop up the debris of cellular "war". Macrophages suppress the immune response as seen with alveolar macrophages which function poorly as accessory cells for T cell activation or which have a diminished capacity to secrete IL-1. Alternatively, macrophages suppress the proliferation of lymphocytes through complement cleavage

products and prostaglanding E, in a nonspecific manner. Macrophages suppress the immune response via the release of mediators influencing the activation of other lymphocytes such as T and B lymphocytes thus influencing the outcome of the immune response in an indirect manner. Macrophages may also be active participants in the induction of suppression in a more direct fashion by secreting suppressor factors which regulate the humoral and cell mediated immune responses. These factors will be discussed in Chapter 3.5.8.

3.4. B SUPPRESSOR CELLS

3.4.1. Introductory statement

The earliest site of development for blood cell precursors is the para-aortic mesenchyme of the embryo. These embryonic stem cells migrate into the yolk sac, where they form blood islands. Pluripotent hemopoietic stem cells give rise to B cells and to all other blood cell types (Phillips et al, 1977).

In mammals B cells are produced in hemopoetic tissues. This process begins in the fetal liver and later shifts to the bone marrow when it becomes the primary blood-forming tissue (Osmond, 1980; Cooper, 1981).

In birds B cells are generated from stem cell precursors which migrate through the blood vessels into the avian bursa of Fabricius (Moore and Owen, 1967). Embryonic bursectomy, or bursectomy at hatching combined with destruction of B cells seeded earlier, completely aborts B cell development and the production of antibodies (Cooper et al, 1980).

By far the most important B cell program along the life span of a B cell, is its ability to synthesize and secrete antibodies of a myriad variety in a complex system of antigen B cell interaction. This interaction may be a direct interaction of antigen and B cell in the case of T-independent antigens such as the repeating units of lipopolysaccharides (LPS) of some bacterial outer membrane antigens, or an indirect interaction of B cells and antigen via the mediation of macrophages and T-ARC cells in the presentation to and stimulation of virgin B cells to become committed to specific antibody synthesis and secretion in the presence of T helper derived B cell augmenting factors in the case of protein or T-dependent antigens.

B cells may act as suppressor cells by synthesizing and secreting anti-idiotype antibodies. These will combine with high afinity to the antibody produced against the antigen and so will neutralize its activity. By doing so these "alternative" B cells are causing the suppression of the immune response to the particular immunizing antigen.

3.4.2. B suppressor cells in the human

Singhal and his associates (Singhal and Duwe, 1976; Singhal et al, 1978; Bains et al, 1982) have described a human bone marrow derived B suppressor cell capable of suppressing the primary PFC response of normal allogeneic spleen or tonsillar MNC and autologous circulating MNC in vitro. Calkins (1982) has shown that plasma cells (lymphoblasts), which could be demonstrated to secrete antibody, were able to activate a suppressor T cell in vitro, in cell populations which have not previously been exposed to the antigen. Suzuki et al (1985), have identified an FcR-bearing human circulating B cell which was able to suppress the polyclonal Ig production by B cells in response to pokeweed mitogen stimulation in vitro. Gilbert and Hoffman (1982) have reported on a lymphokine-induced, IL-1 and T-cell replacing factor or TRF, human suppressor B cell in vitro only if both were added early to the culture. This B suppressor cell suppressed the phytomitogen-induced blastogenic response of allogenic human circulating MNC in vitro.

Human bone marrow MNC stimulated in vitro with PWM and cultured for 7 days, produced a B suppressor cell capable of suppressing Ig synthesis and secretion by normal allogeneic human circulating MNC in vitro (Alley et al, 1983). Fraks et al (1986) have described a PHA-induced human suppressor B cell capable of inhibiting the phytomitogen (Con A, PHA)-induced blastogenic response as well as DNA synthesis by allogeneic human

circulating MNC in vitro.

3.4.3. B suppressor cells in the experimental animal

Symons and Clarkson (1983) have reported the suppression of Ig secretion by pig lymphoblasts with anti-Ig derived from pig circulating B cells in vitro. Naysmith et al (1979) have reported that the regulation of anti-erythrocyte auto-antibody production in mice was via the induction of a suppressor T cell. Yet non-T cell population had similar effect in these mice, they postulated a B cell regulation acting by an antibody-feedback inhibition mechanism. Asherson et al (1980) have reported on a B suppressor cell which seems to regulate the suppression of the efferent stage of contact sensitivity by blocking the production or action of the T-suppressor cell. Shimamura et al (1984) have demonstrated in the mouse a Lyt-1$^-$ B cell which acts as a suppressor inducer cell. These cells were found to induce feedback suppression of the immune response in vivo. Earlier studies by Shimamura et al (1982a) have revealed a splenic B cell which activated an antigen specific T cell and that this B dependent induction of suppression was different from that of antibody-mediated suppression. Further more, the induction of the suppressor-inducer B lymphocyte was dependent on endogenous synthesis of prostaglandins (Shimamura et al, 1982b). McGarry and Singhal (1982) have reported that the in vitro primary antibody responses of spleen cells, in the mouse, can be suppressed in a dose-dependent manner by the addition of bone marrow cells. These suppressor cells were not affected by treatment with anti-Thy-1, anti-Lyt or anti-IJ antisera and complement. Howles and Cox (1983) reported on the induction of B suppressor cells that suppressed the production of autoantibodies against erythrocytes by mice injected with rat erythrocytes, but did not inhibit anti-rat erythrocyte antibody

production.

B cells induce suppression by a variety of mechanisms. One is via the release of suppressive mediators (B cell suppressor factors will be discussed in Chapter 3.5.9.). B cells act via a feedback inhibition mechanism to shut down antibody synthesis and secretion by lymphoblasts and plasmablasts, or as suppressor inducer cells activating antigen specific T suppressor cells. They may also exert their suppression by producing anti-idiotype antibodies causing the abrogation of the immune response in an indirect yet highly specific fashion.

3.5. SUPPRESSOR FACTORS

3.5.1. Introduction

As has been discussed in Chapter 3.2., the immune response appears to be regulated by a complex network of cellular interactions which results in the activation of suppressor cells. Current evidence indicates that the suppressor cells act via the secretion of a soluble suppressor factor(s) which may act by itself or in collaboration with the suppressor cells to bring about the cessation of the immune response. Due to the overwhelming number of publications in this field, it is impossible to discuss all of the suppressor factors described in the literature. I have therefore restricted the discussion to the endogenous antigen-specific and non-antigen specific suppressor factors and have omitted suppressor factors which are derived from exogenous microbial and parasitic sources (i.e. bacteria, viruses, protozoa, helminths). Reference will be made to the suppressor factors in the circulation of normal healthy pregnant women and animals and in individuals with malignancies and metabolic diseases.

Evidence, frequently of a controversial nature, has been presented which indicates that individuals in these three categories may present with symptoms of an immunodeficiency syndrome. However, the primary intent is to identify the physiological regulators of the immune response in the otherwise unaffected animal or human.

3.5.2. Non-specific suppressor factors in the circulation of normal healthy individuals

A number of regulatory (helper and suppressor) factors have been detected in the circulation of normal healthy individuals. These factors, which induce and suppress various immunological activities in vitro cultures and in vivo following their administration to allogeneic hosts, may constitute the regulatory mechanism which governs the synthesis and

secretion of "normal" non-antibody immunoglobulins resulting in their constant concentration in the circulation, a state which is termed homeostatis (discussed in Chapter 1.1.). It is now universally recognized that this regulatory mechanism involves normally circulating non-specific helper and suppressor T cells (which undoubtedly are representative of similar cells in the antibody synthesizing lymphoid tissues) which function by secreting helper factors and suppressor factors, respectively.

The first report that soluble constituents in the circulation of normal animals have immune suppressive activity was described by Kamrin in 1959. He showed that a fraction prepared from normal rat plasma was capable of prolonging allograft survival when injected IV into recipient rats. Following Kamrin's observations, Mowbray (1963) showed that the suppressive plasma component was an α-2 glycoprotein. Mannick and Schmid (1967) isolated this component from normal human serum and referred to it as immunoregulatory-α globulin (IRA). Active IRA fractions were subsequently obtained from sera of cows, rabbits, mice, rats and guinea pigs (Cooperband et al, 1976). IRA appears to act on the T cells but not on the B cells (Menzoian et al, 1974). It must be added to cultures in vitro or injected in vivo within hours of the antigen administration in order to observe suppression of the immune response (Habicht and Miller, 1977; Kern, 1978).

Another α-globulin suppressor factor found in normal human serum is "normal immune suppressive protein" or NIP (Nelken, 1973). In contrast to IRA, NIP has been shown to suppress T, B and natural killer (NK) cells (Goren & Nelken, 1981; Hanna et al, 1975) as well as the proliferation of both human and murine tumor cell lines in vitro (Nelken et al, 1979,a,b; Goren & Nelken, 1980).

There are at least six known plasma protease inhibitors. The two

major ones are α-2 macroglobulin (α_2M) and α-1 antitrypsin (α_1AT). Ford et al (1973) were the first to present evidence that α_2M could block recognition of PPD by human lymphocytes. Streilen and Hart (1976) have shown that hamster α_2M inhibits responses of normal hamster cells to B cell mitogens (LPS and dextran sulfate) but not to T cell mitogens and could cross the species barrier (Stein-Streilen and Hart, 1978; Akerstrom and Lennart, 1986). Arora et al (1978) showed that α_1AT injected into mice along with the antigen (SRBC)inhibited the in vitro anti-SRBC PFC response by the splenic cells.

Morse et al (1977) carried out a systematic study on the ability of human lipoproteins to suppress lymphocyte stimulation in vitro. They divided the serum lipoproteins into very low (VLDL), low (LDL), intermediate (IDL) and high density lipoproteins (HDL); the remaining plasma was referred to as lipoprotein-deficient plasma (LPDP). They found that all classes of lipoproteins were capable of suppressing the lymphocyte response in the order of IDL > VLDL > LDL. The HDL were the least effective at concentrations normally found in the sera of healthy individuals. In contrast to human HDL, mouse HDL is just as suppressive as LDL and VLDL in vitro (Hsu et al, 1981) and in vivo (Hsu et al, 1982).

Fibrinogen-derived peptides, but not the intact protein, have been reported to be suppressive (Girmann et al, 1975; Harvey et al, 1977; Plow et al, 1982). The inhibitory activity of fibrinopeptides occurs early in the response of human circulating MNC to mitogens (PHA) in vitro (Plow et al, 1982).

Complement has also been implicated as an immunosuppressive agent. Although evidence implicating intact complement components is controversial (Weiler et al, 1982), Needleman et al (1981) reported that C'3 inhibits both

mitogen (PHA) and antigen (PPD)-induced blastogenesis of human lymphocytes in in vitro cultures. The earlier C'3 was added to the cultures, the more suppressive was its effect (Hobbs et al, 1982). Although several studies indicate that C'3a is the active suppressive fragment, other evidence points to an acidic protein, possibly another subfragment termed C'3d (Weiler et al, 1982) or a C3-fragment preparation (Walker et al, 1986), as the active suppressor molecule.

3.5.3. Non-specific suppressor factors in the circulation of individuals following inflammation

Acute phase proteins are included in this section because they are normally present in low concentration in the circulation but increase dramatically in a wide variety of acute inflammatory conditions. They include serum amyloid A (SAA), α_1AT, haptoglobin, ceruloplasmin, α_1-acid glycoprotein, and C-reactive protein (CRP). They are all synthesized in the liver. CRP, the prototype of acute phase proteins, was first described in human sera in 1930 by Tillet and Francis (Tillet and Francis, 1930) and is so named because of its ability to precipitate (in the presence of Ca^{2+}) a polysaccharide present in the cell wall of Streptococcus pneumoniae. Analogous proteins have subsequently been described in rabbit (Anderson and McCarthy, 1951), mouse (Bodemes and Siboo, 1977), monkey and dog sera (Riley and Coleman, 1970). The structure and biological properties of CRP have been reviewed by Gewurz et al (1982). The basic structure of CRP is similar in all animal species in which it has been described (Riley and Coleman, 1970; Osmond et al, 1977; Young and Williams, 1978; James et al, 1981a,b, 1982). It is a protein devoid of carbohydrate or lipid with a M.W. of 118,000 daltons. It is composed of five identical noncovalently linked subunits, each with a M.W. of 21,500 daltons, arranged in a cyclic pentameric symetry (Oliveira et al, 1979).

It has been proposed that CRP regulates B cell function and not T cell function (Croft et al, 1976; Mortensen et al, 1977). More recently, Mortensen (1982) has presented evidence that human CRP suppresses PWM-driven differentiation of human B cells into antibody secreting cells and that this suppression is mediated by CRP-activated T cells and/or adherent monocytes. The inhibition appears to be confined to the terminal differentiation of the B cells since CRP does not suppress the proliferative response of the B cells to polyclonal B cell activators (Mortensen, 1979, 1982).

3.5.4. Non-specific suppressor factors in the circulation of patients with infectious diseases

Numerous investigators have disclosed circulating factors in humans and animals infected with bacterial, mycotic, viral and parasitic pathogenic agents capable of suppressing a number of immune and non-immune related activities in vitro. Infected individuals do not exhibit symptoms of an immune deficiency state in that they are not prone to recurrent infections with other pathogens during or after their primary infection. It would therefore appear that the circulating suppressor factors in these individuals play no role in vivo or may affect the immune system minimally without incapacitating it in any way. It is also possible that these circulating suppressor factors are unable to function in vivo in the manner suggested by the in vitro findings. However, a not insignificant percentage of individuals infected with parasites and viruses present with symptoms indicative of generalized suppression of the immune system since they subsequently present with recurrent infections, mainly of bacterial origin. One must therefore assess and interpret the demonstration of suppressor factors in the sera of infected individuals with a great deal of skepticism unless there is corroborating evidence of an immune suppressed

state in these individuals.

Treponema pallidum has been reported to induce a soluble suppressor factor in sera of infected humans and rabbits (Ware et al, 1980). This factor suppresses the in vitro PHA induced blastogenic response of circulating MNC from allogeneic uninfected subjects (Ware et al, 1980).

Patients with lepromatous leprosy have both specific and non-specific immune defects (Godal et al, 1971, 1972). Sera from untreated patients have been shown to suppress the blastogenic responses of normal circulating allogeneic MNC stimulated with mitogens (i.e. PHA, Con-A) (Mehra et al, 1972), and third-person MNC in the MLR blastogenic response (Nelson et al, 1975). Treatment of the patients results in the disappearance of these suppressive factors from their sera. The origin and nature of the serum suppressor factors have not been determined.

Fungal diseases are often associated with immunological deficiencies. Both specific and non-specific suppression can be obtained with the sera of patients with mucocutaneous candidiasis (Fisher et al, 1978). The latter sera and the sera from mice infected with Candida albicans, incubated with normal human T lymphocytes, suppress the antigen-driven and mitogen (PHA, Con-A)-driven blastogenic responses of these cells (Rogers and Balish, 1978; Stobo et al, 1976). The sera of mice infected with Histoplasma capsulatum (histoplasmosis) suppress the PHA driven blastogenic response of uninfected allogeneic mouse MNC (Artz and Bullock, 1979; Tewari et al, 1982). The soluble suppressor factor isolated from cultures of splenic MNC of mice infected with Histoplasma capsulatum was characterized as a protein with a M.W. of approximately 25,000 daltons. It was also shown to be trypsin sensitive, RNase and DNase resistant, and is inactivated at pH 2.2 and 56^{o}C.

Patients infected with hepatitis B virus possess a unique low density lipoprotein (LDL) in the circulation which is not found in the sera of normal uninfected individuals (Chisari and Edgington, 1975). This distinct LDL, which is associated with hepatitis B infection, is capable of interfering with the innate ability of normal human circulating T lymphocytes to rosette with SRBC; however, it is not able to inhibit the mitogen (PHA, Con-A, PWM)-driven blastogenic responses by these cells (Curtiss and Edgington, 1976; Curtiss et al, 1977; Curtiss and Edgington, 1981a,b).

Mice infected with malaria possess a circulating suppressor factor capable of inhibiting the in vitro PHA-induced blastogenic response by normal uninfected syngeneic splenic MNC (Khansori et al, 1981). The authors did not identify or characterize this factor.

Patients with Chagas disease caused by Trypanosoma cruzi (Cunningham et al, 1980; Curotto de Lafaille and Abrahamsohn, 1986)), schistosomiasis caused by Schistosoma mansoni (Colley et al, 1977), visceral leishmaniasis (Carvalho et al, 1986), trichinosis caused by Trichinella spiralis (Jones et al, 1976) and mice infected with Rickettsia (Jerrells and Hickman, 1986) all possess a serum factor which following its injection into mice, inhibits antibody responses to autologous and heterologous antigens by these mice. This factor can also inhibit the PHA and Con-A induced proliferative responses of normal uninfected human circulating MNC in vitro.

3.5.5. Non-specific suppressor factors in the circulation of patients with metabolic diseases

Suppressor factors capable of suppressing the phytomitogen-induced blastogenic response of allogeneic normal human circulating MNC in vitro have been found in the sera of patients with primary biliary cirrhosis (MacSween and Thomas, 1973), idiopathic nephrotic syndrome (Iitaka and

West, 1979), and uremia (Birkeland, 1976). None of the above circulating suppressor factors have been characterized.

3.5.6. Non-specific suppressor factors in the circulation of patients with malignant disease

Several factors capable of inducing generalized suppression of the immune response often accompany tumor growth. These include factors secreted by suppressor cells induced by the tumors, factors secreted from the tumor cells themselves (Broder and Waldmann, 1978), inhibitory normal serum constituents such as IRA which are increased in concentration during oncogenesis (Badger et al, 1976), and a number of, uncharacterized, circulating low M.W. peptides associated with oncogenesis (Synderman and Pike, 1976; Kamo and Friedman, 1977). In addition, tumor associated antigens (Nepom et al, 1976) may also contribute to the generalized suppressed state of the immune system. Immunosuppressive factors have been detected in the sera of patients with a variety of malignancies including sarcomas, melanomas, adenocarcinomas, and carcinomas, (Tada and Okumura, 1979; Greene et al, 1977, 1978; Takei et al, 1978; Hellstrom and Hellstrom, 1974; Hellstrom et al, 1977; Nelson et al, 1975; Nepom et al, 1976; Roth et al, 1983; Hess et al, 1980; Remacle-Bonnet et al, 1978; Noworolska et al, 1985; Hanai et al, 1986; Schulz et al, 1986).

Roth et al (1982) have described a suppressor factor in the sera of patients with malignant melanona. This factor was also detected in the serum-free medium of a cultured human melanoma cell line. Thus, the source of the suppressor factor appears to be the tumor cell itself. It is heat stable (56^{o}C), only partially susceptible to trypsin digestion, and susceptible to extremes of pH and reduction with sulphydryl-containing compounds (Roth et al, 1982, 1983). This factor inhibited both H^3-leucine and H^3-thymidine uptake by normal allogeneic circulating MNC stimulated by

phytomitogens (PHA, Con A) in vitro. Roth and his colleagues postulated that the inhibitory factor secreted by the melanoma cells acts directly on the target cells without the generation of suppressor cells and inhibits lymphocyte proliferation in vivo (Roth et al, 1983). A liposarcoma-derived 70,000 dalton M.W. glycoprotein termed circulating suppressor factor (Roth et al, 1982), a factor derived from ascitic effusions of patients with ovarian cancer (Hess et al, 1980), and suppressive factors in the sera of patients suffering from colonic adenocarcinoma (Remacle-Bonnet et al, 1978) inhibit the PHA-driven lymphocyte proliferation in vitro. It is difficult to compare the various tumor-associated immunosuppressants since few have been characterized. However, many appear to be glycoproteins and most seem to suppress lymphoproliferative responses in vitro. Reports on their action in vivo, as suppressors of the immune response, have not been forthcoming.

The oncofetal antigens (OFA), which are a group of tumor-associated serum proteins, are detected normally in high concentration in fetal serum and virtually disappear in the normal adult. They reappear in the circulation in association with certain tumors. The two best examples and the most studied are alpha fetoprotein (AFP) and carcinoembryonic antigen (CEA) (for review see Chism et al, 1978). CEA is associated with cancers of the colon, pancreas, lung, breast, and ovary (Gold and Freedman, 1965a,b; Banwo et al, 1974; Knapp and Hobbs, 1980), while AFP is found to be elevated in hepatocellular carcinoma and teratocarcinoma (Ruoslahti and Seppala, 1971; Ruoslahti et al, 1974; Parmely and Thompson, 1976) and in patients suffering from hepatitis (Beer and Billingham, 1978; Murgita and Wigzell, 1981).

Pregnant women possess circulating AFP (Beer and Billingham, 1978;

Murgita and Wigzell, 1981). Elevated serum AFP levels are also found in patients with acute and chronic hepatitis, germ cell tumors, and fetal abnormalities such as neural tube defects (Yachnin, 1976; Aure and Kess, 1977; Alpert et al, 1978; Chaouat et al, 1982; Uander and Olding, 1981).

Alpert et al (1978) found a selective suppression of in vitro cellular immune responses with AFP isolated from both human hepatoma and fetal sera. AFP suppressed both mitogen (PHA, PWM)- and antigen (PPD)-induced proliferation of allogeneic lymphocytes but not MIF production or Con A-induced lymphocyte cytotoxicity by T cells. Reports on the absence of suppressor activity of human AFP preparations have also appeared (Charpentier et al, 1977; Soubiran et al, 1979). In fact, these reports demonstrated a stimulatory effect of AFP on the in vitro MLR and mitogen (Con-A)-induced lymphocyte proliferation (Charpentier et al, 1977).

Human AFP is a single polypeptide chain with a M.W. of 70,000 daltons of which approximately 4 percent is carbohydrate (Ruoslahti and Seppala, 1971; Ruoslahti et al, 1971; Alpert et al, 1972). AFP is produced by the yolk sac in the fetus and later in the liver. Human AFP has been isolated from cord serum, amniotic fluid, hepatoma serum, ascitic fluid and tumor homogenates (Nørgaard-Pedersen, 1976).

Murgita and Tomasi (1975a) showed that murine AFP was capable of suppressing the antigen (SRBC)-induced primary and secondary antibody responses of mouse spleen cells in vitro and the lymphocyte proliferation induced by mitogen (PHA) or allogeneic cells in vitro (Murgita and Tomasi, 1975b). Others (Sheppard et al, 1977; Littman et al, 1977; and Hassoux et al, 1978) have not been able to confirm these results.

Mouse AFP was found to exert its (suppressive) effect on a subpopulation of T cells of the $Ly1^+$ phenotype and had no effect on the $Ly2^+$ cell

population (Peck et al 1978a,b). In addition, mice undergoing a GVH reaction exhibit AFP on the surface of approximately 1/3 of their spleen cells suggesting the existence of a receptor for AFP on the cells (Peck et al, 1978b). These spleen cells were unreactive to in vitro mitogenic stimulation by PHA. The authors postulated that AFP suppressive activity may be modulated by the binding of AFP to the target cell. Human AFP appears to act by activating T suppressor cells (Murgita et al, 1977; Alpert et al, 1978). Rat AFP appears to lack immunosuppressive properties such as found with human or mouse AFP (Parmely and Thompson, 1976; Sell et al, 1977; Fujinami et al, 1976; Herve et al, 1982). The evidence suggests that, at least in humans and mice, AFP can mediate inhibition of certain T cell functions in vitro and that it might play a role in regulating some immune responses in vivo.

AFP isolated from the sera of mice, rats and cows all have similar peptide maps and antigenic determinants, but they maintain species-specific antigen determinants (Nishi et al, 1975; Jalanko et al, 1978).

CEA is a family of acid glycoproteins of about 200,000 daltons M.W. with an estimated biological half life ranging from 6 to 60 days (Goldenberg, 1978). CEA was originally described in 1965 by Gold and Freedman (Gold and Freedman, 1965) who reported that human colon cancers all contained an identical tumor-specific antigen which was absent from normal control tissue and which could be found in the circulation of patients harbouring the tumor. CEA is expressed in considerable amounts by the fetal intestinal epithelium where it can be detected; it is also present in fetal serum. However, CEA levels in the circulation is very low in normal adults (below 2.5 µg/L). Modest elevations of the circulating CEA may be found in pregnant women, in smokers, and in patients

with certain chronic inflammatory diseases such as ulcerative colitis, Crohn's disease, and pulmonary infections. Raised levels, in excess of 20 µg/L, are strongly suggestive of malignancy (Fuks et al, 1974). At high serum concentrations (2000 µg/L and above), CEA has been shown to suppress the blastogenic response by T lymphocytes to PHA in vitro (Goldenberg, 1978).

3.5.7. Non-specific suppressor factors in the circulation of pregnant women and animals

In discussing immune suppression in the pregnant human or animal it is essential to distinguish between two suppressor mechanisms which may be recruited and function in the host. One is represented by the host suppressing its own immune response against the fetus, the other is represented by the fetus passively evading, actively suppressing or recruiting host derived suppressor mechanisms to suppress the hosts immune response against it.

Medawar, Brent and Billingham in 1955 (Medawar et al, 1955), in their now classical experiments, demonstrated that the rejection of an allograft (characterized by the cessation of function followed by necrossis and atrophy of the grafted tissue or cells) is immunological in nature, and is the consequence of an immune response by the host toward allogeneic antigens (HLA or transplantation antigens) on the transplanted tissue or cells. They, and many other investigators (Beer and Billingham, 1971, 1978; Goodlin and Herzenberg, 1964; Baines et al, 1976; Beer et al, 1975) subsequently demonstrated that the allograft rejection reaction follows all the immunological rules and dictums - there is a finite latent period between implantation of the graft and the immune response to it, during which the graft functions, physiologically, normally, at the height of the rejection antibodies and/or sensitized cells directed specifically towards

the alloantigens can be detected in the host (Clark and McDermott, 1978; Pence et al, 1975; Nymand et al, 1971). These antibodies and/or sensitized cells can also be found in large numbers in the allografted tissue which is undergoing rejection by the host (they in fact play a major role in the physical destruction of the allograft). A second implantation of an allograft from the same donor will provoke a secondary type allograft rejection, which will result in a more rapid rejection of the allograft. This ability to reject an allograft from a specific donor can be transferred to a naive-syngeneic recipient with sensitized cells from the host which is undergoing either primary or secondary allograft rejection (Chaouat and Voisin, 1981; Chouat et al, 1982; Youtananukorn et al, 1974; Wigzell and Hayrij, 1974).

At this time it is universally accepted that allograft rejection is the consequence of an immune response directed to the allograft.

In the experimental animal it has been frequently demonstrated that hemiallograft which carry both its paternal and maternal antigens is rejected to the same degree in the host as is the total allograft which carries only alloantigens and is completely foreign to the host. Skin or kidney allografts from F_1 mice into either of the two paternal inbred strains will be uniformally rejected whereas, as is expected, skin or renal grafts of either parent into the F_1 hybrid will be uniformally accepted. The question which then arises is why is the hemiallogeneic fetal allograft not rejected by its maternal host?

How and why does a fetus which after all possesses the allogeneic antigens of the male (father), survive in what should otherwise be a hostile milieu in the mother's uterus with its extensive blood supply? This matter has provoked extensive controversy over the past 30 years and

its resolution is still not at hand. Nevertheless, several hypothesis have been proposed to explain the exclusion of the fetus from the rules governing allograft rejection. One hypothesis is that the uterus is a privileged site, first proposed by Currie (Currie, 1968) and therefore the hemiallograft fetus implanted into the uterus will not provoke an immune response against it. The second is that the transplantation antigens of the fetus induce antigen-specific suppressor cells in the host, initially proposed by Edidin (Edidin, 1972). The third is that the presence of the fetus in the uterus activates non-specific suppressor mechanisms in the maternal host which affects her immune response to all antigens, originally proposed by Hellstrom (Hellstrom et al, 1969). The fourth hypothesis is that the concepts concerning allograft rejection are in fact wrong and that we are turning nature on its head. The fact is that the fetal allograft is a physiological occurrence and will be accepted by the host irrespective of the species. The rules which we have implaced regarding the rejection of allografts concern the transfer of tissues or cells from one individual to an allogeneic or a xenogeneic recipient which would be expected to initiate pathological responses in the host and which we interpret as rejection.

A few investigators have proposed that the uterus is a privileged site (Currie, 1968; Dixon and Weigle, 1959; Currie et al, 1968), immunologically speaking, with respect to the hemiallograft fetus thus permitting the fetus to grow undisturbed. Dixon and Weigle, 1959) proposed that the synsiciotrophoblast is composed solely of maternal antigens and serves as a filter to prevent paternal antigens from entering the maternal circulation. This work is supported, in part, by most recent investigations (Kilpatrick et al, 1986; Stranick et al, 1986; Billington

and Burrows, 1986) which have disclosed that the syncitiotrophoblast is composed mainly of maternal antigens. Albeit, paternal antigens can be found on the internal (the side facing the fetus) surface. In point of fact however, even fetuses totally allogeneic to the mother can survive and thrive following artificial implantation into the uterus (Mohammed and Saadi, 1986). Cows, horses, sheep (and recently, even human females) have served as surrogate mothers for prized species-specific embryos for decades, prior to our knowledge of transplantation immunology and our explanation why this practice should not succeed, when in fact it does, may be irrelevant or incorrect. Furthermore, there is sound evidence which shows that the uterus is not an immunologically privileged site since antigens injected into the uterine wall provoke normal immune responses (Hellstrom et al, 1969; Olding et al, 1974; Olding and Oldstone, 1974,1976; Durandy et al, 1979; Uander and Olding, 1981). Therefore, if one assumes that the fetus is capable of inducing a suicidal immune response in the host, it is mandatory, in order for the species to survive, to also assume that the fetus is capable of recruiting and activating suppressor mechanisms which will suppress the maternal immune response against it.

Although many investigations relevant to the discussion in this chapter concerning the immune state of pregnant humans or animals demonstrated the presence of circulating antigen-specific and antigen non-specific suppressor cells (Nymand et al, 1971; Olding and Oldstone, 1974, 1976; Olding et al, 1974; Chaouat et al, 1979, 1982; Slapsys et al, 1986; Croy et al, 1986; Loblay et al, 1986), only suppressor factors in the circulation of normal healthy pregnant individuals will be discussed here as so not to exceed the permitted length of the thesis.

A number of investigators have reported that maternal serum is capable

of inhibiting the phytomitogen response of normal (non-pregnant) allogenic circulating lymphocytes in vitro (Goodlin and Herzenberg, 1964; Beer and Billingham, 1971, 1978; Purtillo et al, 1972; Harrison, 1976; Parvia and Stites, 1979; Pence et al, 1975; Hellstrom et al, 1969; Bernard, 1977). Human chorionic gonadotropin (HCG) has been implicated as a potent suppressor (Adcock et al, 1973). Although later studies showed that a dializable contaminant was responsible for the inhibitory properties attributed to HCG (Muchmore and Blaese, 1977), results of recent studies (Rolfe et al, 1983; Zheng, 1986) suggest that early pregnancy factor (EPF), detected in the sera of pregnant women within 2 days of fertilization and which remains in the circulation until at least the end of the second trimester (Morton et al, 1982), is the immunosuppressive molecule in question. EPF can inhibit contact sensitivity after adoptive transfer and inhibits the rosetting of human circulating T cells with SRBC (Noonan et al, 1979). Circulating proteins in maternal serum which have been found to be immunosuppressive include AFP and pregnancy-associated α_2-glycoprotein (PAG) (AFP as an immunosuppressant has been discussed earlier in Chapter 3.5.6.). PAG increases in concentration in the serum throughout pregnancy and then decreases to normal levels within 6 weeks after parturition (Berne, 1976; Stimson, 1975; Stimson and Farquharson, 1978). At PAG levels of 200 to 400 µg/ml normally attained by the third trimester of pregnancy (Damber et al, 1976), both phytomitogen (PHA, Con-A)- and antigen (tetanus toxoid)-induced human lymphocyte transformation are reduced by 50 to 60 percent in vitro. However, even at higher concentrations of PAG, B cell proliferation in response to LPS in vitro

is only lowered by 20 percent (Damber et al, 1975; Stimson, 1976). These results suggest that PAG acts on T-cell-mediated responses.

Four additional pregnancy-associated plasma proteins (PAPPS) have been described (Gall and Halbert, 1972; Lin et al, 1974a). They are specific for pregnancy since they cannot be detected in males, nonpregnant women, cancer patients, or in women receiving estrogen oral contraceptives (Lin et al, 1974a,b). They are a family of high M.W. glycoproteins ranging from 10^5 to 10^6 daltons with the exception of PAPP-D which has a M.W. of only 20,000 daltons (Lin et al, 1976, 1978). These serum factors have been shown to suppress PHA-induced lymphocyte proliferation in vitro (Contractor and Davies, 1973; Cernie et al, 1977; Stimson, 1980).

3.5.8. Suppressor factors derived from macrophages/monocytes

3.5.8.1. Introductory statement

The macrophage plays a major role in the immune response, both as an accessory cell and as a regulatory cell, and thus can either enhance or suppress the immune response. The immunoenhancing function of the macrophage may be attributed, in part, to the secretion of a soluble product, macrophage replacing factor or IL-1, which facilitates the proliferation of T lymphocytes to phytomitogen stimulation in vitro (Oppenheim et al, 1979; Bodel et al, 1980; Amento et al, 1982). Macrophages also produce immunosuppressive agents. These are generally low M.W. mediators such as prostaglandins (Stout and Fisher, 1983a,b) and oxygen metabolites (Aune and Pierce, 1981; Kennard and Zolla-Pagner, 1980).

3.5.8.2. Suppressor factors derived from human macrophages/monocytes

Purified circulating monocytes from healthy normal individuals incubated in the presence of FCS and stimulated with E. Coli LPS for 48 hrs have been shown to secrete a suppressor factor which suppresses the antigen

(tetanus toxoid and KLH) and the phytomitogen (PWM)-induced blastogenic response and Ig synthesis by autologous circulating MNC in vitro (Gerrard and Fauci, 1982; Fauci et al, 1980). This factor was not characterized.

Circulating monocytes from normal healthy aged humans (ranging from 65-84 years, median age 72 years) were shown to suppress Ig synthesis and secretion by autologous circulating MNC in vitro (Antonaci et al, 1983). The authors suggested that the inhibitory effect was due to prostaglandins (Antonaci et al, 1982, 1983).

Fujiwara and Ellner (1986) studied the spontaneous production of a suppressor factor by the human macrophage-like cell line U937. This factor inhibited IL-1, IL-2, and PHA-induced blastogenesis by mouse thymocytes in vitro, and was generated independent of the concentration of IL-1 or PHA (Kleinhenz and Ellner, 1983, Fujiwara and Ellner, 1986). Characterization of the U937 suppressor factor showed that it was non-dialyzable, partially inactivated by heat (56^{o}C), ammonium sulfate precipitable, sensitive to pH 2.5, and resistant to freeze-thawing. It had an estimated M.W. of 85,000 daltons by gel filtration (Fujiwara and Ellner, 1986). Calvo et al (1986) have described an additional suppressor factor, an IgG-binding factor (IgG-BF), secreted by the U937 human macrophage cell line. IgG-BF did not interfere with cell proliferation in vitro but specifically suppressed IgG production by normal human circulating MNC in vitro (Calvo et al, 1986). Gel filtration of IgG-BF gave rise to two peaks with inhibitory activity with apparent M.W. of 30,000 to 40,000 daltons and 60,000 to 73,000 daltons, respectively.

3.5.8.3. Suppressor factors derived from animal macrophages/monocytes

Soluble immune response suppressor (SIRS) (discussed in Chapter 3.5. 10.3.1.) is a protein produced by activated suppressor T lymphocytes

of the mouse. However, in its pure form, SIRS in itself is not suppressive and must undergo activation by macrophages or by H_2O_2 to $SIRS_{ox}$, the active form of SIRS (Aune and Pierce, 1981).

Judas and Peck (1986) have described three monokines secreted in vitro by macrophages residing in spleens of newborn mice. They suppressed both the T-dependent and T-independent immune responses in vitro and in vivo (Judas and Peck, 1984, 1986). These suppressor factors have M.W. of 58,000, 10,800, and 10,000 daltons, appear to be proteins, and are insensitive to heat inactivation. The 58,000 dalton suppressor molecule is antigenically distinct from AFP, while the two lower M.W. molecules are too large to be prostaglandins (Judas and Peck, 1986).

A diffusible suppressor factor (DSF) from splenic macrophages of mice with plasmacytoma was described by Chen and Heller (Chen and Heller, 1982, 1984). DSF suppressed the in vitro primary immune response of normal mouse splenic MNC to SRBC (Chen and Heller, 1982). DSF was characterized as a non-dializable protein, sensitive to heat (56°C) and trypsin proteolysis. DSF binds specifically to the myeloma proteins secreted by the plasmacytoma cells (Chen and Heller, 1984).

3.5.9. Suppressor factors derived from B cells

3.5.9.1. Introductory statement

Although the main function of the B cells is the synthesis and secretion of specific antibodies in response to immunization, and the secretion of non-specific Ig for the maintenance of homeostasis in the normal human and animal, a not insignificant number of investigators have reported that B cells exert a suppressive effect on the immune system. As discussed in chapter 3.4. B-suppressor cells have been demonstrated in humans and animals. In the human, the main source of B suppressor cells

and B suppressor factors appears to be the bone marrow and to a lesser extent the circulation; in animals, only the bone marrow has been shown to be the source of B suppressor cells and B suppressor factors.

This chapter will introduce the reader to suppressor factors secreted by B cells which affect humoral immune responses and cellular proliferation in humans and animals. Reference will be made to antibody mediated suppression of the immune response.

3.5.9.2. Suppressor factors derived from human B cells

Singhal and colleagues (Bains et al, 1982; Singhal and Duwe, 1976; Singhal et al, 1978) have studied human bone marrow (HBM) regulatory cells that can suppress the primary PFC response of normal allogeneic spleen or tonsillar MNC and autologous circulating MNC in vitro. The suppression by HBM cells is mediated by a soluble suppressor factor, bone marrow suppressor factor or BSF, that is maximally effective when added early (during the first 24 hrs) to the cultures of autologous circulating or allogeneic splenic or tonsillar MNC in vitro (Singhal, 1985). BSF suppresses early inductive events in the antibody response, and its target is a non-T cell. BSF does not affect DNA synthesis by tonsillar MNC as measured by the incorporation of H^3-thymidine (Bains et al, 1982).

Human bone marrow MNC stimulated in vitro with PWM for 1 hr, washed and cultured for 7 days without PWM in the culture medium, secrete a suppressor factor into the culture supernatant referred to as marrow-derived suppressive supernatant (MDSS) (Alley et al, 1983). This factor suppressed the spontaneous synthesis and secretion of Ig by normal human bone marrow cells and the PWM-driven Ig synthesis by normal human circulating MNC in vitro (Alley et al, 1983). MDSS was characterized as a non-specific, non-HLA (human leukocyte antigen), heat liable (56°C for 1 hr) protein.

A human B cell derived suppressor factor (SBF), which can suppress autologous and allogeneic Ig production by human circulating MNC in response to PWM or Nocardia mitogens and the proliferation of human T and B tumor cell lines in vitro, has been studied by Suzuki and Miyama-Inaba and associates (Suzuki et al, 1983a,b, 1985; Masuda et al, 1978; Miyama - Inaba et al, 1982; Miyama et al, 1979). SBF is produced by normal human $Fc_\gamma R^+$ B cells in in vitro cultures. The inhibitory effect on tumor cell growth by SBF is cytostatic and not cytotoxic as in the case of lymphotoxin (Suzuki et al, 1985). The suppressive effect of SBF is not HLA restricted. SBF was characterized as a protein of approximately 30,000 to 50,000 daltons M.W. with a peak of activity at 32,000 daltons (Pisko et al, 1986).

A recent investigation by Farkas et al (1986) has demonstrated the existence of a PHA-activated human suppressor B cell. This B cell was capable of inhibiting the MLR response and the PHA-induced DNA synthesis by allogeneic human circulating MNC (Farkas et al, 1986). The authors suggested that the suppression was mediated by a suppressor factor secreted by the B cells. No further characterization of this factor was attempted.

3.5.9.3. Suppressor factors derived from animal B cells

Suzuki et al (1983b) have reported an SBF molecule secreted by mouse marrow B cells ($Fc_\gamma R^+$ B cells) which suppressed the phytomitogen (Con A and PHA)-induced blastogenic response of B cells but not of T cells in vitro, and the primary in vitro response of autologous splenic MNC to dinitrophenol. The target of mouse SBF is the B cell and it acts in an MHC-restricted manner (Suzuki et al, 1983b).

Singhal and colleagues (Roder et al, 1978; Duwe and Singhal, 1979a,b; McGarry and Singhal, 1982; McGarry et al, 1982; Singhal et al, 1978) have

described a suppressor factor (BSF) obtained from mouse bone marrow B cells similar to human BSF. Mouse BSF was shown to suppress nonspecifically the primary in vitro antibody response of autologous splenic MNC to SRBC (McGarry et al, 1982). Mouse BSF was shown to be a low M.W. ($1\text{-}3.5 \times 10^3$ daltons) glycolipid-containing carbohydrate which is thought to be the active component of the suppressor molecule (McGarry and Singhal, 1982; McGarry et al, 1982).

Normal unprimed rabbit bone marrow (BM) cells have been shown to suppress specific immune responses in vitro (Soderberg, 1984a,b, 1985). This factor was obtained from normal $Fc_\gamma R^+$ BM cells following incubation in vitro for 24 hrs. This suppressor factor inhibited the in vitro differentiation of autologous $Fc_\gamma R^-$ BM cells by blocking the production of B-cell growth factor by activated T cells necessary for B cell development (Soderberg, 1985).

Antibodies may be considered as mediators secreted by B cells. The administration of antibodies can cause specific suppression of humoral immune responses (Fitch, 1975). There are undoubtedly several mechanisms by which antibodies mediate the suppression of the immune response. The masking of antigenic determinants or reduction in the amount of free antigen may account, partially, for the observed suppression (Hancock et al, 1985). Another mechanism by which this may occur is via antigen-antibody complexes binding to antigen-reactive cells opsonizing them for destruction by macrophages (reviewed by Hutchison, 1980). A third, may be the secretion of anti-idiotypic antibodies which serve as specific suppressors of the immune response (Charan and Zinkernagel, 1986), presumably by blocking receptors, neutralizing the modulating factors, or specifically stimulating T suppressor cells (Hutchison, 1980).

3.5.10. Suppressor factors derived from T cells

3.5.10.1. Introductory statement

By far the largest number of reports in the literature of suppressor factors are concerned with suppressor factors produced by T suppressor cells. These factors are secreted in vitro following stimulation of the T cells with phytomitogens, antigens, or allogeneic cells. They may also be secreted from T cells into the culture medium by simple incubation of these cells in serum containing- or serum-free medium.

Like the suppressor cells, there are two functionally-distinct classes of suppressor factors - the antigen-specific suppressor factors which are secreted from antigen-specific T suppressor cells and the antigen-nonspecific suppressor factors which are secreted from the antigen-nonspecific T suppressor cells.

In humans, both specific and nonspecific suppressor factors can be obtained from circulating T suppressor cells. In the experimental animal suppressor factors have been obtained from suppressor T cells in the spleen and in the circulation. Although much work has been done in the past decade characterizing, isolating and purifying the suppressor factors, the primary focus of the majority of the studies has been the ability of these factors to suppress the immune response in vitro. There have been few efforts to correlate the in vitro action of the suppressor factors with any role they may play in the termination of the immune response in vivo.

3.5.10.2. Suppressor factors produced by unstimulated T cells

There are several reports which propose that lymphocytes produce soluble suppressor factors during in vitro incubation of the cells in the absence of antigen, phytomitogen (Con A, PHA, PWM) allogeneic cells, serum or serum factors.

Shou et al (1980) reported that human circulating T lymphocytes secrete a soluble suppressor factor (SSF) into the culture medium during a 4-day in vitro incubation. This factor was shown to inhibit the synthesis of IgG and IgM by normal autologous or allogeneic circulating lymphocytes stimulated with PWM in vitro (Shou et al, 1980). SSF was characterized as a glycoprotein with two active peaks determined by gel filtration - one at 13,700 daltons and the other between 13,700 and 25,000 daltons. It is stable at 56°C but loses its activity at 70°C (Shou et al, 1980). SSF's suppressive activity is blocked by L-rhamnose, L-fucose, and α-methyl-D-mannoside. The authors suggested the existence of receptor sites for SSF on the T helper cell which seems to be the target of SSF (Nair and Schwartz, 1982). Lederman et al (1981) have reported a similar factor which they termed immune response suppressor factor (IRSF). IRSF suppressed PWM- and antigen-induced Ig synthesis by normal human circulating MNC in vitro.

Lethibichthuy et al (1980) reported that unstimulated human circulating T lymphocytes shed soluble receptors for Fc of IgG (IgG-binding factor or IgG-BF) capable of interfering with the differentiation of circulating B cells into Ig-secreting cells in cultures stimulated with PWM or Nocardia extracts. The authors suggested that this suppressor factor may represent the human analogue of murine IBF (discussed in Chapter 3.5.10.3.1. produced by alloactivated murine T cells.

In a recent publication, Lethibichthuy and Revilland (1985) showed that the suppressive effect of IgG-BF isolated from cell-free supernatants of unstilulated T lymphocyte cultures was dependent on the presence of T4 cells in the cultures. Selective depletion of T4 helper or T8 suppressor subsets in the Nocardia mitogen (a T cell independent polyclonal B cells

activator) stimulated cultures showed that IgG-BF required the presence of T_4 lymphocytes to induce suppression.

A factor secreted from a human T cell hybridoma capable of suppressing PWM-induced B cell proliferation into antibody synthesizing and secreting cells in in vitro culture has been reported (Grillot-Courvalin et al, 1982). This factor must be added early (within the first 48 hours) to the culture in order to exert its suppressive effect. The target of this factor is a T4 cell and it has an apparent M.W. of 45,000 daltons (Grillot-Courvalin et al, 1982).

3.5.10.3. Suppressor factors produced by non-specifically (non-antigen) stimulated T cells

3.5.10.3.1. Suppressors of immunoglobulin and antibody synthesis

Con A activates murine nonspecific suppressor T cells (Rich and Pierce, 1973; Shou et al, 1976). These suppressor cells secrete a suppressor factor referred to as soluble immune response suppressor or SIRS (Rich and Pierce, 1974). SIRS has been reported to inhibit the PFC response (IgM and IgG) but not T cell proliferative responses to phytomitogen or the generation of cytotoxic T cells in vitro (Pierce and Kapp, 1976). It must be added early (day 0 to 3) to the 5-day cultures to exhibit its suppressive effects. The murine T cell responsible for the production of SIRS has been identified as the $Lyl^{-}23^{+}$ phenotype (Kapp et al, 1978). SIRS has been characterized as a heat-labile glycoprotein with a M.W. of 50,000 to 55,000 daltons. It is not an Ig and has no MHC restriction (Tadakuma and Pierce, 1978; Aune et al, 1983). The target for SIRS appears to be the macrophage (Tadakuma and Pierce, 1976, 1978) which produces another soluble factor which, in fact, is oxidized SIRS or SIRS-ox and which is more active than the unmodified SIRS (Aune and Pierce, 1981a,b, 1982, 1983; Irons et al, 1984).

Pierce and co-workers have also described a soluble immune suppressor factor derived from a T cell hybridoma. This SIRS was found to consist of two entities which could be separated by chromatography, $SIRS_{\alpha}$ and $SIRS_{\beta}$, both of which had nearly identical M.W. of approximately 11,000 daltons. These two forms of SIRS were able to suppress the antibody-forming cell response to SRBC in SRBC primed mice (Webb et al, 1985; Schnaper and Aune, 1986).

A human SIRS counterpart to murine SIRS, secreted by human suppressor T cells activated by leukocyte interferon or Con A, has been described by Schnaper et al (1984). The human SIRS has a M.W. of 110,000 to 150,000 daltons and is sensitive to protease and acid (pH 2) treatment. A SIRS-like suppressor factor secreted by normal human spleen cells activated with Con-A in vitro has also been described (Krakauer et al, 1980). This factor was able to suppress polyclonal Ig synthesis (IgG, IgM and IgA) by autologous normal spleen cells stimulated with the T-cell dependent mitogen PWM or the T-cell independent mitogens LPS and Nocardia in in vitro cultures (Krakauer et al, 1980; Beer et al, 1982).

Two soluble constituents released from human Con A-stimulated circulating MNC in vitro (Greene et al, 1981; Fleisher et al, 1981) are referred to as SISS-T and SISS-B (soluble immune suppressor supernantant). SISS-T will be discussed in Chapter 3.5.10.3.2. SISS-B inhibits immunoglobulin synthesis by B cells. SISS-B was characterized as a protein of 60,000 to 80,000 dalton M.W., stable at pH 2.5 but sensitive to heat (56°C) treatment (Green et al, 1982). Warrington et al (1983) have described another suppressor factor derived from Con A-stimulated human T cells referred to as T_{γ}. It was characterized as a 20,000 to 25,000 dalton M.W. glycoprotein stable at pH 2.0 and at 56°C. This factor, like SISS-B,

inhibits the secretion of Ig by PWM-stimulated human B cells in vitro. Like SIRS, it must be added early (days 0 to 3) to the cultures to have its suppressive effect. The authors suggest that T_γ is the human counterpart of the murine SIRS. They base their assumption on the fact that T_γ resembles another lymphokine, MIF, which is similar to SIRS and cannot be distinguished from it (Block et al, 1978; Kuhner and David, 1976; Fox and Rajaraman, 1981).

Another suppressor factor synthesized by phytomitogen or antigen-stimulated T cells is the immunoglobulin binding factor (IBF) first described by Gisler and Fridman in 1975 (Gisler and Fridman, 1975) and subsequently by Fridman and co-workers (Neauport-Sautes et al, 1975; Gisler and Fridman, 1976; Fridman et al, 1977; Yagello et al, 1979; Fridman et al, 1981; Fridman et al, 1984; Teillaud et al, 1986). IBF acts by inhibiting the differentiation of B cells into Ig and antibody synthesizing plasmablasts and lymphoblasts (Gisler and Fridman, 1976). IBF is a glycoprotein and is composed of two polypeptide chains of 40,000 and 20,000 dalton M.W. It binds to the Fc portion of homologous and heterologous IgG (Joskowicz et al, 1978; Sandor et al, 1986). IBF is believed to be the soluble form of the receptor for FcG on the T cell surface membrane which is shed from the cell surface of both human (Molenaar et al, 1977) and mouse (Fridman et al, 1977; Neauport-Sautes et al, 1975) stimulated T cells at 37°C.

Millerioux et al (1981) have described a suppressor factor, spleen derived immunosuppressive protein, referred to as SDIP, which is synthesized by bovine splenic T cells stimulated in vitro by Con A. SDIP was shown to inhibit the PFC response of normal mouse spleen cells to SRBC in vitro (Millerioux et al, 1981). Unlike IBF, SDIP must be extracted

from the stimulated cells (Lenfant et al, 1980). The functional suppressive moiety of SDIP was present in a low M.W. constituent (M.W. 2,000 daltons) of a larger 50,000-dalton fraction (Lenfant et al, 1980). SDIP appears to act directly on B cells, affecting its differentiation into the antibody-forming blast cells (Lenfant et al, 1983).

3.5.10.3.2. Suppressors of cell proliferation

Several nonspecific suppressor factors of T cell origin operate by inhibiting cell proliferation. An inhibitor of the proliferative response in the MLR has been studied by Rich and Rich (1974, 1975, 1976, 1977). This factor was produced by injecting allogeneic lymphocytes into the footpads of mice, removing the spleen 4 days later and culturing the splenic MNC in vitro in the presence of the injected allogenic cells. The factor was secreted into the culture medium but could also be extracted from the cells (Rich and Rich, 1977). The suppressor factor was characterized as a glycoprotein, non-immunoglobulin in nature, with a M.W. of 25,000 to 75,000 daltons.

Another allospecific suppressor factor capable of suppressing the MLR proliferative response has been studied by Eisenthal et al (1979). This factor was produced by mouse thymocytes stimulated in vitro with irradiated allogeneic spleen cells. The synthesis of the factor appeared to necessitate the cooperation between Lyt-1^+ and Lyt-2^+ cells and required the presence of adherent cells. This factor was not characterized.

Mayumi et al (1985) have studied the mechanisms of "immunologic-escape" of embryos during the implantation period in mice. They found a suppressor factor which is secreted or could be extracted from the eight-cell stage fertilized ova or blastocyte but not from unfertilized ova. This factor was able to suppress both the MLR response and the generation of cytotoxic T lymphocytes by the maternal circulating and splenic MNC.

An inhibitor of DNA synthesis (IDS) was first described in rats (Namba et al, 1977). IDS inhibits both T cell and B cell proliferative responses apparently through the elevation of cAMP resulting from the activation of adenylate cyclase (Jegasothy et al, 1978). Pharmacological agents which raise intracellular cAMP (PGE, dibutyryl cAMP) can mimic the action of IDS (Waksman and Wagshal, 1978). IDS is produced by mitogen (Con A) stimulated normal mouse splenic lymphocytes in vitro and by mouse spleen or lymph node MNC following in vivo immunization. It has been characterized as a 20,000 dalton M.W. glycoprotein and appears to aggregate to a higher M.W. of a dimeric or trimeric form which retains its suppressive activity (Jegasothy and Battles, 1979). Human IDS has been purified from the supernatants of human circulating MNC cultures stimulated with Con A (Jegasothy and Battles, 1981). Human IDS appears similar to rat IDS in its activity and M.W. profile; however, human IDS always appears in the higher M.W. aggregate form and may be capable of inhibiting DNA-polymerase activity in vitro (Jegasothy and Battles, 1981).

SISS-T, a suppressor molecule of 30,000 to 45,000 dalton M.W., which inhibits both phytomitogen and antigen-induced T cell proliferation, has been described by Greene et al (1981). This factor is secreted by human circulating T cells incubated with Con A in vitro. This factor, like its SISS-B counterpart which suppresses Ig synthesis by PWM-stimulated B cells in vitro (described above), is unstable at 56°C. SISS-T, like SISS-B, has not been further characterized.

A soluble inhibitory factor (SIF) derived from human tonsil T lymphocytes in vitro has been described (Wolf et al, 1978). This factor can suppress B cell and T cell proliferation in vitro. An SIF-like molecule, recovered from normal human serum, is composed of two components:

a protein and a glycolipid which possesses the suppressor activity and is referred to as lipid suppressor substance (LSS) (Wolf and Andreoni, 1982). Serum-derived SIF (SIF-serum) resembles SIF derived from cell culture supernatants (SIF-sup) by chromatographic and functional analysis. They both suppress PHA-induced autologous or allogeneic normal T lymphocytes proliferation in vitro. SIF-sup has an apparent M.W. of 40,000 to 80,000 daltons whereas SIF-serum has a M.W. between 100,000 and 150,000 daltons (Wolf and Adreoni, 1982).

SIF-sup can activate circulating human T cells to suppress the responses in both the autologous and allogeneic MLR (Kasakura et al, 1983). This SIF was characterized as an 18,000 to 29,000 dalton M.W. protein which could be recovered by isoelectric focusing in the pH range from 6.9 to 7.3 (Kasakura et al, 1983). Thus, SIF may play a role in both active suppression and activation of suppressor cells in vitro. The presence of SIF in normal serum may suggest an in vivo role for SIF in immune regulation.

Histamine induces a suppressor factor (HSF) capable of inhibiting antigen-induced proliferation and the production of MIF by sensitized T lymphocytes in vitro. HSF production could be clocked by H_2 but not H_1 receptor antagonists. HSF was shown to be a glycoprotein of approximately 23,000 to 40,000 daltons M.W. (Rocklin, 1977). HSF appears to be produced by T cells that bear both histamine (H_2) receptors and receptors for the Fc portion of IgG (FcG) (Rocklin et al, 1980).

Lymphotoxin(s) (LT) are detected in culture supernatants of antigen- or phytomitogen-stimulated T lymphocytes (Ruddle and Waksman, 1968; Granger et al, 1969). Investigations with murine cells indicate that the cell source of LT is a T cell but B cells may produce LT as well

(Eardley et al, 1980). Guinea pig LT and murine LT have an approximate M.W. of 50,000 daltons and are sensitive to trypsin treatment (Sawada et al, 1975).

Unlike the guinea pig LT and the murine LT, human LT has been found to be composed of α- and β-LT components. The α component has an approximate M.W. of 75,000 daltons and the β component 45,000 daltons (Walker et al, 1976). The β component resembles guinea pig and murine LT. The mechanism of action of LT is not completely understood. LT action is not genetically (MHC) restricted or tissue-specific. DNA, RNA and protein synthesis appear normal in the target cells prior to their undergoing lysis (Okamoto and Mayer, 1978a). However, a three-to fourfold increase in Ca^{2+} uptake has been noted in LT-damaged cells (Okamoto and Mayers, 1978b). Thus, LT damages the Ca^{2+} uptake system which appears to be essential for the normal functioning of the T cells.

3.5.10.4. Suppressor factors produced by specifically (antigen) stimulated T cells

Heijnen, Uytdehaag and Ballieux (Heijnen et al, 1979; Uytdehaag et al, 1981; Ballieux and Heijnen, 1982) have described an antigen specific suppressive factor secreted by cultured antigen-stimulated circulating human T cells (TSF-120). These cells were found to be $Fc_\gamma R^+$ cells (Uytdehaag et al, 1981). TSF-120 has to be added to cultures of autologous or allogeneic circulating MNC early (the first 24-48 hrs) in order to maximally suppress the antigen-induced blastogenesis of these cells in vitro (Uytdehaag et al, 1981).

Two antigen-specific suppressor factors secreted by the splenic T cells of mice immunized with SRBC have been described by Cantor and associates (Cantor et al, 1978; Eardley et al, 1979; Yamauchi et al, 1981a,b). These factors are referred to as the T cell suppressor inducer

secreted by the Ly-1 T cell (TsiF) and the T cell suppressor factor (TSF) secreted by the Ly-2 T cell. Both are obtained from the suppressor cells following the cessation of antibody synthesis (day 20 to 30 post-immunization), and must be added early (first 24-48 hrs) to the cultures of autologous or syngeneic antigen-primed splenic MNC in order to suppress the immune response (Yamauchi et al, 1981a,b; Flood et al, 1982a,b). TsiF induces, in an MHC-restricted manner, the transformation of an Ly2,3 T cell into a suppressor effector cell (Yamauchi et al, 1981a; Noguchi et al, 1985) which exerts its suppressive effect by secreting an antigen-specific suppressor effector factor (Ferguson and Iverson, 1986). TsiF was characterized as a protein with an apparent M.W. of 68,000 daltons by SDS-PAGE analysis (Ferguson and Iverson, 1986). TSF acts, in an MHC-restricted manner to inhibit Lyl helper T cell activity (Flood et al, 1982).

Pierce, Kapp and colleagues (Kapp et al, 1976, 1977, 1980, 1983; Kapp, 1978; Jenkins et al, 1985; Lopez et al, 1986) have described a GAT-induced antigen-specific T suppressor factor (GISF) from GAT-immunized mice. GISF can be extracted obtained from splenic, lymph node and thymic T cell homogenates within a few days following the initiation of the immune response (Kapp et al, 1976, 1978). GISF acts in an MHC non-restricted manner but is strain specific (Kapp et al, 1980, 1983). GISF inhibits GAT-specific PFC responses of GAT-immune splenic MNC in vitro (Pierce et al, 1984, 1985) and must be added early (first 24 hrs) to the cultures in order to maximally suppress the in vitro immune response (Kapp et al, 1977, 1980).

4. MATERIALS AND METHODS

4.1. Materials

4.1.1. Reagents

α-naphthyl acetate - obtained from Sigma Chemical Co., St. Louis, Missouri, and stored at 18°C.

Ammonium chloride - obtained from British Drug Houses, Montreal, Quebec, and stored at 18°C.

Antisera - 1) The chromatographically pure rabbit IgG and IgM antibodies to ORBC and HRBC were obtained from Cappel Laboratories, Westchester, Pennsylvania, and stored at 4°C.

2) The chromatographically pure rabbit IgM, 19S fragment against SRBC was obtained from Cordis Laboratories, Miami, Florida, and stored at -20°C.

Carbonyl iron grade SF - obtained from Dyestuffs and Chemicals, Toronto, Ontario, and stored at 18°C.

Complement - The source of C'3 was serum from a normal human volunteer, guinea pig or normal rabbit serum.

Concanavalin-A - obtained in a lyophilized powdered form in 250 mg quantities from Calbiochem, La Jolla, California, and stored at 4°C.

Culture medium - The culture medium was RPMI 1640 fortified with 10% FCS serum, penicillin (100 units/ml), streptomycin (100 µg/ml), gentamicin (50 µg/ml) and 25 mm Hepes.

Erythrocytes - Heparinized ORBC, SRBC and HRBC were obtained fresh weekly from Qualicum Laboratories, Ottawa, and stored at 4°C until required.

FCS - was obtained in 100 ml quantities from Microbiological Associates and stored at 4°C. FCS was decomplemented (heated at 56°C, 30 min) and filtered through a 0.20 µm Nalgene filter before use.

Ficoll 400 - (500 g) obtained from Pharmacia Fine Chemicals AB, Upsala, Sweden and stored at 18°C.

Gentamicin - obtained from Schering Inc., Pointe Claire, Quebec, and stored at 4°C.

H^3-Thymidine - (µCi/ml) - was obtained from New England Nuclear, Boston, Massachusetts and stored at -20°C.

Hanks Balanced Salt Solution (HBSS) (500 ml) - was obtained from Microbiological Associates, Bethesda, Maryland, and stored until use at 4°C.

Heparin (mucosa) sodium - (1% Benzylalcohol as preservative) was obtained in concentrations of 1000 USP units/ml from Organon Canada Ltd., Organon, Ontario, and stored at 4°C.

HEPES buffer - obtained from Microbiological Associates, Bethesda, Maryland, and stored until use at 18°C.

Hypaque sodium (diatriozate) - 50% w/v pH = 6.5-7.7 was obtained from Winthrop Laboratories, Aurora, Ontario. The 30 ml ampules were stored in the dark at 18°C.

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

Mitomycin-C - obtained in 2 mg vials (powder form) from Sigma, St. Louis, Missouri, and stored in the dark at 4°C. Immediately

before use the powder was dissolved in 2 ml of RPMI 1640 to give a solution of 1 mg/ml.

Pararosanilin - obtained from Sigma Chemical Co., St. Louis, Missouri, and stored at 18°C.

Phytohemagglutinin-M (PHA) - obtained lyophilized from Difco Laboratories, Detroit, Michigan and stored at 4°C.

Pokeweed mitogen (PWM) - obtained lyophilized from Gibco Laboratories, Chagrin Falls, Ohio, and stored at 4°C.

Potassium - penicillin G (5000 units/ml) and streptomycin sulphate (5000 ug/ml) - were obtained pre-mixed in 100 ml quantities from Microbiological Associates, Bethesda, Maryland, and stored until use at -20°C.

Saline solution - (0.9% NaCl) in pyrogen free sterile water was obtained in 1000 ml quantities from Abbott Laboratories Ltd., Montreal, Quebec, and stored at 18°C.

Scintillation liquid - premixed cocktail obtained from Beckman Instruments, Fullerton, California and stored at 18°C.

Sodium acetate - obtained from Fisher Scientific, Fair Lawn, New Jersey and stored at 18°C.

Sodium nitrite - obtained from Fisher Scientific, Fair Lawn, New Jersey and stored at 18°C.

Sodium Pentothal (Pentothal 2.5% solution, 25 mg/ml, 500 ml) - was obtained from Abbott Laboratories, Montreal, Quebec, Canada and stored at 18°C.

Tissue culture medium RPMI-1640 (500 ml) - was obtained from Microbiological Associates, Bethesda, Maryland, and stored until use at 4°C.

Trypan blue - obtained from Grand Island Biological Co., Grand Island, New York, and stored at 18°C.

4.1.2. Supplies

Culture tubes - were 17x100 mm and 15 ml Falcon 2001 plastic sterile disposable tubes were obtained from Falcon Plastics, Cockesville, Maryland.

Eppendorf repeater 4780 - automatic pipette and sterile disposable tips were obtained from Brinkman Instruments, Westbury, New York, and Eppendorf, Hamburg, West Germany.

Liquid scintillation vials - these plastic vials were obtained from Fisher Scientific, Fair Lawn, New Jersey.

Microtiter plates and lids - Micro Test III flat bottom plates were obtained from Becton Dickinson Labware, Oxnard, California.

Nalgene 0.22 µm filters - obtained from Nalgene Labware, Rochester, New York.

Pipette aid (Automatic Pipette) - obtained from Drummond Scientific Co., Broomall, Pennsylvania.

Pipettes - 1, 2, 5 and 10 ml Pyrex sterile disposable serological pipettes were obtained from Corning Glass Works, Corning New York. A pro-pipette or a Pipette aid was always used to manipulate fluids.

Syringes - 1, 3, 5, 10 and 30 ml Plastiplate sterile disposable plastic syringes obtained from Becton Dickinson Labware, Oxnard, California.

4.1.3. Equipment

CO_2 Incubator - cultures were maintained in a National incubator (NAPCO, Portland, Oregon).

Coulter counter - obtained from Coulter Electronics Inc., Mialeah, Florida.

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

Harvester - Titertek - obtained from Flow Laboratories, Rockville, Maryland.

Liquid Scintillation Coulter LS-230 - obtained from Beckman Instruments, Fullerton, California.

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

Refrigerated centrifuge CRU 5000 - obtained from Fisher Scientific, Fair Lawn, New Jersey.

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

4.1.4. Animals

4.1.4.1. Rabbits - New Zeland White outbred normal rabbits, immunologically mature (3 months of age, 4 to 5 lb), were obtained

from La Ferme LAPRO Inc., Montreal, Quebec, Canada, and kept in our animal facility at constant temperature (19-21°C) and humidity (50% Rhm) with food and water ad lib.

4.1.4.2. Guinea pigs - White laboratory outbred guinea pigs (COBS Hartley crl x COBS (HA) BR) were purchased from Charles River Breeding Labs, St. Constant, Quebec, Canada, and kept under the same conditions as the rabbits above.

4.2. Methods

4.2.1. Preparation of erythrocytes - Heparinized ORBC, SRBC and HRBC were obtained fresh weekly from Qualicum Laboratories, Ottawa, and stored at 4°C until required. The RBC were washed three times in HBSS or saline at 900 g for 10 min at 15°C and resuspended in RPMI 1640 or saline to the desired concentration. Human volunteers and rabbits were bled by venipuncture into heparinized syringes. The bloods were centrifuged at 900 g at 15°C for 10 minutes, the supernatants were decanted, the cells were suspended in medium and were centrifuged once more at 900 g for 10 minutes. The erythrocytes were washed once more in medium and suspended in medium (RPMI 1640) to the desired cell concentration.

4.2.2. Complement - The source of C'3 was normal guinea pig, rabbit serum or normal healthy volunteer serum. A volume of this serum was absorbed with a 0.1 volume of packed ORBC, SRBC or HRBC at 4°C for 1 hour, centrifuged at 490 g for 10 min at 18°C and absorbed once more with the RBC at 37°C for 60 min. The absorbed serum did not agglutinate the respective RBC even in the undiluted state.

4.2.3. E indicator erythrocytes - SRBC were washed with HBSS three times at 490 g for 10 minutes at 18°C and resuspended in RPMI 1640 to the required cell concentration.

4.2.4. EAG indicator erythrocytes - ORBC were washed three times with HBSS at 490 g for 10 minutes at 18°C. One ml of a 5% ORBC suspension was mixed with 1 ml of rabbit antibodies to human IgG (1/200, which was found to be the maximum subagglutinating concentration) incubated for 1 hour at 37°C and washed three times with HBSS at 490 g for 10 minutes at 18°C. The pelleted cells EAG were resuspended in RPMI 1640 to the desired cell concentration.

4.2.5. EAC indicator erythrocytes - One ml of 5% ORBC was mixed with 1 ml rabbit antibodies to human IgM (1/100). The cells were incubated for 30 minutes at 37°C, washed three times in HBSS at 490 g for 10 minutes at 18°C and resuspended in 1 ml of RPMI 1640. One ml of human C (1/8) was added. After 30 minutes of incubation, the cells were washed three times with HBSS at 490 g for 10 minutes at 18°C and resuspended in RPMI 1640 to the desired cell concentration. Neither the rabbit IgM antiserum nor the human serum used as a source of complement, in the concentrations used in the preparation of EAC, were capable of independently sensitizing the ORBC sufficiently to impart to them the ability to form rosettes.

4.2.6. EAM indicator erythrocytes - Two ml of 2% ORBC were mixed with 2 ml of rabbit antibodies to human IgM (1/64, which is the maximum subagglutinating concentration) and incubated for 30 minutes at 4°C. The cells were washed three times with HBSS at 490 g for 10 minutes at 18°C and resuspended in RPMI 1640 to the desired cell concentration.

4.2.7. Mitomycin C treatment of mononuclear cells - Mononuclear cells (10^6 cells per tube) were incubated with 75 µg mitomycin-C at 37°C for 1 hour. These cells were subsequently washed three times with HBSS at 490 g for 10 minutes at 18°C to remove residual mitomycin-C and resuspended in culture medium to the appropriate concentration.

4.2.8. Assessment of cell viability - The viability of the cells was determined by the conventional trypan blue dye exclusion test.

4.2.9. Macrophage/monocyte-depleted mononuclear cell (MNC-$M\emptyset_1$ and MNC-$M\emptyset_2$) preparation - Macrophage/monocyte-depleted MNC were prepared by incubating 10^7 MNC with 5 ml of RPMI 1640 containing 50% autologous serum and 10 mg of carbonyl iron. These cells were incubated on a tissue culture rotator for 45 minutes at 39°C. They were then layered on a Ficoll-Hypaque discontinuous gradient (SG 1.10) and centrifuged at 1200 g for 40 minutes at 18°C. The non-phagocytic cells isolated from the interphase were washed twice in HBSS at 500 g for 10 minutes at 18°C and are referred to as once depleted mononuclear cells or MNC-$M\emptyset_1$. The MNC-$M\emptyset_1$ cells were cultured overnight in culture medium and subjected to monocyte depletion with carbonyl iron as described above. These twice monocyte-depleted cells are referred to as MNC-$M\emptyset_2$. Ninety-six to ninety-eight percent of the MNC-$M\emptyset_1$ cells were lymphocytes, as identified by differential staining of the cells for non-specific esterase (NSE), whereas >99% of the MNC-$M\emptyset_2$ cells were identified as lymphocytes.

4.2.10. Non-specific esterase staining - The morphology of monocytes and lymphocytes was identified by staining for non-specific esterase. The staining reagent was prepared as follows: 10 ml of distilled water was added to 0.4 sodium nitrite, 10 ml of ethylene glycol monoethyl ether (EGME) was added to 0.2 g α-naphtyl acetate, 7.5 ml of pararosanilin solution was added to 7.5 ml of 4% sodium nitrite solution following exactly 1 minute, 12 ml of EGME solution was added. Following the addition of 178 ml of PBS a buff colored precipitate formed and was filtered. The pH was adjusted to 6.1 using HCl or 1N NaOH. Air dried cytocentrifuged cells were stained for 30 minutes with the above mentioned reagent. The slides were washed twice in distilled water, fixed in methanol for 60 seconds and dried in cold air. The slides were counterstained with 1% methyl green for 1 minute, washed twice with distilled water and air dried at 18°C. Methyl green was prepared as follows: 100 ml of distilled water was added to 1.4 g sodium acetate and 1.0 methyl green. The pH was adjusted to 4.2 with acetic acid.

4.2.11. Immunization of rabbits and preparation of lymphoid cells - Immunologically mature (3 months of age, 4 to 5 lb) normal, outbred New Zealand White rabbits, were immunized IV with 10^9, $4x10^5$ SRBC or HRBC or with SSSF or HSSF equivalents. At predetermined intervals of time thereafter, the rabbits were sacrificed by the IV injection of pantothal. The abdominal and chest cavities were immediately entered, blood was withdrawn from the heart into heparinized syringes (50 units herapin per ml of blood), and the thymus (THY), spleen (SPL), appendix (APP), saccylus rotundus (SR), Peyer's patches (PP) and popliteal lymph node (PLN),

were excised and placed into sterile plastic tubes (Corning 50ml) containing Medium RPMI 1640. The PLN were excised from the popliteal fossae. The lymphoid organs were cut into small fragments and placed onto wire mesh screens (50 mesh). The cells were expressed via the application of slight pressure to the fragments and collected in medium in sterile plastic tubes. The tubes were centrifuged at 500 g for 10 minutes at 18°C, the cells were suspended in medium (RPMI 1640), washed and resuspended to the desired cell concentration in medium (RPMI 1640). Bone marrow (BM) MNC were obtained by splitting the femur and collecting the bone marrow into a sterile plastic tube containing medium (RPMI 1640). The tube was shaken vigorously several times and centrifuged at 500 g for 10 minutes at 18°C. The fatty upper layer was removed and the cells were washed in medium. The SPL, BM and circulating (PB) MNC were centrifuged through Ficoll-Hypaque (SG 1.10) (Milthorp and Richter, 1979) in the manner as originally described by Boyum for human circulating MNC (Boyum, 1968). The MNC cells at the interface were collected, washed twice with medium (RPMI 1640) and resuspended to the desired cell concentration in medium.

4.2.12. The hemolytic plaque-forming (PFC) assay to detect AFC - The PFC assay was carried out in liquid medium as described by Cunningham and Szenberg (Cunningham and Szenberg, 1968) and modified by Richter et al (1981). Anti-SRBC antibodies (rabbit) were added to the assay mixture in a sub-hemolytic concentration, thereby increasing the sensitivity of the assay about 10 fold. The PFC assay used is a modification of the one originally described by Jerne and Nordin (1963).

4.2.13. The secondary immune response in vitro - The secondary immune response in vitro was carried out as previously described (Richter et al, 1986). The lymphoid cells (4×10^6) were incubated with an equal number of SRBC in a volume of 4 ml in sterile plastic tubes. The medium was RPMI 1640 fortified with fetal calf serum (10 percent final conc.), penicillin (100 units per ml) and streptomycin (100 µg per ml). After incubation for 5 days at 37°C in 5 percent CO_2 in air, the tubes were centrifuged at 500 g for 10 minutes and the cells were suspended in medium (RPMI 1640) to the desired cell concentration.

4.2.14. Inhibition of immune splenic PFC by the immune thymus suppressor cells (ITSC) - Spleen MNC obtained from rabbits 7 days post-1° IMM IV with SRBC or HRBC were coincubated at 37°C for 1, 2 or 4 hours with autologous or allogeneic 7-day post 1° IMM thymus cells, in varying ratios of splenic MNC to thymus cells. The cells were then centrifuged at 500 g for 10 minutes, suspended in medium (RPMI 1640) to the desired cell concentration and assayed for PFC.

4.2.15. Inhibition of immune splenic PFC by immune thymus suppressor factor (ITSF) - ITSF was prepared by incubating thymus cells (10^7 cells per ml, 5 ml), obtained from rabbits at different days post-immunization, in Medium RPMI 1640 at 37°C in 5 percent CO_2 in air, for 1, 2 or 4 hours. The tubes were then centrifuged at 500 g for 10 minutes and the cell-free supernatant, containing the ITSF, was collected and filtered through a millipore (0.20 µ) filter. One ml ITSF was added to 1 ml of autologous or allogeneic 7 day immune spleen MNC (10^6 cells per ml) and the tubes were incubated at 37°C for 1, 2 or 4 hours. The tubes were then centrifuged at 500 g for 10 minutes and the pelleted cells were resuspended in medium and assayed for PFC.

4.2.16. The isolation of ITSC from suspensions of thymocytes obtained from immunized rabbits - Thymocytes obtained from rabbits 7 days post-1^0 IMM IV with SRBC (or HRBC) (10^7 thymocytes per ml, 10 ml) were mixed with an equal volume of a 3 percent suspension of SRBC (or HuRBC, RRBC or HRBC) in Medium RPMI 1640. The tubes were incubated in a 37°C water bath for 15 minutes, centrifuged at 150 g for 5 minutes and incubated at 2°C to 4°C for 2 hours (further incubation overnight at 4°C did not alter the results). The pelleted cells were gently resuspended and centrifuged through Ficoll-Hypaque (SG 1.10) at 18°C at 1200 g for 40 minutes. The MNC at the interface (the rosette-depleted MNC) and the pelleted (rosetted) MNC were collected, washed twice with medium, and suspended to the desired cell concentration in medium. The RBC in the rosetted cells were lysed by incubation with hypotonic ammonium chloride for 1 minute (Sklar et al, 1980).

4.2.17. The identification of ITSC - The thymocytes of rabbits immunized 7 days previously with 10^9 SRBC or HRBC IV were rosetted with EAG or EAC in the manner described previously (Richter et al, 1983; Banerjee et al, 1981). The RBC in the rosetted cells were lysed with hypotonic ammonium chloride (Sklar et al, 1980) and washed with Medium RPMI 1640. The non-rosetted and rosetted thymocytes were each suspended to the desired concentration in medium.

Thymocytes were subjected to MNC-$MØ_1$ and $MØ_2$ depletion (see section 4.2.9.) in order to determine whether MØs are the suppressor cells within the thymus, and are the cells which secrete the ITSF.

The thymocytes were also incubated for 1 hour at 37°C with horse anti-rabbit thymocyte (T cell) serum (HARTS) and complement (fresh guinea pig serum, final dilution 1:5) or horse anti-rabbit bone marrow (B cell) serum (HARBMS) and complement in order to lyse the T or B cells, respectively. These antisera, appropriately absorbed with RRBC (HARTS) and RRBC and rabbit thymocytes (HARBMS) are capable of specifically lysing 90 to 100 percent of the rabbit T cells (De La Noue et al, 1972) and 90 to 95 percent of the rabbit B cells (De La Noue and Richter, 1974), respectively. The former does not lyse rabbit bone marrow MNC (De La Noue et al, 1972) and the latter fails to lyse rabbit thymus cells (De La Noue and Richter, 1974).

4.2.18. Preparation of ITSF for in vivo injections and in vitro suppression of the immune response - Rabbits were immunized with 10^9 SRBC and/or HRBC IV and sacrificed 7-8 days later. The thymuses were removed, cut into small fragments, and placed onto sterile gauze pads. The thymocytes were expressed into Medium RPMI 1640 in sterile plastic tubes by the application of pressure accompanied by vigorous shaking. The tubes were centrifuged at 500 g for 15 minutes and the thymocytes were suspended in medium (RPMI 1640) (5×10^7 cells per ml). Thirty ml of this thymocyte cell suspension were incubated for 24 hours at 37°C in 5 percent CO_2 in air in a 50 ml sterile Falcon plastic tube in a stationary upright position. The cells were then centrifuged at 500 g for 10 minutes and the cell-free supernatant was collected and filtered through a 0.2 μ Nalgene filter into sterile falcon tubes. The supernatant, containing the ITSF, was tested for its suppressive capacity in the manner described previously (Richter and Talor, 1986) and stored at -20°C.

4.2.19. Preparation of NTS - NTS was prepared from NTC in the manner as described above for ITSF. The thymocytes were obtained from the thymuses of unimmunized rabbits.

4.2.20. The inhibition of the in vivo primary immune response by ITSC - Rabbits were immunized IV on day 0 with 4×10^5 SRBC, or with 4×10^5 SRBC and 4×10^5 HRBC. The rabbits were injected IV with 2×10^9 ITSC (SRBC) and/or 2×10^9 ITSC (HRBC), or with 2×10^9 NTC in sterile saline on days 0 and 3 or days 0, 3 and 5 following immunization. The rabbits were sacrificed on day 7 and the MNC of the various lymphoid organs were prepared and assayed for hemolytic plaque formation immediately after sacrifice and following 5 days in culture with the immunizing antigen. The agglutination titers to SRBC and HRBC at the time of sacrifice were determined.

4.2.21. The inhibition of the in vivo primary immune response by ITSF - Rabbits were immunized with 4×10^5 SRBC and/or 4×10^5 HRBC IV on day 0 and injected IV with 10 ml of ITSF, NTS or medium RPMI 1640 daily for 5 days (days 0 to 5). The rabbits were sacrificed 2 days later (day 7) and the MNC of the various lymphoid organs were assayed for hemolytic plaque formation immediately after sacrifice and following 5 days in culture with the immunizing antigen(s). The hemagglutinating antibody titers to SRBC and HRBC at the time of sacrifice were determined.

4.2.22. The inhibition of the in vitro-induced secondary immune response by ITSC - Rabbits were immunized IV with 10^9 SRBC and/or 10^9 HRBC and sacrificed 7 or 60 days later. Mixtures of MNC (2×10^6 cells) and ITSC (2×10^6 cells) or NTC (2×10^6 cells) were suspended in 4 ml

culture medium in sterile 10 ml plastic Falcon 2001 tubes. SSSF or HSSF (equivalent to 4 x 10^6 SRBC or HRBC, respectively) was added to the tubes (0.1 ml). The cells were cultured for 5 days at 37°C in 5 percent CO_2 in air. On day 3 of culture, 2 x 10^6 ITSC or NTC were added to the cultures. The cells were harvested at the termination of culture and assayed for their capacity to generate PFC to SRBC or HRBC in the hemolytic plaque-forming assay.

4.2.23. The inhibition of the in vitro-induced secondary immune response by ITSF - Rabbits were immunizing IV with 10^9 SRBC or HRBC and sacrificed 7 or 60 days later. MNC (4 x 10^6 cells) were suspended in 4 ml of ITSF fortified with FCS (final conc. 10 percent) in sterile 10 ml plastic Falcon tubes. SSSF or HSSF (equivalent to 4 x 10^6 SRBC or HRBC, respectively) was added to the cultured cells (0.1 ml). The cultures were maintained in a stationary upright position for 5 days at 37°C in 5 percent CO_2 in air. The culture medium was replaced on day 3 of culture with fresh ITSF fortified with FCS. Controls consisted of splenic MNC cultured with SSSF in the presence of NTS containing FCS or Medium RPMI 1640 containing FCS. These cells were treated in the same way as the cells cultured with ITSF. The cells were harvested at the termination of culture and assayed for their capacity to generate PFC in the hemolytic plaque-forming assay.

4.2.24. The one-way mixed leucocyte culture reaction (MLR) - MNC treated with mitomycin-C were designated as stimulator cells. One ml cultures consisting of 0.5 ml responder (5 x 10^5) cells and 0.5 ml allogeneic stimulator (5 x 10^5) cells were incubated in 10 ml sterile Falcon plastic tubes at 37°C for 5 days in a humidified atmosphere of

5 percent CO_2 in air. Control cultures consisted of responder cells co-cultured with the appropriate number of mitomycin-C treated autologous cells. The culture medium consisted of the culture medium supplemented with heat-inactivated (56°C, 30 minutes) normal rabbit serum to a final concentration of 2.5 percent.

4.2.25. Preparation of SRBC and HRBC stromal sonicate filtrates (SSSF and HSSF, respectively) - The SRBC or HRBC were washed three times with HBSS and suspended in HBSS to a cell conc. of 2×10^9 per ml. One part RBC suspension was mixed with 5 parts sterile water in 50 ml sterile plastic tubes and the tubes were shaken vigorously for 1 hour, with occasional vortexing, to facilitate the lysis of the RBC. The tubes were centrifuged at 37,000 g for 40 minutes and the supernatants were discarded. The cell lysate was suspended in sterile water in plastic tubes and further shaken and vortexed for an additional 20 minutes. The tubes were centrifuged at 37,000 g and the supernatants were discarded. The washing of the lysate was repeated until the supernatants were clear following centrifugation. The RBC stroma was then suspended in saline, vortexed repeatedly for 15 minutes and centrifuged at 37,000 g for 40 minutes. The supernatant was decanted and the sedimented stroma was suspended in 5 ml sterile saline in sterile 10 ml Falcon tubes, cooled to 4°C, and placed in an ice bath. The cooled stroma were sonicated at 60,000 cycles per minute for 2 minutes following which the sonicates were filtered through 0.2 μ Nalgene filters and stored in 1 ml aliquots at -20°C.

4.2.26. Detection of Ig synthesis and Ig secreted into the culture medium - Ig synthesis by the rabbit splenic MNC (intracytoplasmic Ig) was determined by the conventional immunofluorescent (IF) assay using

fluorescein (FITC)-conjugated $F(ab)_2$ fragment of sheep anti-rabbit pan Ig. The cells were then examined by fluorescence microscopy.

Ig secreted by the immune splenic MNC into the culture medium was determined by the ELISA assay. Inert discs coupled with goat anti-rabbit IgG antibodies were incubated with aliquots of the cell culture supernatants (Step 1). After a 60 minute incubation period, the discs, were washed five times in PBS and incubated with the appropriate amount of goat anti-rabbit IgG antibodies complexed with alkaline phosphatase enzyme (Step 2). After another 60 minute incubation period, the discs were washed five times in PBS and transferred to tubes containing the chromogen, P-nitrophenyl phosphate. Following incubation for another 60 minutes, aliquots of the supernatant fluid were read in a colorimeter at 405 nm.

4.2.27. The effects of ITSF and NTS on antibody and Ig synthesis by immune rabbit splenic MNC - The splenic MNC of rabbits immunized with SRBC or HRBC 7 days previously were cultured in ITSF or NTS conditioned medium. The cells were stimulated in culture with SSSF, which is the solubilized form of the original immunizing antigen, SRBC. The reason for using SSSF rather than the whole SRBC is that SSSF does not neutralize or absorb ITSF whereas the SRBC do (Richter and Talor, 1986). Ig synthesis and secretion by the rabbit splenic MNC was determined by the IF and ELISA assays described above. The secretion of antibodies by these cells was determined by the ability of the cells to generate hemolytic plaques in the PFC assay (Richter and Talor, 1986).

4.2.28. Desalting and delipidation process of proteins in preparation for isoelectric focusing - An aliquot containing 150-300 µg protein was delipidated and desalted by 5 ml of freshly made mixture of acetone-

ethanol 1:1 (v/v) at -15°C. The contents of the tube were thoroughly mixed with a vortex-type mixer and allowed to stand overnight at -15°C. The protein was sedimented by centrifugation (15 min, 1300 g) and the solvent was aspirated. The precipitated protein was resuspended in the same volume of solvent mixture and stored at -15°C for 2 hrs, after which the protein was again sedimented and the solvent was aspirated. This last step was repeated again with a 1 hr incubation period. The protein was resuspended in 5 ml of diethyl ether at -15°C for 1 hr, and centrifuged, the solvent was aspirated and the protein dried under a weak stream of nitrogen and immediately dissolved with 200 µl of 8 M urea containing Tris (hydroxymethyl) aminomethane - HCl (10 mmol/L, pH 8.2) and dithiothreitol (30 mmol/L).

4.2.29. Gel isoelectric focusing - The gel was prepared from a stock solution made of; 20 g acrylamide and 0.55 g of N,N - methylene-bisacrylamide dissolved in deionized water to a final volume of 50 ml. 6.0 ml of the stock solution was added to 15.2 g urea and dissolved in 16.4 ml of deionized water. To this solution 1.4 ml of carrier ampholyte mixture was added. The ampholyte mixture was prepared by using Ampoline pH 4 - 6 and Ampholine pH 5 - 7 in a 1:1 ratio. The solution was de-aerated under reduced pressure. 34 µl of N,N - tetramethylene diamine (TEMED) and 136 µl of fresh 100 g/L ammonium persulphate solution were added to the latter solution with mixing. Immediately, the solution was added to a height of 110 mm in 5 mm (i.d.) x 125 mm long tubes (The polymerization progressed at room temperature for 1 hr).

The focusing was carried out in a Bio-Rad Mode - 175 gel electrophoresis cell, at 4°C. The lower chamber (anode) was filled with 10 mmol/L phosphoric acid and the upper chambers (cathode) was filled

with 20 mmol/L NaOH. The gels were prefocused for 2 hrs with a current of 1mA per gel rod, and the power supply, was sent to a limiting value of 500 v. Following prefocusing, the dissolved protein specimens were applied to the top of the gels and the focusing was carried out for 22 hrs at 500 v, at current of 2mA per gel rod. Following which the gels were removed from the tubes into tubes containing 0.4 g/L solution of Coomassie Brilliant Blue G 250 in perchloric acid solution (35 g/L). The contents of the tubes were continuously mixed by inversion on a rotator for 2 hrs. The excess stain was removed from the gels by transferring them into tubes containing dilute (100 ml/L) acetic acid with gentle shaking for 48 hrs.

4.2.30. Agglutinating antibody titer determination

Nonimmune or immune rabbits were bled (5 ml) by venipuncture. The blood was placed in a 10 ml sterile plastic tube, let to coagulate at room temperature ($21^{o}C \pm 2^{o}C$) and centrifuged at 150 g for 10 minutes. The serum was collected and spun at 1200 g for 10 minutes to eliminate any residual cells. The cell-free serum was collected into a 5 ml sterile plastic tube and decomplemented by heating (56^{o} for 30 minutes). Doubling dilutions (i.e. 1:5; 1:10; 1:20...etc.) were made of the decomplemented serum. 200 µl of the diluted serum was placed in a round-bottom well microtiter-plate and 20 µl of a 1% solution of RBC (SRBC, HRBC, HuRBC or RRBC) in saline (v/v) was added. The control consisted of 200 µl of saline and 1% of RBC solution. The contents of the wells were mixed well and left to develop, at a stationary phase, at room temperature for 8 to 12 hrs. The agglutinating antibody titer (i.e. the highest dilution of the serum which still gave a latice arrangement at the bottom of the well) was determined. The agglutinating antibody (Ab) titer was

calculated using the formula:

$$\text{agglutinating AB titer} = \frac{1}{\frac{1}{\text{dilution}}}$$

5. RESULTS

5.1. Introductory statement

The overall objectives of this investigation were as follows: (i) to determine whether antigen-specific suppressor cells can be detected in the thymus following primary and secondary IV immunization, (ii) to identify and characterize the suppressor cells, (iii) to determine whether the suppressor cells exert their suppressive effect by the secretion of a soluble suppressor factor, and (iv) to define a physiological role for the suppressor cells and the suppressor factor(s) in vivo.

For ease of presentation, the results section is divided into seven major subsections. The first deals with the demonstration of suppressor cells in the thymus of the IV immunized rabbit post-primary and post-secondary immunization capable of secreting a factor which can suppress the secretion of antibodies from autologous and allogeneic AFC in vitro. The second deals with the antigenic specificity of the suppressor cells and the suppressor factor(s) secreted by the suppressor thymocytes. The third deals with the phenotypic characterization of the suppressor cell, and the target cell(s) for the suppressor factor. The fourth addresses the questions of the temporal relationship between immunization, the dose of antigen injected IV, and the appearance of suppressor cells in the thymus in vivo and their ability to secrete a suppressor factor(s) upon incubation in vitro. The fifth deals with the ability of both the suppressor cell and the suppressor factor to suppress the primary immune response in vivo and the secondary immune response in vitro. The sixth deals with the ability of the suppressor factor(s) to inhibit antibody but not non-specific Ig synthesis by immune rabbit splenic MNC in vitro. The last section deals with initial attempts to characterize the suppressor

factor(s).

All of the experiments with few exceptions were repeated a minimum of five times unless otherwise stated. The majority of the experiments were repeated more than 10 times. Each experiment was carried out in duplicate. For ease of presentation, the results of representative experiments are presented in the tables and the figures.

It should be noted that the mean variation of the results between experiments and within any given experiment, expressed as the number of PFC per 10^6 MNC or splenic MNC, did not at any time exceed 5 percent and was therefore omitted from the data presented in the tables.

In reading the data presented in the tables, <5 PFC per 10^6 MNC means that only 1 to 5 PFC per 10^6 MNC or splenic MNC were detected.

In our system , there was no cross-reactivity detected between the following antigens: SRBC, HRBC, HuRBC, and RRBC as can be seen in the schematic representation presented below:

	Parameters tested							
	SRBC		HRBC		HuRBC		RRBC	
	Ab[b]	PFC[b]	Ab	PFC	Ab	PFC	Ab	PFC
Rabbits immunized with[a]								
SRBC	++	++	-	-	-	-	-	-
HRBC	-	-	++	++	-	-	-	-
HuRBC	- (+)?	-	-	-	++	++	-	-
RRBC	-	-	-	-	-	-	++	++

[a] Rabbits were immunized IV with 10^9 SRBC, HRBC, HuRBC or RRBC and sacrificed 7 days later.

[b] The serum agglutination antibody titers and the number of PFC were determined at sacrifice.

5.2. The demonstration of suppressor cells in the thymus of the immunized rabbit capable of secreting a suppressor factor in vitro

5.2.1. Rational and objectives

As discussed in Chapter 2., AFC are detected primarily in the spleen following 1^{o}IMM IV in vivo and never in the thymus. Furthermore, only the spleen possesses memory cells during the first 3-4 months following 1^{o}IMM IV since a 2^{o}IM in vitro can only be induced with splenic MNC. However, the thymus contains memory cells by 6 months post 1^{o}IMM IV since a 2^{o}IM in vitro can be induced at this time with thymic and splenic MNC. These results suggested that the failure to detect AFC in the thymus in the former instance might be due to the activation of suppressor cells in the thymus which would suppress any incepient immune response in that organ.

The OBJECTIVES in this part of the investigation were to (i) demonstrate the presence of suppressor cells in the thymus of the IV immunized rabbit and (ii) to demonstrate whether the suppressor cells secrete a suppressor factor.

5.2.2. Experimental protocols

Immunologically mature outbred New Zealand White rabbits were immunized IV with 10^{9} SRBC (1^{o}IMM) as described in Chapter 4.2.11. In addition a number of rabbits were given a second IV injection of 10^{9} SRBC (2^{o}IMM) 3 months following 1^{o}IMM. The animals were sacrificed at various times following 1^{o} and 2^{o}IMM IV and MNC suspensions were prepared from the different lymphoid organs and the blood as described in Chapter 4.2.11. The MNC were assessed for their capacity to generate hemolytic plaques in the PFC assay as described in Chapter 4.2.12.

The MNC of the different lymphoid organs were coincubated in vitro with autologous or allogeneic splenic MNC prior to the PFC assay in order

to demonstrate the presence of suppressor cells as described in Chapter 4.2.14.

The MNC of the different lymphoid organs were incubated in vitro and the cell-free culture supernatants were investigated for the presence of a suppressor factor as described in Chapter 4.2.15.

Each of the two lobes of the thymus obtained 7 days post 1^{o}IMM IV with 10^{9} SRBC was cut into equal parts and the thymocytes prepared from each of these thymic segments were assessed for the presence of suppressor cells and their ability to secrete a suppressor factor as described in Chapters 4.2.14. and 4.2.15., respectively.

The MNC were cultured for 5 days with the antigen in order to induce a 2^{o}IM in vitro as described in Chapter 4.2.13. These cultured MNC were assessed for PFC in the same manner as described above.

5.2.3. Experimental results

Following IV immunization of outbred rabbits with 10^{9} sheep erythrocytes (SRBC), antibody-forming cells (AFC) detected by the hemolytic plaque-forming cell (PFC) assay were detected primarily in the spleen between days 4 and 40, with the peak on day 7. Some PFC were detected in the blood on days 7 and 10 post-1^{o}IMM and a few PFC were detected in the bone marrow on days 7, 10 and 14 post-1^{o}IMM. No PFC were detected in any of the other lymphoid organs (Table 1).

The splenic MNC generated by far the most PFC following culture with the SRBC in vitro. It should be noted that there is a marked diminution in the number of PFC generated in the 2^{o}IM in vitro by the splenic MNC obtained from rabbits sacrificed between days 14 and 22 following 1^{o}IM. The circulating MNC gave rise to many PFC in culture only on day 7 post-1^{o}IMM. None of the other lymphoid cells generated PFC in culture

(Table 1).

When the 7-day post-1°IMM splenic MNC were coincubated with autologous thymus cells for 4 hrs, the spleen MNC were unable to generate PFC (Table 2). The cells of the other autologous lymphoid organs and the circulating MNC were uniformly incapable of inhibiting the splenic AFC from generating PFC (Table 2). Identical results were obtained with cells of rabbits assayed 7 days post-2°IMM IV (Table 3). A minimum of 4 hrs coincubation with autologous thymus cells was necessary in order to totally inhibit the formation of PFC by the immune splenic AFC (Table 4). Coincubation with the thymus cells for 2 hrs resulted in an 80 percent suppression of the PFC by the splenic AFC. None of the cells of the other lymphoid organs or the circulation were able to suppress the generation of splenic PFC following 4 hrs of coincubation (Table 4). Furthermore, no inhibition of splenic PFC was observed even when the period of coincubation of these cells was extended to 8 hrs (results not presented in Tables).

When constant numbers of immune splenic MNC were coincubated with decreasing numbers of autologous immune thymus cells for 4 hrs, a 10 percent "contamination" of thymus cells was sufficient to inhibit the splenic PFC by over 60 percent (Table 5).

The thymus cells of rabbits immunized IV 7 days prior to sacrifice inhibited the generation of PFC by both autologous and allogeneic 7-day-immune splenic MNC; the thymus cells of unimmunized rabbits exhibited no suppressive effects on the generation of PFC by allogeneic immune splenic PFC (Table 6). The suppressor cells in the thymus of the immunized rabbit which suppress PFC formation by autologous splenic PFC are referred to as immune thymus suppressor cells or ITSC.

The lymphoid cells of the different lymphoid organs of 7-day SRBC immune rabbits were incubated for 1, 2 or 4 hrs at 37°C in the absence of SRBC, centrifuged, and the cell-free supernatants were incubated with autologous 7-day immune splenic MNC for 4 hrs prior to assay for PFC. Only the cell-free supernatants of the cultured immune thymus cells were able to suppress the generation of PFC by the immune splenic MNC (Table 7). The 1 hr thymus cell culture supernatant suppressed the generation of PFC by 70 to 80 percent whereas the 4 hr thymus cell culture supernatant consistently totally suppressed the generation of PFC (Table 7). The suppressor factor(s) secreted by the immune thymocytes (ITSC) in vitro is referred to as immune thymus suppressor factor or ITSF.

The splenic MNC from SRBC immunized rabbits were incubated for 1, 2 or 4 hrs with the 4 hr culture supernatants of autologous thymocytes (4 hr autologous ITSF) in order to determine the minimum incubation time required for ITSF to inhibit the generation of SRBC PFC. A 1 hr incubation was sufficient to inhibit the generation of PFC by 70 to 80 percent and a 2 hr incubation was almost totally suppressive (Table 8). Identical results were obtained with a 4-hr allogeneic ITSF (results not presented in Tables). No suppressor factor was detected in the supernatants of 4 hr cultures of autologous (Table 8) and allogeneic (results not presented in Tables) lymphoid cells of the other lymphoid organs. Furthermore, none of the cells of the lymphoid organs of the unimmunized rabbit secreted a suppressor factor in culture (results not presented in Tables).

Thymocytes were coincubated with autologous or allogeneic immune splenic MNC (7 days post-1°IMM with 10^9 SRBC IV) for 4 hrs at 4°C, 21°C, 37°C or 39°C. The cells were then centrifuged, washed and assayed for

PFC. As can be seen in Table 9 the cells coincubated at 4°C generated normal numbers of PFC. However, the cells coincubated at 21°C generated less than 10 to 15 percent of the PFC generated by splenic MNC incubated by themselves at 21°C. The cells coincubated at 37°C and 39°C generated no PFC (Table 9). Similarly, the thymocytes incubated by themselves for 4 hrs at 4°C failed to secrete ITSF since these thymocyte culture supernatants failed to inhibit immune splenic PFC following their incubation with the splenic MNC for 4 hrs at 37°C prior to the PFC assay. The thymocytes incubated by themselves at 21°C secreted ITSF capable of inhibiting the PFC response by over 90 percent and thymocytes incubated by themselves at 37°C and at 39°C secreted ITSF capable of totally inhibiting the PFC (Table 9). At no time were thymocytes obtained from unimmunized rabbits capable of inhibiting the PFCs or secreting ITSF (Table 9).

ITSC generated in the thymus 7 days post-IV 1°IMM with 10^{9} SRBC appear to be disseminated throughout the thymus since ITSC of equal suppressive activity capable of secreting ITSF were detected in all four segments of the thymus (Table 10).

5.2.4. Commentary

Seven days following primary or secondary IV immunization with 10^{9} SRBC thymocytes capable of inhibiting the generation of hemolytic plaques by autologous or allogenic splenic AFC in the PFC assay were detected only in the thymus and in no other lymphoid organ nor in the circulation. The thymus suppressor cells or ITSC are capable of secreting a suppressor factor referred to as immune thymus suppressor factor or ITSF, following a short term (4 hr) incubation period.

ITSF by itself is capable of inhibiting the generation of hemolytic

plaques by autologous or allogeneic splenic AFC. Since the immune splenic AFC 7 days post immunization are synthesizing and secreting antibodies most actively as they generate the maximum number of PFC during the 1^{o}IM and 2^{o}IM, it is concluded that both ITSC and ITSF are capable of inhibiting the generation of hemolytic plaques by immune splenic AFC by inhibiting the secretion of antibodies from the antibody-synthesizing cells.

It is interesting to note that ITSC capable of secreting ITSF are not localized or restricted to any one focus in the thymus 7 days post-1^{o}IMM but rather appear to be dispersed throughout the thymus. These results suggest a polyclonal origin rather than a monoclonal origin for the ITSC.

Although, ITSC must be coincubated with the immune splenic AFC for 4 hrs in order to totally suppress the generation of PFC, ITSF must be incubated for only 2 hrs with the immune splenic AFC in order to totally suppress the generation of PFC. This result would be expected since it takes about 2 hrs under optimal culture conditions for ITSC to secrete detectable amounts of ITSF.

Suppression of PFC by ITSC, and the secretion of ITSF by ITSC, are active processes since they are temperature dependent. It must be noted that the failure by ITSC to inhibit PFC at 4^{o}C correlates with their inability to secrete ITSF. These results and those presented above strongly suggest that the suppression by ITSC is mediated by ITSF.

Note should be taken of the marked drop in the number of PFC induced in the 2^{o}IM in vitro by cultured splenic MNC obtained from rabbits between days 14 and 24 post-1^{o}IMM IV. These results suggest the presence of activated suppressor cells in the spleen at these times. This matter will be discussed in Chapter 6.

TABLE 1

IN VIVO GENERATED PFC (AFC) AND IN VITRO INDUCED PFC (MEMORY CELLS) BY CELLS OF THE DIFFERENT LYMPHOID ORGANS OF THE RABBIT FOLLOWING PRIMARY IV IMMUNIZATION WITH 10^9 SRBC

Cells of organs assayed	The number of PFC per 10^6 MNC at the following days after primary IV immunization with 10^9 SRBC								
	4	7	10	14	18	22	30	40	60
Spleen (SPL)	125 (850)[a]	450 (3454)	70 (1446)	33 (212)	24 (151)	27 (156)	53 (1374)	18 (1476)	5 (2480)
Circulation (PB)	0 (0)	185 (1990)	15 (78)	0 (0)	0 (0)	0 (0)	0 (0)	0 (<5)	0 (<5)
Bone Marrow (BM)	0 (0)	68 (0)	10 (0)	8 (0)	0 (0)	<5 (0)	<5 (0)	0 (0)	0 (0)
Thymus (THY)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Popliteal lymph node (PLN)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Appendix (APP)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Peyer's patches (PP)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Sacculus rotundus (SR)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)

a The figures outside and inside the brackets represent the numbers of immediate PFC detected in the freshly isolated cells and the PFC detected following culture of the cells with the antigen for 5 days in vitro, respectively.

TABLE 2

THE CAPACITY OF THYMUS CELLS, BUT NOT OTHER LYMPHOID CELLS, OF THE IMMUNIZED (PRIMARY IMMUNIZATION) RABBIT TO SUPPRESS THE GENERATION OF HEMOLYTIC PLAQUES BY AUTOLOGOUS SPLENIC AFC IN THE PFC ASSAY

Cells of organs assayed	The number of PFC per 10^6 MNC 7 days post-primary IV immunization		
	I	II	III
(10^6 per ml)			
SPL	170	460	360
THY	0	0	0
PLN	0	<5	0
BM	<5	<5	<5
PB	100	200	160
SR	0	0	0
APP	<5	<5	0
PP	0	0	0
	The number of PFC per 10^6 splenic MNC		
($5x10^5 + 5x10^5$ cells per ml)[a]			
SPL + THY	0	<5	<5
SPL + PLN	170	330	340
SPL + BM	160	340	360
SPL + APP	280	400	330
SPL + PP	180	360	320
SPL + SR	150	330	330
SPL + PB	180	440	ND[b]

a The cells were coincubated in Medium RPMI 1640 at 37°C in 5% CO_2 in air for 4 hours prior to their incorporation into the PFC assay.

b ND = not done

TABLE 3

THE CAPACITY OF THYMUS CELLS, BUT NOT OTHER LYMPHOID CELLS OF THE IMMUNIZED (SECONDARY IMMUNIZATION) RABBIT TO SUPPRESS THE GENERATION OF HEMOLYTIC PLAQUES BY AUTOLOGOUS SPLENIC AFC IN THE PFC ASSAY

Cells of organs assayed	The number of PFC per 10^6 MNC 7 days post-secondary IV immunization		
	I	II	III
(10^6 per ml)			
SPL	300	229	285
THY	0	0	0
PLN	15	30	10
BM	150	120	165
PB	70	55	80
SR	0	0	0
APP	0	0	0
PP	0	0	0
	The number of PFC per 10^6 splenic MNC		
($5x10^5$ + $5x10^5$ cells per ml)[a]			
SPL + THY	<5	<5	0
SPL + PLN	330	250	270
SPL + BM	380	310	350
SPL + APP	280	210	255
SPL + SR	265	235	265
SPL + PP	290	220	280
SPL + PB	315	295	330

a The cells were coincubated in Medium RPMI-1640 at 37°C in 5% CO_2 in air for 4 hours prior to their incorporation into the PFC assay.

TABLE 4

"IMMUNE" THYMUS CELLS INHIBIT THE GENERATION OF HEMOLYTIC PLAQUES BY AUTOLOGOUS SPLENIC AFC IN THE PFC ASSAY AS A FUNCTION OF THE TIME OF COINCUBATION OF THE AUTOLOGOUS LYMPHOID MNC PRIOR TO THE ASSAY

Cells of organs assayed	The number of PFC per 10^6 MNC[a] preincubated for the following periods of time (hours)			
	0	1	2	4
(10^6 cells per ml)				
SLP	460	360	540	380
THY	0	0	0	0
BM	<5	<5	<5	<5
PLN	0	<5	0	0
APP	<5	<5	0	<5
SR	0	0	0	0
PP	0	0	0	0
PB	110	95	135	100
	The number of PFC per 10^6 splenic MNC			
($5x10^5 + 5x10^5$ per ml)				
SPL + THY	420	360	60	5
SPL + BM	410	470	400	340
SPL + PLN	310	270	420	330
SPL + APP	470	380	286	340
SPL + SR	560	480	476	490
SPL + PP	540	556	510	560
SPL + PB	490	520	630	450

a The cells were obtained from the rabbit 7 days following 1^{o}1M with 10^9 SRBC IV.

TABLE 5

"IMMUNE" THYMUS CELLS INHIBIT THE GENERATION OF HEMOLYTIC PLAQUES BY AUTOLOGOUS SPLENIC AFC IN THE PFC ASSAY AS A FUNCTION OF THE RATIO OF SPLENIC TO THYMIC CELLS INCUBATED PRIOR TO THE ASSAY

Ratio of splenic MNC(S) to thymocytes(T) (S:T)[b]	The number of PFC per 10^6 splenic MNC[a] following the incubation (4 hours) of splenic MNC with thymocytes in varying ratios			
	I	II	III	IV
1:0	190	210	490	455
1:1	<5	<5	0	<5
2:1	<5	<5	<5	<5
4:1	30	<5	<5	25
8:1	50	40	75	70
10:1	85	75	120	105
16:1	185	155	215	230
0:1	0	0	0	0

a The cells were obtained from the rabbit 7 days following $1^{o}1M$ with 10^9 SRBC IV.

b The number of splenic MNC is constant throughout (10^6 per ml). The number of thymocytes is varied.

TABLE 6

THE ABILITY OF THYMUS CELLS OF IMMUNIZED, BUT NOT OF UNIMMUNIZED, RABBITS TO SUPPRESS THE GENERATION OF HEMOLYTIC PLAQUES BY ALLOGENEIC IMMUNE SPLENIC AFC IN THE PFC ASSAY

The number of PFC per 10^6 splenic MNC following incubation (4 hours) of splenic MNC with normal allogeneic thymocytes, or autologous or allogeneic "immune" thymocytes obtained 7 days post-primary IV immunization.

	I	II	III	IV
SPL (A)[a]	190	490	565	260
SPL (B)[a]	210	455	480	305
SPL (C)[a]	0	0	0	0
THY (A)	0	0	0	0
THY (B)	0	0	0	0
THY (C)	0	0	0	0
SPL (A) + THY (A)	<5	<5	<5	<5
SPL (B) + THY (B)	<5	<5	<5	0
SPL (A) + THY (B)	<5	0	<5	0
SPL (B) + THY (A)	<5	<5	<5	<5
SPL (A) + THY (C)	190	360	590	190
SPL (B) + THY (C)	270	320	470	220

a Rabbits A and B were immunized IV with 10^9 SRBC. They were sacrificed 7 days later. Rabbit C was not immunized.

TABLE 7

THE SUPERNATANTS OF ITSC BUT NOT OF THE CELLS OF THE OTHER LYMPHOID ORGANS INCUBATED FOR 4 HOURS INHIBIT THE GENERATION OF HEMOLYTIC PLAQUES BY AUTOLOGOUS SPLENIC AFC IN THE PFC ASSAY

Cells of lymphoid organs incubated	Supernatants collected following incubation for[b]	The number of PFC per 10^6 splenic MNC[a] following incubation of the splenic MNC with the cell-free supernatants for 4 hours prior to the PFC assay.	
		I	II
THY[a]	0 hr	140	375
	1 hr	50	70
	2 hr	20	<5
	4 hr	0	<5
SPL[a]	4 hr	180	425
PLN[a]	4 hr	180	340
BM[a]	4 hr	165	395
PB[a]	4 hr	170	400
APP[a]	4 hr	185	420
SR[a]	4 hr	170	395
PP[a]	4 hr	165	405

a The cells were obtained 7 days following IV immunization with 10^9 SRBC.

b The cells were incubated in 5 ml Medium RPMI 1640 (5×10^7 cells per ml) at 37°C in 5% CO_2 in air.

TABLE 8

ITSF INHIBITS THE GENERATION OF HEMOLYTIC PLAQUES BY AUTOLOGOUS SPLENIC AFC IN THE PFC ASSAY AS A FUNCTION OF THE TIME OF COINCUBATION OF THE SPLENIC AFC WITH THE ITSF

Supernatants of the cells of the following lymphoid organs incubated for 4 hr[b]	The number of PFC per 10^6 splenic MNC[a] following incubation of the splenic MNC with the cell-free supernatants for the following periods of time (hours) prior to the PFC assay.			
	0	1	2	4
THY[a]	285	85	10	5
SPL[a]	310	390	285	300
PLN[a]	295	275	265	280
BM[a]	300	280	320	315
PB[a]	325	275	290	285
APP[a]	300	310	290	290
SR[a]	310	290	280	280
PP[a]	320	300	320	300

a The cells were obtained from the rabbit 7 days following IV immunization with 10^9 SRBC.

b The cells were incubated in 5ml Medium RPMI 1640 (5×10^7 cells per ml) at 37°C in 5% CO_2 in air.

TABLE 9

ITSC INCUBATED AT 4°C FOR 4 HOURS FAIL TO INHIBIT THE GENERATION OF HEMOLYTIC PLAQUES BY IMMUNE SPLENIC APC IN THE PFC ASSAY AND FAIL TO SECRETE ITSF

Immune splenic MNC[a] incubated with (I) ITSC at the following temperature, or (II) ITSF secreted by ITSC at the following temperature	The number of PFC per 10^6 splenic MNC following incubation of the immune splenic MNC with ITSC or ITSF obtained from the thymus of rabbits.			
	A[b]	B[b]	C[b]	D[c]
4°C				
I	350	320	360	340
II	360	330	310	315
21°C				
I	40	30	10	290
II	20	<5	<5	280
37°C or 39°C				
I	<5	0	<5	355
II	0	<5	<5	340

a The rabbit was immunized with 10^9 SRBC IV and sacrificed 7 days later.

b The thymus was obtained from a rabbit (autologous or allogeneic) immunized with 10^9 SRBC IV and sacrificed 7 days later.

c The thymus was obtained from a normal, unimmunized rabbit.

TABLE 10

THE INTRATHYMIC DISTRIBUTION OF ITSC

Cells assayed for suppressor activity[a]	The number of PFC per 10^6 splenic MNC following incubation of the immune splenic MNC with either autologous ITSC or ITSF for 4 hrs at 37°C prior to the PFC assay[b].					
	I		II		III	
	ITSC	ITSF	ITSC	ITSF	ITSC	ITSF
THY_1	5	0	10	<5	<5	<5
THY_2	10	<5	0	<5	<5	0
THY_3	0	0	<5	0	10	<5
THY_4	<5	<5	<5	0	<5	<5
SPL (Control)	340		365		290	

a The rabbits were immunized IV with 10^9 SRBC and sacrificed 7 days later. The thymus was removed and each lobe was cut in half to yield four segments of equal size referred to as THY_1, THY_2, THY_3 and THY_4.

b The splenic MNC ($5x10^5$ in 0.5ml culture medium) obtained 7 days post-IV immunization were incubated for 4 hr at 37°C with either (i) autologous thymocytes from THY_1, THY_2, THY_3 or THY_4 (ITSC) ($5x10^5$ cells in 0.5ml culture medium) or (ii) ITSF secreted by the ITSC (0.5ml ITSF).

5.3. The antigenic specificity of the immune thymus suppressor cell (ITSC) and the immune thymus suppressor factor (ITSF)

5.3.1. Rational and objectives

In the preceding section it was demonstrated that suppressor cells are detected in the thymus of the rabbit immunized IV 7 days previously with 10^9 SRBC. These suppressor cells inhibited the secretion of antibodies from the autologous or allogeneic immune splenic AFC since they could inhibit the generation of hemolytic plaques by the antibody secreting AFC in the PFC assay following a short term (4 hrs) coincubation of the suppressor cells and the immune splenic AFC in vitro. The suppressor cells were detected only in the thymus of the immunized rabbit and in no lymphoid organ of the unimmunized rabbit. The immune thymus suppressor cell or ITSC, during short term culture (4 hr, $37^{\circ}C$) in antigen-free and serum-free mediums, secreted a factor with suppressor activity identical to that exhibited by the ITSC. This factor is referred to as immune thymus suppressor factor or ITSF.

The OBJECTIVE in this part of the investigation was to demonstrate the antigenic specificity of the ITSC and the ITSF.

5.3.2. Experimental protocol

The rabbits were immunized IV with SRBC and/or HRBC as described in Chapter 4.2.11. The rabbits were sacrificed 7 days post immunization and the MNC were prepared from the different lymphoid organs and the blood as described in Chapter 4.2.11. The immune splenic MNC were tested for AFC by the hemolytic PFC assay as described in Chapter 4.2.12. The ITSC and ITSF were assayed for their capacities to inhibit the generation of hemolytic plaques by the immune splenic AFC as described in Chapters 4.2.14. and 4.2.15, respectively. The thymocytes were depleted of cells which rosetted with SRBC and/or HRBC as described in Chapter 4.2.17.

ITSF was incubated with 10^3, 10^4, 10^5, 10^6, 10^7, 10^8 and 10^9 SRBC and with 10^7, 10^8, 10^9 HRBC, HuRBC or RRBC for 1 hr at.37°C, centrifuged and the cell-free supernatants were assayed for ITSF activity as described in Chapter 4.2.15.

5.3.3. Experimental results

Evidence for the antigenic specificity of the ITSC is provided in Table 11. The thymus cells of rabbits, immunized IV with SRBC and HRBC simultaneously 7 days previously, depleted of cells which rosetted with SRBC lost the capacity to suppress the generation of SRBC PFC but not HRBC PFC by the autologous immune spleen cells (Table 11). Similarly, the thymus suppress the generation of HRBC PFC but not SRBC PFC by the autologous immune spleen cells (Table 11). As expected, the SRBC and HRBC rosetted thumus cells could, by themselves, suppress the generation of SRBC PFC and HRBC PFC, respectively, by the autologous immune spleen cells. The thymus cells depleted of both SRBC and HRBC rosetted cells could not inhibit the generation of either SRBC PFC or HRBC PFC by the autologous immune spleen cells (Table 11).

The ITSF, like its parent ITSC suppressor cell, is antigen specific. The ITSF generated by thymus cells of SRBC-immunized rabbits was markedly diminished in its capacity to suppress PFC to SRBC following prior absorption with as few as 10^4 SRBC (Table 12). On the other hand, 10^9 HRBC, HuRBC or RRBC (autologous or allogeneic) were not as effective as 10^4 SRBC in their capacity to absorb SRBC-directed ITSF (Table 12). Similarly, ITSF generated by HRBC-immune thymus cells was diminished in its activity following absorption with 10^4 HRBC and not by 10^9 SRBC, RRBC or HuRBC (results not presented in tables). The ITSF secreted by thymus cells of rabbits immunized simultaneously with two non-cross-reactive

antigens, SRBC and HRBC, lost the capacity to inhibit the generation of SRBC PFC by autologous immune spleen MNC if it was absorbed with 10^9 SRBC and not with 10^9 HRBC (Table 13). Similarly, the ITSF lost the capacity to inhibit the generation of HRBC PFC if it was absorbed with 10^9 HRBC and not with 10^9 SRBC (Table 13).

5.3.4. Commentary

The results of the above experiments demonstrate unequivocally the antigenic specificity of both the ITSC and the ITSF. The thymocytes from rabbits immunized simultaneously with both SRBC and HRBC and depleted of cells which rosetted with SRBC retained the capacity to inhibit the generation of HRBC PFC but lost the ability to inhibit the generation of SRBC PFC by autologous (Table 11) or allogeneic (results not presented in Tables) immune splenic AFC. Similarly, the thymocytes depleted of cells which rosetted with HRBC retained the capacity to inhibit the generation of SRBC PFC but lost the ability to suppress HRBC PFC.

ITSF generated by thymocytes of rabbits immunized with SRBC was removed from the cell-free culture supernatants of these cells by absorption with SRBC but not with the non-cross reacting antigens HRBC, HuRBC or RRBC. Furthermore, the ITSF generated by thymocytes of rabbits immunized simultaneously with SRBC and HRBC and absorbed with SRBC (or HRBC) retained the capacity to inhibit the generation of HRBC PFC (SRBC PFC) but lost the ability to inhibit the generation of SRBC PFC (HRBC PFC).

TABLE 11

THE ANTIGENIC SPECIFICITY OF THE ITSC OBTAINED FROM RABBITS SEVEN DAYS POST-PRIMARY INTRAVENOUS IMMUNIZATION WITH SRBC AND HRBC

Splenic MNC incubated with the following autologous thymocytes (1:1) for 4 hrs prior to the PFC assay[a]	The number of PFC per 10^6 splenic MNC							
	SRBC PFC				HRBC PFC			
	I	II	III	IV	I	II	III	IV
Untreated thymocytes	0	0	<5	0	0	0	0	<5
Thymocytes depleted of SRBC rosetted cells	270	200	190	245	0	<5	<5	<5
Thymocytes depleted of HRBC rosetted cells	<5	<5	<5	0	220	206	155	170
Thymocytes depleted of SRBC and HRBC rosetted cells	290	240	205	195	220	140	145	175
SRBC rosetted thymocytes	<5	<5	<5	0	240	120	160	200
HRBC rosetted thymocytes	350	220	135	210	<5	<5	<5	<5
SRBC and HRBC rosetted thymocytes	<5	<5	<5	0	<5	0	<5	<5
CONTROLS	The number of PFC per 10^6 MNC							
Spleen cells alone	330	240	225	265	180	145	185	210
Thymus cells alone (untreated)	0	0	0	0	0	0	0	0

a Rabbits were immunized with 10^9 SRBC and 10^9 HRBC IV and sacrificed 7 days later.

TABLE 12

THE ANTIGENIC SPECIFICITY OF SRBC-DIRECTED ITSF. THE FAILURE OF NON-CROSS-REACTING ERYTHROCYTE ANTIGENS TO ABSORB ITSF

ITSF absorbed with [a]		The number of PFC per 10^6 MNC following incubation of the SRBC-immune splenic MNC[b] with the absorbed autologous ITSF for 4 hr at 37°C prior to the PFC assay.	
		I	II
Not absorbed		<5	<5
SRBC	10^3	45	75
	10^4	105	155
	10^5	145	185
	10^6	195	240
	10^7	225	235
	10^8	240	255
	10^9	255	280
HRBC	10^7	0	0
	10^8	4	<5
	10^9	50	65
HuRBC	10^7	<5	<5
	10^8	<5	50
	10^9	80	60
RRBC	10^7	<5	<5
	10^8	60	50
	10^9	70	80

CONTROLS		Number of PFC per 10^6 MNC	
		I	II
SPL	MNC alone	275	340

a ITSF was incubated with the RBC for 1 hr at 37°C in 5% CO_2 in air followed by centrifugation. The cell-free supernatants were then tested for ITSF activity by their ability to inhibit PFC following coincubation with autologous splenic AFC.

b The rabbits were immunized with 10^9 SRBC IV and sacrificed 7 days later.

TABLE 13

THE ANTIGENIC SPECIFICITY OF ITSF. THE SIMULTANEOUS IMMUNIZATION WITH TWO NON-CROSS-REACTIVE ANTIGENS, SRBC AND HRBC, RESULTS IN THE GENERATION OF A DISTINCT ITSF TO EACH OF THESE ANTIGENS

Immune splenic MNC[a] incubated for 4 hrs with	The number of PFC per 10^6 MNC					
	SRBC PFC			HRBC PFC		
	I	II	III	I	II	III
Medium	340	250	230	190	150	170
Unabsorbed ITSF	0	<5	0	0	0	<5
ITSF absorbed with SRBC[b]	265	220	200	0	<5	<5
ITSF absorbed with HRBC[b]	0	<5	0	175	160	145
ITSF absorbed with SRBC and HRBC[b]	290	240	210	160	125	140

a The rabbits were immunized with 10^9 SRBC and 10^9 HRBC IV and sacrificed 7 days later.

b The ITSF was incubated with 10^9 SRBC and/or 10^9 HRBC for 1 hr at 37°C in 5% CO_2 in air followed by centrifugation. The cell-free supernatants were then tested for ITSF activity by their ability to inhibit the PFC following coincubation with autologous splenic AFC.

5.4. The phenotypic characterization of the suppressor cell, and the identity of the target cell(s) for the suppressor cell (ITSC) and the suppressor factor (ITSF)

5.4.1. Rational and objectives

The results presented in the preceding section indicate that the suppressor cell (ITSC) detected in the thymus of the rabbit 7 days post-primary IV immunization, and the suppressor factor (ITSF) operated by these thymocytes display identical antigenic specificity with respect to their suppressor activity. However, no data was presented in the previous sections regarding the phenotypical characteristics of the ITSC.

Evidence was also presented indicating that both ITSC and ITSF suppress the generation of hemolytic plaques by the immune splenic MNC. The splenic MNC consist of a number of distinct cells of different functions in addition to the AFC B cell - macrophage, T cell and Null cell. It must therefore be established whether the ITSC and ITSF act directly on the AFC B cell resulting in the suppression of the PFC, or whether they act on one or more of the other cells which then mediate(s) the suppression of the PFC.

The OBJECTIVES in this part of the investigation were (i) to identify the ITSC in terms of its surface membrane receptors and (ii) to identify the target cell for the ITSC and ITSF.

5.4.2. Experimental protocol

The rabbits were immunized with 10^9 SRBC IV as described in Chapter 4.2.11. The rabbits were sacrificed 7 days post-1^{o}IMM IV and the thymocytes and splenic MNC were prepared as described in Chapters 4.2.11. and 4.2. 16. The thymocytes were incubated with horse anti rabbit T cell serum (HARTS) and complement or with horse anti rabbit B cell serum (HARBMS) and complement in order to eliminate the T and B cells,

respectively, as described in Chapter 4.2.17.

The thymocytes were rosetted with EAG and EAC indicator erythrocytes and depleted of EAG (Null cells) and EAC (B cells) cells rosetting cells as described in Chapter 4.2.17. Thymocytes were also depleted of macrophages as described in Chapter 4.2.9.

The assays for PFC by the immune splenic MNC and the inhibitor of the generation of the PFC by ITSC and ITSF were carried out as described in Chapters 4.2.12., 4.2.14., 4.2.15., respectively.

5.4.3. Experimental results

The EAG-rosetted thymocytes were capable of suppressing the splenic PFC whereas the EAC-rosetted thymocytes were incapable of suppressing the splenic PFC (Table 14). Conversely, the thymocytes depleted of EAG-rosetting cells were unable to suppress the splenic PFC whereas the thymocytes depleted of EAC-rosetting cells retained all of the suppressor activity. Furthermore, the thymocytes depleted of T cells following incubation for 1 hr with HARTS and guinea pig complement no longer possessed detectable suppressor cells (ITSC) and the cells did not secrete detectable ITSF (Table 14). On the other hand, the thymocytes incubated for 1 hr with HARBMS and guinea pig complement retained essentially all suppressor cell (ITSC) activity and the cells secreted ITSF. It should also be noted that elimination of macrophages from the thymocytes once, or twice over a 24 hr period, did not affect the thymic ITSC suppressive activity nor the capacity of the ITSC to secrete ITSF (Table 14).

As can be seen in Table 15 the target cell in the immune splenic MNC for the ITSC and the ITSF is the AFC/B cell since the immune splenic MNC depleted of MØ, T cells and Null cells failed to generate PFC if

first incubated for 4 hrs with ITSC or ITSF. These MØs, T cell and Null cell depleted immune splenic MNC gave large numbers of PFC as they were incubated for 4 hrs in medium (Table 15).

5.4.4. Commentary

The results in this part of the investigation demonstrate conclusively that the ITSC is a T cell with a receptor for Fc(G) since it could only be eliminated from the immune thymocytes following rosetting with the EAG indicator cells or treatment with anti rabbit T cell serum and complement. Since the ITSC rosette with the immunizing antigen (SRBC or HRBC), as demonstrated in Chapter 5.3., it may be concluded that the ITSC has receptors for both the immunizing antigen and Fc(G).

The results also demonstrate that the target cell for the ITSC and the ITSF in the immune splenic MNC is the AFC/B cell since the immune splenic MNC depleted of T cells, Null cells and MØs generated many more PFC than did the untreated splenic MNC. This result was anticipated since the elimination of T cells, Null cells and MØs from the immune splenic MNC results in an increased concentration of the B cells in the remaining cells.

TABLE 14

THE IDENTIFICATION OF ITSC AS A T CELL WITH A RECEPTOR FOR Fc(G)

Autologous thymocytes assayed[a]	The number of PFC per 10^6 MNC following coincubation of the splenic MNC[a], with the autologous thymocytes (THY-C) or the culture supernatants of these cells (THY-S)[b] for 4 hrs at 37°C prior to the PFC assay.					
	I		II		III	
	THY-C	THY-S	THY-C	THY-S	THY-C	THY-S
Untreated thymocytes	0	0	<5	0	0	<5
EAG-rosetted thymocytes	0	<5	<5	10	0	<5
Depleted of EAG rosetted thymocytes	230	245	360	380	335	320
EAC-rosetted thymocytes	265	280	375	355	340	350
Depleted of EAC-rosetted cells	10	<5	<5	<5	<5	0
Incubated with anti-B cell antiserum and complement	25	30	30	20	40	25
Incubated with anti-T cell antiserum and complement	245	260	340	370	290	330
Depleted of $MØ_1$[c]	<5	10	<5	<5	0	<5
Depleted of $MØ_2$[d]	10	<5	0	<5	<5	<5

CONTROL	The number of PFC per 10^6 MNC		
Spleen cells alone	285	395	360

a The cells were obtained 7 days following IV immunization with 10^9 SRBC.

b The thymocytes ($5x10^7$ cells per ml) were incubated in 5ml Medium RPMI 1640 for 4 hr at 37°C. The cell-free supernatants (THY-S) were assessed for ITSF activity.

c Thymocytes were depleted of $M\emptyset_1$ by incubation with carbonyl iron filings followed by centrifugation through ficoll-hypaque.

d Thymocytes were treated as in b, then incubated in culture medium for 24 hrs and depleted of MØs a second time.

TABLE 15

ITSF ACTS DIRECTLY ON THE B LYMPHOCYTE AND NOT ON THE T LYMPHOCYTE, NULL LYMPHOCYTE OR MACROPHAGE, TO INHIBIT THE GENERATION OF HEMOLYTIC PLAQUES BY AUTOLOGOUS IMMUNE SPLENIC AFC IN THE PFC ASSAY

Immune splenic MNC treated as follows prior to incubation with either ITSC or ITSF followed by	The number of SRBC PFC per 10^6 MNC after incubation of the treated cells for four hours with Nil		ITSC or ITSF[a]	
	I	II	I	II
Nil	235	265	0	0
Depleted of T cells[b]	210	250	<5	0
Depleted of MØ[c]	245	295	0	<5
Depleted of MØ and T cells	295	330	0	<5
Depleted of MØ and EAG rosetted cells	370	405	<5	0
Depleted of MØ, T cells and EAG rosetted cells	380	430	<5	<5
Depleted of AFC/memory cells[d]	0	0	0	0
Depleted of AFC/memory cells, MØ and EAG rosetted cells	0	0	0	0
Purified AFC/memory cells[e]	410	460	<5	10
Purified AFC/memory cells depleted of MØ and EAG rosetted cells[f]	390	420	<5	0

a Rabbits were immunized IV with 10^9 SRBC and sacrificed 7 days later.

b Splenic MNC were incubated with horse anti-rabbit thymocyte serum (HARTS) and complement, resulting in the lysis of the T cells.

c Splenic cells were incubated with carbonyl iron followed by centrifugation in Ficoll-Hypaque. The interphase consists of the macrophage (MØ)-depleted cells.

d Splenic MNC were incubated with the antigen, SRBC, resulting in rosetting of the AFC/memory cells with the SRBC.

e AFC/memory cells, the cells which rosetted with the antigen, SRBC.

f AFC/memory cells were isolated from splenic MNC following depletion of the splenic MNC of macrophages and EAG rosetted cells.

5.5. The generation of the suppressor cells in the thymus (ITSC) of the immunized rabbit capable of secreting a suppressor factor (ITSF) is a function of the time elapsed following IV immunization

5.5.1. Rational and objectives

As was demonstrated in Chapter 5.2. suppressor cells (ITSC) capable of inhibiting the generation of hemolytic plaques by autologous immune splenic AFC are detected in the thymus of the IV immunized rabbit 7 days following immunization. These cells secrete a suppressor factor (ITSF), when incubated in antigen- and serum-free medium for 4 hrs, capable by itself of suppressing the generation of hemolytic plaques. The suppression was attributed to the abilities of ITSC and ITSF to inhibit Ab secretion from the splenic AFC.

The OBJECTIVES for this part of the investigation were to (i) establish the temporal relationship between the appearance of ITSC capable of secreting ITSF in the thymus and the time following immunization (ii) to determine whether this temporal relationship varies with the dose of antigen administered IV.

5.5.2. Experimental protocol

Immunologically mature outbred New Zealand White rabbits were immunized IV with varying doses of the antigen (SRBC) and sacrificed at predetermined intervals of time following immunization. The MNC were prepared from the different lymphoid organs and the blood as described in Chapter 4.2.11. The MNC were assessed for their capacity to generate hemolytic plaques in the PFC assay as described in Chapter 4.2.12. The ITSF was prepared from the ITSC as described in Chapter 4.2.15. ITSC and ITSF were assayed for their capacity to suppress the generation of hemolytic plaques by immune splenic AFC in the PFC assay as described in Chapters 4.2.14 and 4.2.15, respectively.

The different MNC preparations were rosetted with the antigen, SRBC (E),

and with EAG indicator erythrocytes as described in Chapters 4.2.16 and 4.2.17, respectively.

The agglutinating antibody titers were determined as described in Chapter 4.2.30.

5.5.3. Experimental results

The ITSC were not detected in the rabbit during the first 4 days following 1^{o}IMM IV with 10^{9} SRBC. They were detected in the thymus by day 5 post-1^{o}IMM but their ability to secrete ITSF was not optimal until day 7 post-1^{o}IMM (Table 16). The ITSC began to lose their suppressive activity beginning at day 12 post-1^{o}IMM. However, even by day 40 post-1^{o}IMM, the ITSC were still capable of suppressing the PFC by 50 percent (Table 16). They were no longer detected at day 60 post-1^{o}IMM. Interestingly, the ITSC began to lose their capacity to secrete ITSF by days 10 to 11 post-1^{o}IMM and secreted negligible amounts of ITSF by day 21 post-1^{o}IMM (Table 16).

As can be seen in Table 17 the cells in the thymus which rosetted with the antigen (SRBC) and the EAG indicator cells grossly correlated with the appearance and disappearance of suppressor cells from the thymus (Table 16). No rosetted cells were detected in the thymus prior to day 3 post-1^{o}IMM IV, the SRBC and EAG rosetted MNC rose in parallel, peaked around day 10, decreased to insignificant numbers by day 30, and could no longer be detected by day 60 post-1^{o}IMM IV (Table 17). It should be noted that in the spleen, the appearance of MNC which rosetted with SRBC preceded the appearance of the MNC which rosetted with EAG by 1 to 2 weeks. The percentage of SRBC rosetted cells rose sharply in the spleen between days 2 and 5, plateaued between day 5 and 10, decreased slowly thereafter and could no longer be detected by day 60 post-1^{o}IMM IV. On-

the other hand, insignificant numbers of EAG rosetted MNC were detected in the spleen up to day 8, they rose sharply on day 10, peaked on day 20 and declined until they could no longer be detected by day 60 post-1^{o}IMM IV (Table 17). Insofar as the circulating MNC (PB) are concerned only much lower numbers of SRBC and EAG rosetted MNC were detected during the course of the 1^{o}IM and both appeared to peak between days 7 and 10 post-1^{o}IMM IV (Table 17). It must be stressed that unimmunized rabbits do not possess cells capable of rosetting with SRBC or EAG (Table 17).

Suppressor cells capable of secreting ITSF appear to be induced in the thymus at the same time post-2^{o}IMM IV as they are post-1^{o}IMM IV. As can be seen from Table 18 suppressor cells were not detected in the thymus until the fifth day following 2^{o}IMM IV. These cells did not attain the capacity to secrete ITSF capable of totally suppressing PFC until day 7 post-2^{o}IMM IV (Table 18). At no time were suppressor cells or cells capable of secreting ITSF detected in any of the other lymphoid organs or the circulation post-2^{o}IMM IV (Table 18).

The generation of ITSC capable of secreting ITSF is a function of the dose of antigen (SRBC) administered (Table 19). ITSC capable of secreting ITSF were detected in rabbits immunized IV 7 days previously with 10^{7}, 10^{8} or 10^{9} SRBC whereas they were not detected following IV immunization with 10^{3}, 10^{4}, or 10^{5} SRBC (Table 19). Limited numbers of ITSC capable of secreting ITSF were detected in rabbits immunized IV with 10^{6} SRBC (Table 19).

The percentages of cells in the thymus and spleen which rosetted with the antigen (SRBC) and EAG on day 7 post-IV immunization is a function of the dose of antigen (SRBC) injected (Table 20.) Almost equal numbers of thymocytes rosetted with SRBC and EAG following immunization with 10^{7},

10^8 or 10^9 SRBC. Essentially no rosetting cells were detected in the thymus of rabbits immunized IV with 10^3, 10^4 or 10^5 SRBC (Table 20). The spleen of rabbits immunized IV with 10^5, 10^6, 10^7, 10^8 or 10^9 SRBC possessed cells capable of rosetting with SRBC but not with EAG. The spleens of rabbits injected with 10^3 or 10^4 did not possess cells capable of rosetting with either SRBC or EAG (Table 20). Very few rosetting cells were observed in the circulation irrespective of the dose of immunization (Table 20).

5.5.4. Commentary

As was demonstrated in this part of the investigation ITSC are not detected until day 5 post-1°IMM IV or post-2°IMM IV with 10^9 SRBC. They were maximally suppressive between days 5 and 11 and were no longer detected after day 60 post-1°IMM IV. ITSF capable of totally suppressing the PFC was not secreted by the ITSC until day 7 post-1°IMM and post-2°IMM IV. The ITSC began to lose their ability to secrete ITSF by day 11 and lost the ability to secrete ITSF by day 40 post-primary immunization. The appearance of the ITSC in the thymus correlated with the appearance of cells capable of rosetting with the antigen (SRBC) and the EAG indicator erythrocyes. These results were anticipated on the basis of the results presented in the two previous sections which demonstrated that the ITSC rosettes with both the immunizing antigen, SRBC and with the EAG indicator cells. It is instructive to note that whereas the numbers of thymocytes which rosetted with SRBC and EAG coincided temporarily following immunization, such was not the case with the splenic MNC. Cells capable of rosetting with SRBC were present in maximum numbers in the spleen before the appearance of cells capable of rosetting with the EAG indicator cells. Both AFC/memory cells and

suppressor cells rosette with the immunizing antigen, and only the suppressor cells rosette with the EAG. It is therefore apparent, that the rosetting cells detected up to day 8 post immunization in the spleen capable of rosetting with the antigens are primarily if not only AFC whereas the cells which appear subsequently and rosette with both the immunizing antigen and the EAG indicator cells are suppressor cells. Thus, the conditions exist in the spleen for the initial induction of an immune response by the AFC and the subsequent termination of the immune response by the suppressor cells.

As one would expect, there is a correlation between the amount of antigen injected and the subsequent appearance of the suppressor cells in the thymus. Immunization with as few as 10^7 SRBC injected IV resulted in the generation of optimal number of suppressor cells. Rabbits immunized with fewer than 10^6 SRBC lose the ability to generate suppressor cells. This conclusion is supported by the fact that cells capable of rosetting with EAG are markedly diminished in the thymus of rabbits immunized with fewer than 10^6 SRBC.

TABLE 16

SUPPRESSOR CELLS DETECTED IN THE THYMUS AS A FUNCTION OF THE TIME ELAPSED BETWEEN IMMUNIZATION AND SACRIFICE

Thymocytes obtained at the following days post-primary IV immunization with 10^9 SRBC	The number of PFC per 10^6 MNC following coincubation of the allogeneic immune spleen MNC[a] with					
	I			II		
	Nil	ITSC	ITSF	Nil	ITSC	ITSF
-1	490	360	410	565	590	540
0[b]	260	190	220	305	270	295
1	360	280	310	360	320	350
2	290	230	260	290	270	285
3	320	280	310	320	260	290
4	220	160	220	220	210	190
5	285	<5	95	285	<5	130
6	410	<5	70	410	<5	80
7	245	<5	<5	340	<5	0
8	290	0	<5	290	<5	<5
9	220	<5	<5	220	<5	<5
10	310	<5	20	310	0	40
11	270	<5	120	270	<5	110
12	360	40	170	360	50	190
13	305	35	150	405	45	150
14	330	40	140	330	60	170
21	315	70	250	315	60	280
30	310	25	170	310	35	210
40	385	180	350	385	170	380
60	280	230	270	280	250	260

a The rabbits were immunized with 10^9 SRBC IV and sacrificed 7 days later.

b These rabbits were immunized with 10^9 SRBC IV and sacrificed 15 minutes later.

TABLE 17

THE APPEARANCE OF (i) SRBC AND (ii) EAG ROSETTING CELLS IN THE THYMUS, SPLEEN AND PERIPHERAL BLOOD AS A FUNCTION OF TIME BETWEEN IMMUNIZATION AND SACRIFICE

Number of days post IV immunization	Cells of organs assayed (percent rosetted cells) THY (i)	THY (ii)	SPL (i)	SPL (ii)	PB (i)	PB (ii)	Agglutinating Ab titer
-1[a]	0	0	0	0	0	0	0
0[b]	0	0	0	0	0	0	0
1	0	0	0	0	0	0	0
2	0	0	2%	0	3%	0	0
3	3%	4%	4.5%	0	4%	0	0
4	5%	6%	8%	0	5%	1%	160
5	6%	10%	12%	1%	5%	2%	640
7	11%	11%	10%	2%	7%	3%	1280
8	12%	9%	12%	2%	7%	2%	1280
10	13%	8%	11%	5%	6%	4%	2560
15	7%	6%	8%	9%	4%	1%	1280
20	4%	3%	6%	12%	3%	0	160
30	2%	1%	6%	7%	3%	0	80
60	0	0	1%	0	0	0	20

a Rabbits were immunized IV with 10^9 SRBC and sacrificed at the indicated days post primary IV immunization.

b Rabbits were immunized IV with 10^9 SRBC and sacrificed 15 mins later.

TABLE 18

THE GENERATION OF SUPPRESSOR CELLS IN THE THYMUS BUT NOT IN THE OTHER LYMPHOID ORGANS, FOLLOWING 2^{o} IMMUNIZATION IN VIVO, AS A FUNCTION OF TIME BETWEEN 2^{o} IMMUNIZATION AND SACRIFICE

The number of PFC per 10^6 splenic MNC following incubation of the immune splenic MNC[a] with the allogeneic lymphoid cells[c] or the cell-free culture supernatants of these cells[d] of rabbits sacrificed at the following days post-2^{o} immunization.

Cells of organs assayed[b]	4		5		7		10	
	Cells	Sup	Cells	Sup	Cells	Sup	Cells	Sup
SPL	310	280	300	320	310	300	300	320
THY	280	290	10	190	<5	<5	<5	<5
BM	350	320	360	295	330	315	300	340
PB	295	340	310	330	315	320	315	355
APP	275	290	295	320	330	320	335	370
SR	295	290	340	295	295	310	305	315
PP	320	280	355	335	320	290	315	345
PLN	300	295	320	335	325	310	325	360
SPL alone	310		340		325		355	

a Rabbits were immunized with 10^9 SRBC IV and sacrificed 7 days later.

b Rabbits were immunized with 10^9 SRBC IV (1^{o} IMM) reimmunized with 10^9 SRBC IV 3 months post-1^{o} IMM (2^{o} IMM in vivo) and sacrificed 4, 5, 7 or 10 days following 2^{o} IMM.

c Immune splenic MNC[a] (5×10^5 cells per ml) were incubated with the cells of the different lymphoid organs (5×10^5 cells per ml) obtained at various times post 2^{o} IMM for 4 hrs at 37^{o}C in 5% CO_2 in air prior to their incorporation into the PFC assay.

d The cells of the different lymphoid organs were incubated in 5 mls medium (RPMI 1640 (5×10^7 cells per ml) at 37^{o}C in 5% CO_2 in air for 4 hrs, centrifuged and the cell-free supernatant containing the suppressor factor was collected and incubated with the immune splenic MNC[a] for 4 hrs prior to the PFC assay.

TABLE 19

THE GENERATION OF SUPPRESSOR CELLS IN THE THYMUS CAPABLE OF SECRETING A SUPPRESSOR FACTOR 7 DAYS POST PRIMARY IMMUNIZATION IS A FUNCTION OF THE DOSE OF ANTIGEN (SRBC) INJECTED IV

Thymocytes obtained 7 days following 1° IMM IV with SRBC (number of SRBC injected)	The number of PFC per 10^6 MNC following incubation of the allogeneic splenic MNC[a] with					
	I			II		
	Nil	ITSC	ITSF	Nil	ITSC	ITSV
10^9 $\frac{(345)}{(2560)}$[b]	345	0	<5	310	<5	0
10^8 $\frac{(300)}{(2560)}$[b]	290	<5	<5	320	<5	<5
10^7 $\frac{(200)}{(1280)}$[b]	345	15	10	370	25	20
10^6 $\frac{(150)}{(640)}$[b]	290	75	70	320	95	115
10^5 $\frac{(45)}{(160)}$[b]	290	275	245	320	260	300
10^4 $\frac{(5)}{(20)}$[b]	290	295	310	320	320	295
10^3 $\frac{(0)}{(0)}$[b]	290	300	290	320	310	315

a The rabbits were immunized with 10^9 SRBC IV and sacrificed 7 days later.

b The numerator and the denominator are the number of PFC per 10^6 splenic MNC and the agglutinating antibody titer at the time of sacrifice (day 7 post primary immunization).

TABLE 20

THE APPEARANCE OF (i) SRBC AND (ii) EAG ROSETTING CELLS IN THE THYMUS, SPLEEN AND PERIPHERAL BLOOD 7 DAYS POST-PRIMARY IMMUNIZATION IS A FUNCTION OF THE DOSE OF THE ANTIGEN (SRBC) INJECTED IV

Cells obtained 7 days following primary IV immunization with SRBC (no. of SRBC injected)	Cells of organs assayed (percent rosetted cells)						
	THY		SPL		PB		Agglutinating Ab titer
	(i)	(ii)	(i)	(ii)	(i)	(ii)	
10^9	10%	8%	11%	1%	3%	2%	2560
10^8	9%	7%	8%	1%	2%	1%	2560
10^7	8%	6%	7%	1%	1%	0	1280
10^6	4%	5%	5%	1%	1%	0	640
10^5	2%	1%	4%	0	0	0	160
10^4	0	0	1%	0	0	0	20
10^3	0	0	<1%	0	0	0	0
0 (unimmunized)	0	0	0	0	0	0	0

5.6. The inhibition by ITSC and ITSF of the primary immune response in vivo and the secondary response induced in vitro

5.6.1. Rational and objectives

The results of the experiments presented in the previous sections of this Chapter demonstrate that antigen-specific suppressor cells (ITSC), capable of secreting an antigen-specific suppressor factor (ITSF) in vitro, are generated only in the thymus of the rabbit immunized IV with SRBC and/or HRBC. These ITSC are not detected in any of the lymphoid organs of the unimmunized rabbit. The ITSC are not detected prior to the fifth day post immunization, at a time when the immune response has been initiated. The ITSC attain maximum suppressive activity between days 10 and 15 and can no longer be detected after day 40 post IV immunization. These results strongly indicate that the ITSC and/or the ITSF constitute the suppressor arm of the physiological immunoregulatory mechanism.

The OBJECTIVES in this part of the investigation were to demonstrate (i) that ITSC and ITSF suppress the primary immune response in vivo, and (ii) that ITSC and ITSF suppress the secondary immune response induced in vitro.

5.6.2. Experimental protocol

The immunization of the rabbits, the preparation of the thymocytes and splenic MNC, the preparation of the ITSF, the NTS, the hemolytic PFC assay, the in vitro cultures for the induction of the 2^{o}IM, and the determination of the agglutinating antibody titers were all carried out as described in the previous sections of this Chapter. Splenic MNC and thymocytes were treated with mitomycin-C, so as to prevent their undergoing blastogenesis upon stimulation with allogeneic cells in the

MLR, as described in Chapter 4.2.24. SSSF and HSSF were prepared as described in Chapter 4.2.25. In some of these experiments SSSF and HSSF were used in place of SRBC and HRBC, respectively. They were used in doses equivalent to the numbers of SRBC and HRBC used for IV immunization or culture. In preliminary experiments (results not presented in Tables) it was consistantly observed that immunization with SSSF (HSSF) induced immune responses both in vivo and in vitro comparable to those induced with SRBC (HRBC).

5.6.3. Experimental results

As can be seen in Table 21, the IV administration of allogeneic ITSC into SRBC immunized rabbits on days 0 and 3 post-immunization resulted in a much diminished immune response, as compared with the rabbits injected with NTC or medium. The antibody titers at day 7 post-immunization, at the time of sacrifice, were approximately 5 to 10 percent of those of the NTC or medium injected rabbits. Similarly, the number of immediate PFC and the number of PFC after 5 days in culture of splenic MNC of the rabbits given ITSC were reduced by approximately 75 percent and 80 percent, respectively, as compared to the PFC generated by the splenic MNC of the rabbits given NTC. On the other hand, the SRBC immunized rabbits injected with allogeneic ITSC on days 0, 3 and 5 post-immunization failed to give an immune response on day 7 post-immunization (Table 22). The SRBC immunized rabbits injected with NTC on days 0, 3 and 5 post-immunization responded very well with both titers of circulating antibodies and large numbers of PFC in the spleen (Table 22).

The ITSC demonstrate antigenic specificity as indicated by the data in Table 23. Rabbits were immunized with both SRBC and HRBC IV. The rabbits given ITSC depleted of SRBC rosetting cells were capable of

generating an immune response to HRBC but responded normally to SRBC. Conversely, the rabbits given ITSC depleted of cells which rosetted with HRBC failed to give an immune response to SRBC but responded normally to HRBC. As could be predicted on the basis of the above results, immunized rabbits given ITSC depleted of cells which rosetted with both SRBC and HRBC gave immune responses to both of these antigens comparable to the immune responses generated by control immunized rabbits given NTC instead of ITSC (Tables 22 and 23).

As can be seen in Table 24, no MLR was observed when mitomycin-C treated thymocyte stimulator cells were cultured with allogeneic splenic responder cells. A minimal MLR was observed when thymocyte responder cells were cultured with mitomycin-C treated allogeneic splenic stimulator cells. In contrast, a marked MLR was observed when mitomycin-C treated splenic stimulator cells were cultured with allogeneic splenic responder cells.

ITSF, obtained from allogeneic ITSC, injected daily IV into immunized rabbits on days 0 to 5 following immunization was capable of totally suppressing the in vivo immune response to the antigen, SRBC, NTS, obtained from allogeneic NTC, injected on days 0 to 5 following immunization, was capable of suppressing the in vivo immune response (Table 25).

As can be seen in Table 26 rabbits immunized initially with SSSF and HSSF and injected IV daily for 5 days following 1^{o}IMM IV with ITSF (SRBC) (ITSF obtained from thymocytes of rabbits immunized with 10^{9} SRBC IV 7 days previously), did not generate an immune response to SRBC even after a secondary IV immunization on day 28 post-1^{o}IMM IV. They did not respond with marked circulating antibody titers 7 days following tertiary IV

immunization on day 80 post-1°IMM IV. This recovery of the capacity to respond to SRBC is antigen specific since the immune response to HRBC was not affected at any time following 1°IMM IV. The distribution of the AFC 7 days following IV immunization with SSSF and HSSF on day 80 post-1°IMM IV can be seen in Table 27. The majority of the AFC were localized to the spleen, fewer numbers were detected in the blood and very few were present in the bone marrow. None of the other lymphoid organs (thymus, popleteal lymph node, sacculus rotundus and Peyer's patches) possessed AFC as detected by the hemolytic PFC assay.

ITSC and ITSF were then assessed for their capacity to inhibit the secondary immune response of immune (AFC and memory) splenic MNC in vitro. The splenic MNC, obtained from rabbits immunized IV with 10^9 SRBC 7 or 60 days previously, were cultured for 5 days in the presence of the immunizing antigen. As can be seen from the data presented in Table 28, both the continuation of the primary immune response by day 7 AFC and the secondary immune response by day 60 splenic memory cells in vitro were totally suppressed by the allogeneic ITSC while the allogeneic NTC exerted no detrimental effect on the in vitro immune response.

The ITSC demonstrated the same antigen-specific suppressor activity in in vitro cultures as it did when injected in vivo. Rabbits were immunized with both SRBC and HRBC and sacrificed 7 or 60 days later. The splenic MNC were cultured in vitro with SSSF or HSSF in order to stimulate a continuation of the primary response initiated in vivo (day 7 splenic MNC) or a secondary response in vitro (day 60 splenic MNC). In both instances, untreated ITSC added to the cell cultures on days 0 and 3 of the 5 day culture totally suppressed the in vitro immune response. The addition to the cultures of ITSC depleted of SRBC rosetted cells

suppressed the in vitro immune response to HRBC but not to SRBC whereas the addition to the cultures of ITSC depleted of HRBC rosetted cells suppressed the in vitro immune response to SRBC but not to HRBC (Table 29).

ITSF, obtained from thymocytes of rabbits immunized with SRBC, added to the cultures of immune (day 7) or memory (day 60) splenic MNC on days 0 and 3, was capable of totally suppressing the 2^{o}IM in vitro to the immunizing antigen, SRBC (Table 30) just as it was able to suppress the 1^{o}IM to SRBC in vivo (Table 22 and 23). Similarly, ITSF obtained from thymocytes of rabbits immunized with both SRBC and HRBC suppressed the 2^{o}IM in vitro to both SRBC and HRBC. Absorption of the ITSF with SRBC (or HRBC) resulted in the loss of its capacity to subsequently suppress the 2^{o}IM in vitro to SRBC (or HRBC) but not to HRBC (or SRBC) (Table 31). NTS had no detrimental effect on the 2^{o}IM in vitro (Table 30).

5.6.4. Commentary

The results presented in this section of the investigation demonstrate unequivocally that ITSC and ITSF can totally suppress in an antigen-specific manner the 1^{o}IM induced in vivo and the 2^{o}IM induced in vitro. It was further demonstrated that rabbits injected with ITSF for 5 days following 1^{o}IMM IV recovered the capacity to synthesize antibodies to the original immunizing antigen 7 days following tertiary immunization IV on day 80 post-1^{o}IMM IV. Thus, the suppression of antibody synthesis by ITSF in vivo is not permanent. It must be emphasized that ITSF was not at all cytotoxic to the lymphoid cells in vitro and produced no signs of morbidity following its administration IV on five consecutive days.

TABLE 21

THE ABILITY OF ALLOGENEIC ITSC, BUT NOT ALLOGENEIC NTC, TO ONLY PARTIALLY INHIBIT THE IN VIVO PRIMARY IMMUNE RESPONSE WHEN INJECTED IV ON DAYS 0 AND 3 FOLLOWING IV IMMUNIZATION WITH SRBC ON DAY 0

Rabbit no.	Rabbits injected IV with ITSC[b]	NTC[c]	Medium	Agglutinating Ab titers at the time of sacrifice (day 7)	The number of PFC per 10^6 splenic MNC
1	+	-	-	40	50 (200)[d]
2	+	-	-	80	120 (214)
3	+	-	-	40	160 (566)
4	+	-	-	20	100 (320)
5	-	+	-	640	260 (1300)
6	-	+	-	1280	365 (1210)
7	-	+	-	1280	380 (1368)
8	-	+	-	640	300 (1720)
9	-	-	+	640	215 (1618)
10	-	-	+	640	320 (1465)
11	-	-	+	320	340 (1864)
12	-	-	+	320	240 (2104)

a 4×10^5 SRBC injected IV on day 0.

b 2×10^9 ITSC (thymocytes of rabbits 7 days post primary immunization IV with 10^9 SRBC) injected on days 0 and 3 post-immunization.

c 2×10^9 NTC (thymocytes of unimmunized rabbits) injected on days 0 and 3 post-immunization.

d The figures outside and within the brackets are the number of PFC detected immediately after sacrifice and after 5 days of culture with SRBC, respectively.

TABLE 22

THE SUPPRESSION OF THE IN VIVO PRIMARY IMMUNE RESPONSE TO SRBC BY THE IV INJECTION OF ALLOGENEIC ITSC, BUT NOT ALLOGENEIC NTC, ON DAYS 0, 3 AND 5 FOLLOWING IV IMMUNIZATION WITH SRBC ON DAY 0

Rabbit no.	Rabbits injected IV with SRBC[a]	ITSC[b]	NTC[c]	Ab titers[d]	The number of PFC per 10^6 splenic MNC at time of sacrifice (day 7)
1	-	-	-	0 (0)	0 (0)[e]
2	-	-	-	0 (0)	0 (0)
3	-	-	-	0 (<5)	0 (<5)
4	-	-	-	0 (<5)	<5 (0)
5	+	-	-	80 (2560)	165 (1960)
6	+	-	-	40 (1280)	190 (2106)
7	+	-	-	80 (5120)	175 (1882)
8	+	-	-	160 (5120)	220 (2161)
9	+	+	-	0 (10)	<5 (20)
10	+	+	-	0 (<5)	<5 (30)
11	+	+	-	0 (0)	0 (0)
12	+	+	-	0 (<5)	0 (<5)
13	+	-	+	40 (1280)	140 (2009)
14	+	-	+	20 (1280)	155 (1450)
15	+	-	+	40 (2560)	170 (1864)
16	+	-	+	80 (5120)	195 (1316)

a 4×10^5 SRBC injected IV on day 0.

b 2×10^9 ITSC (thymocytes of rabbits 7 days post-primary immunization IV with 10^9 SRBC) injected on days 0, 3 and 5 post immunization.

c 2×10^9 NTC (thymocytes of unimmunized rabbits) injected on days 0, 3 and 5 post-immunization.

d The circulating agglutinating antibody titers on day 5 following immunization (outside the brackets) and at time of sacrifice (day 7) (within the brackets).

e The figures outside and within the brackets are the number of PFC detected immediately after sacrifice and after 5 days of culture with SRBC, respectively.

TABLE 23

THE ANTIGENIC SPECIFICITY OF THE ITSC IN VIVO. THE ITSCs OBTAINED FROM RABBITS IMMUNIZED WITH SRBC AND HRBC ARE ONLY CAPABLE OF INHIBITING THE PRIMARY IMMUNE RESPONSES TO SRBC AND HRBC, RESPECTIVELY

Rabbit no.[a]	Allogeneic ITSC treated as follows[b]	Ab titers to[c] SRBC	Ab titers to[c] HRBC	The number of PFC per 10^6 splenic MNC directed to SRBC	The number of PFC per 10^6 splenic MNC directed to HRBC
1	Untreated	<5	0	<5	0
2	Depleted of SRBC rosetting cells	640	<5	145	<5
3	Depleted of HRBC rosetting cells	0	1280	<5	110
4	Depleted of both HRBC and SRBC rosetting cells	1280	1280	160	135
5	Control (NTC)[d]	2560	1280	145	115

a Rabbits were immunized with 4×10^5 SRBC and 4×10^5 HRBC IV (day 0) and injected with 2×10^9 untreated and treated allogeneic ITSC on days 0, 3 and 5 post-immunization.

b The ITSC were obtained from the thymus of a rabbit immunized IV with 10^9 SRBC and 10^9 HRBC 7 days previously.

c The circulating agglutinating antibody titers at time of sacrifice (day 7).

d Rabbits injected with 2×10^9 allogeneic NTC instead of ITSC.

TABLE 24

THYMUS MNC DO NOT STIMULATE A BLASTOGENIC RESPONSE IN ALLOGENEIC SPLENIC MNC IN THE ONE-WAY MLR

Cells cultured for 5 days[a]		^{3}H-thymidine incorporated into the responding cells
Responder cells	Stimulator cells	(cpm)
SPL(A)	-	182
SPL(B)	-	128
-	mTHY(A)	20
-	mTHY(B)	32
SPL(A)	mTHY(A)	180
SPL(B)	mTHY(B)	158
SPL(A)	mTHY(B)	188
SPL(B)	mTHY(A)	150
-	mSPL(A)	86
-	mSPL(B)	66
THY(A)	-	186
THY(B)	-	116
THY(A)	mSPL(A)	296
THY(B)	mSPL(B)	300
THY(B)	mSPL(A)	2193
THY(A)	mSPL(B)	948
SPL(A)	mSPL(A)	275
SPL(B)	mSPL(B)	198
SPL(A)	mSPL(B)	11,860
SPL(B)	mSPL(A)	13,600

a mTHY denotes mitomycin-C treated THY cells.
mSPL denotes mitomycin-C treated SPL MNC cells.

TABLE 25

ITSF(SRBC) BUT NOT NTS INHIBITS THE PRIMARY IMMUNE RESPONSE TO SRBC IN VIVO

Rabbit no.[a]	Rabbits injected IV with ITSF[b]	NTS[c]	Medium[d]	Ab titers[e]	The number of PFC per 10^6 splenic MNC[f]
1	+	-	-	0 (0)	0
2	+	-	-	0 (<5)	0
3	+	-	-	0 (0)	0
4	-	+	-	40 (160)	115
5	-	+	-	80 (640)	110
6	-	+	-	20 (320)	135
7	-	-	+	40 (320)	130
8	-	-	+	80 (1280)	105
9	-	-	+	80 (640)	120

a The rabbits were immunized with 4×10^5 SRBC IV on day 0.

b The cell-free/culture supernatant (ITSF) of thymocytes obtained from a rabbit immunized with 10^9 SRBC IV 7 days previously. ITSF (10 ml) was injected IV daily on days 0 to 5 following immunization.

c The cell-free culture supernatant (NTS) of thymocytes obtained from an unimmunized rabbit. NTS (10 ml) was injected IV daily on days 0 to 5 following immunization.

d Medium RPMI 1640 (10 ml) was injected IV daily on days 0 to 5 following immunization.

e The circulating agglutinating Ab titers on day 5 following immunization (outside the brackets) and at time of sacrifice (day 7) (within the brackets).

f The number of PFC per 10^6 splenic MNC at the time of sacrifice (day 7).

TABLE 26

THE RECOVERY OF ANTIBODY SYNTHESIZING CAPACITY FOLLOWING THE IV ADMINISTRATION OF ITSF(SRBC) DAILY FOR 5 DAYS BEGINNING WITH THE PRIMARY IV IMMUNIZATION WITH SSSF AND HSSF

Day of immunization IV with SSSF and HSSF		ITSF(SRBC)[a] injected IV on following days	The agglutinating antibody titer of rabbits I[b], II[b], III and IV to							
			I		II		III		IV	
			SRBC	HRBC	SRBC	HRBC	SRBC	HRBC	SRBC	HRBC
	-1	-	0	0	0	0	0	0	0	0
I^{o}_{IMM}[c]	0	-	-	-	-	-	-	-	-	-
	0 to 5	+	-	-	-	-	-	-	-	-
	7	-	0	320	0	640	0	320	0	640
	10	-	0	640	0	1280	0	640	0	1280
	14	-	<5	1280	0	2560	<5	2560	0	5120
	27	-	0	40	0	40	0	40	0	80
2^{o}_{IMM}[c]	28	-	-	-	-	-	-	-	-	-
	35	-	0	2560	<5	2560	<5	2560	0	1280
	38	-	10	5120	20	5120	10	5120	<5	2560
	42	-	40	5120	20	5120	20	5120	20	5120
	79	-	<5	20	<5	10	10	20	0	<5
3^{o}_{IMM}[d]	80	-	-	-	-	-	-	-	-	-
	87	-	5120	2560	1280	640	1280	640	2560	1280
	90	-	-	-	-	-	2560	1280	5120	2560
	94	-	-	-	-	-	5120	2560	10240	5120

a ITSF was prepared by incubating 5×10^7 thymocytes of 7-day immune rabbits per ml of Medium RPMI 1640 for 24 hr at 37°C. Ten ml of ITSF was injected IV daily for 5 days commencing with the immunization with SSSF and HSSF.

b The rabbits were sacrificed on day 87 post-immunization.

c The rabbits were immunized IV with SSSF and HSSF equivalent to 4×10^5 SRBC and HRBC, respectively.

d The rabbits were immunized IV with SSSF and HSSF equivalent to 10^9 SRBC and HRBC, respectively.

TABLE 27

THE IN VIVO DISTRIBUTION OF SRBC AND HRBC PFC IN THE RABBITS INJECTED WITH ITSF(SRBC) DAILY FOR 5 DAYS BEGINNING WITH THE PRIMARY IMMUNIZATION IV WITH SSSF AND HSSF AND SACRIFICED ON DAY 87 (7 DAYS FOLLOWING TERTIARY IV IMMUNIZATION WITH SSSF AND HSSF)

Cells of organs assayed[a]	The number of PFC per 10^6 MNC of rabbits I[b] and II[c] to			
	I		II	
	SRBC	HRBC	SRBC	HRBC
SPL	200	145	215	175
THY	0	0	0	0
BM	<5	<5	<5	<5
PB	50	75	75	95
PLN	0	0	0	0
APP	0	0	0	0
SR	0	0	0	0
PP	0	0	0	0

a Rabbits were immunized with SSSF and HSSF equivalent to 4×10^5 SRBC and HRBC, respectively, on days 0 and 28 and SSSF and HSSF equivalent to 10^9 SRBC and HRBC, respectively, on day 80. They were injected with 10 ml ITSF IV daily from days 0 to 5.

b The agglutinating antibody titers to SRBC and HRBC at time of sacrifice were 5120 and 2560, respectively.

c The agglutinating antibody titers to SRBC and HRBC at time of sacrifice were 1280 and 640, respectively.

TABLE 28

THE ABILITY OF ALLOGENEIC ITSC FROM SRBC-IMMUNIZED RABBITS, BUT NOT ALLOGENEIC NTC, TO TOTALLY SUPPRESS THE IN VITRO SECONDARY IMMUNE RESPONSE TO SRBC WHEN ADDED ON DAYS 0 AND 3 OF THE CULTURE

SPL and PB MNC obtained from SRBC-immunized rabbits sacrificed on following day post-1^{o}1M with 10^{9} SRBC IV[a]	The number of PFC per 10^{6} freshly isolated SPL and PB MNC before and after culture in the presence of SSSF[b] and								
	Allogeneic ITSC[c]			Allogeneic NTC[c]			Culture medium		
	I	II	III	I	II	III	I	II	III
Day 7									
SPL	150 (30)[d]	135 (10)	170 (<5)	150 (940)	135 (795)	170 (861)	150 (1000)	135 (962)	170 (845)
PB	60 (0)	45 (<5)	75 (0)	60 (340)	45 (400)	75 (424)	60 (410)	45 (315)	75 (390)
Day 60									
SPL	0 (10)	5 (0)	0 (<5)	0 (1112)	5 (990)	0 (1090)	0 (1242)	5 (1305)	0 (1122)
PB	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)

a Only SPL and PB MNC were assayed for PFC and cultured in vitro for the induction of a 2^{o}1M since only these organs possess detectable AFC or memory cells at either 7 (SPL, PB) or 60 (SPL) days post-1^{o}1M.

b SSSF equivalent to 4 x 10^{6} SRBC was added to the MNC at the beginning of the 5 day culture.

c The allogeneic ITSC or NTC (4 x 10^{6} cells) were added to the MNC cultures at days 0 and 3 of culture.

d The figures outside and within the brackets are the number of PFC detected immediately following sacrifice and after 5 days in culture with the antigen, respectively.

TABLE 29

THE ANTIGENIC SPECIFICITY OF THE ITSC IN VITRO. THE ITSCs OBTAINED FROM RABBITS IMMUNIZED WITH SRBC AND HRBC ARE ONLY CAPABLE OF INHIBITING THE SECONDARY IMMUNE RESPONSES INDUCED IN VITRO TO SRBC AND HRBC, RESPECTIVELY

ITSC treated as follows prior to addition to immune splenic MNC cultures	Splenic MNC obtained from rabbits sacrificed on the following day post-$1^{O}1M$[a]	The number of PFC per 10^6 splenic MNC cultured for 5 days with ITSC[b] and					
		I	SSSF II SRBC PFC	III	I	HSSF II HRBC PFC	III
Untreated	7	159 (10)[c]	170 (<5)	191 (20)	110 (0)	101 (0)	130 (30)
	60	10 (30)	0 (21)	<5 (40)	0 (19)	0 (10)	<5 (26)
Depleted of SRBC-rosetting cells	7	159 (1014)	170 (952)	191 (893)	110 (<5)	101 (20)	130 (0)
	60	10 (1214)	0 (1300)	5 (1110)	0 (10)	0 (<5)	<5 (21)
Depleted of HRBC-rosetting cells	7	159 (<5)	170 (10)	191 (30)	110 (890)	101 (963)	130 (1100)
	60	10 (40)	0 (10)	<5 (12)	0 (1150)	0 (1210)	<5 (998)
Depleted of both SRBC and HRBC-rosetting cells	7	159 (1100)	170 (1212)	191 (990)	110 (1000)	101 (1116)	130 (920)
	60	10 (1240)	0 (1274)	<5 (1010)	0 (1200)	0 (1000)	<5 (1140)
No ITSC (control)	7	159 (1190)	170 (1278)	191 (1100)	110 (980)	101 (1090)	130 (948)
	60	10 (1266)	0 (1310)	<5 (1200)	0 (1174)	0 (1150)	<5 (1092)
Agglutinating Ab titers at time of sacrifice	7	640	1280	640	640	640	320
	60	<5	20	10	0	<5	0

a The rabbits were immunized with 10^9 SRBC and 10^9 HRBC IV on day 0.

b ITSC (4×10^6 cells) were added to the allogeneic splenic MNC cultures on days 0 and 3 of culture.

c The figures outside and within the brackets are the number of PFC at the time of sacrifice and after culture for 5 days in vitro with the antigen, SSSF or HSSF, respectively.

TABLE 30

THE SUPPRESSION, BY ITSF BUT NOT NTS, OF THE SECONDARY IMMUNE RESPONSE INDUCED IN VITRO

Rabbits were sacrificed on the following days after immunization[a]	Agglutinating Ab titer at day 7 and/or at sacrifice	The number of PFC per 10^6 splenic MNC cultured for 5 days with SSSF and		
		ITSF[d]	NTS[e]	Medium[f]
7	640[b]	149 (10)[g]	149 (790)	149 (845)
7	640[b]	170 (20)	170 (910)	170 (1100)
7	1280[b]	185 (<5)	185 (1003)	185 (1119)
60	1280 (<5)[c]	<5 (20)	<5 (938)	<5 (1032)
60	640 (10)[c]	0 (30)	0 (1213)	0 (1194)
60	1280 (<5)[c]	<5 (14)	<5 (1128)	<5 (1002)

a The rabbits were immunized with 10^9 SRBC IV on day 0.

b The circulating agglutinating Ab titers on day 7 at time of sacrifice.

c The circulating agglutinating Ab titers on days 7 (outside the brackets) and 60 (within the brackets, day of sacrifice).

d ITSF was added to the cultured cells on days 0 and 3 of culture.

e NTS was added to the cultured cells on day 0 and 3 of culture.

f Culture medium was replaced on day 3 of culture.

g The figures outside and within the brackets are the number of PFC at the time of sacrifice and after culture for 5 days in vitro with the antigen, SSSF, respectively.

TABLE 31

THE ANTIGENIC SPECIFICITY OF THE ITSF IN VITRO. THE ITSFs OBTAINED FROM RABBITS IMMUNIZED WITH SRBC AND HRBC ARE ONLY CAPABLE OF INHIBITING THE SECONDARY IMMUNE RESPONSES INDUCED IN VITRO TO SRBC AND HRBC, RESPECTIVELY

ITSF treated as follows prior to addition to immune splenic MNC cultures	Splenic MNC were obtained from rabbits sacrificed on the following day post-1°1MM[a]	The number of PFC per 10^6 splenic MNC cultured for 5 days with ITSF[b] and					
		SSSF			HSSF		
		I	II	III	I	II	III
		SRBC PFC			HRBC PFC		
Untreated	7	180 (5)[c]	210 (30)	145 (<5)	100 (<5)	98 (0)	110 (20)
	60	0 (24)	<5 (40)	0 (15)	0 (17)	0 (10)	<5 (32)
Absorbed with SRBC	7	180 (1093)	210 (984)	145 (899)	100 (<5)	98 (20)	110 (0)
	60	0 (1119)	<5 (1201)	0 (1053)	0 (10)	0 (<5)	<5 (0)
Absorbed with HRBC	7	180 (<5)	210 (10)	145 (43)	100 (875)	98 (936)	110 (1010)
	60	0 (30)	<5 (15)	0 (18)	0 (1110)	0 (1021)	<5 (998)
Absorbed with SRBC and HRBC	7	180 (1214)	210 (909)	145 (1000)	100 (1121)	98 (1011)	110 (926)
	60	0 (1371)	<5 (1100)	0 (1209)	0 (1314)	0 (1212)	<5 (1410)
No ITSF (control)	7	180 (1291)	210 (1193)	145 (1089)	100 (1112)	98 (984)	110 (1010)
	60	0 (1201)	<5 (1170)	0 (1239)	0 (1051)	0 (1150)	<5 (1096)
Agglutinating Ab titers at time of sacrifice	7	1280	640	640	640	1280	640
	60	20	<5	10	<5	10	0

a The rabbits were immunized with 10^9 SRBC and 10^9 HRBV IV on day 0.

b ITSF was added to the allogeneic splenic MNC cultures on days 0 and 3 of culture.

c The figures outside and within the brackets are the number of PFC at the time of sacrifice and after culture for 5 days in vitro with the antigen, SSSF or HSSF, respectively.

5.7. ITSF inhibits the secretion of specific antibodies, but not the synthesis and secretion of non-specific immunoglobulins (Ig) by cultured immune splenic MNC

5.7.1. Rational and objective

The results of the investigation to this point have demonstrated that the antigen-specific suppressor cells (ITSC) and antigen-specific suppressor factor generated by the ITSC (ITSF) invariably inhibit the secretion of specific antibodies from immune splenic AFC (Chapters 5.3. and 5.4.), the induction of the 1^{o}IM in vivo and the 2^{o}IM induced in vitro (Chapter 5.6.). What was not determined, however, is whether ITSF also inhibits the synthesis and secretion of non(antigen)-specific Ig by immune splenic MNC.

The OBJECTIVE in this part of the investigation was to determine whether and to what extent ITSF inhibits the synthesis and secretion of non(antigen)-specifig Ig by immune splenic MNC culture in vitro.

5.7.2. Experimental protocols

The immunization of the rabbits, the preparation of splenic MNC and thymocytes, the preparation of ITSF and NTS and the hemolytic PFC assay were all carried out as described previously. The percentage of cells with intracytoplasmic Ig was determined by the immunofluorescent assay (IF) as described in Chapter 4.2.26. The quantity of non-specific Ig secreted into the culture medium by the immune splenic MNC was determined as described in Chapter 4.2.26.

5.7.3. Experimental results

As can be seen in Table 32, ITSF but not NTS inhibited generation of hemolytic plaques by the immune splenic MNC by the PFC assay following incubation of the splenic MNC with ITSF or NTS. On the other hand, the amount of Ig secreted by the ITSF suppressed splenic MNC was equal to

that secreted by the cells incubated with either NTS or culture medium.

Immune splenic MNC incubated with ITSF, NTS or culture medium for periods of 1, 8, 24 or 72 hrs, generated identical percentages of IF positive cells and secreted similar quantities of Ig into the culture supernatants (Table 33).

5.7.4. Commentary

The results of this part of the investigation demonstrate that ITSF inhibits the secretion of specific antibodies from the immune splenic AFC but does not inhibit the synthesis or the secretion of non(antibody)-Ig by the cultured immune splenic MNC.

TABLE 32

ITSF INHIBITS ANTIBODY SECRETION BUT NOT NON-SPECIFIC IMMUNOGLOBULIN SECRETION BY ALLOGENEIC IMMUNE SPLENIC MNC

Immune splenic MNC[a] were incubated with the following[b]	The number of PFC per 10^6 MNC	Ig secreted into culture supernatant (ng per ml)[c]
Culture medium	283	38
ITSF[d]	<5	39
NTS[d]	295	35

a Rabbit was immunized IV with 10^9 SRBC 7 days prior to sacrifice.

b Splenic MNC were incubated with ITSF or NTS for 4 hours, washed once with culture medium and incubated for an additional 24 hours in culture medium. The cells were then assayed for PFC and the cell culture supernatants were assayed for Ig.

c Determined by the ELISA assay.

d ITSF and NTS were assayed for the presence of rabbit Ig and were found to contain rabbit Ig at concentrations of 46 and 57 ng per ml, respectively.

TABLE 33

ITSF DOES NOT INHIBIT THE SYNTHESIS OF INTRACYTOPLASMIC Ig NOR THE SECRETION OF NON-SPECIFIC Ig INTO THE CULTURE SUPERNATANTS BY IMMUNE SPLENIC MNC

Immune splenic MNC[a] were incubated with the following	Percentage of cells immunofluorescent positive				Ig secreted into culture supernatant (ng per ml)[c]			
	1 hr	8 hrs	24 hrs	72 hrs	1 hr	8 hrs	24 hrs	72 hrs
Culture medium	1%	3%	8%	13%	<30	<30	38	156
ITSF[d]	<1%	2%	7%	12%	<30	<30	39	154
NTS[d]	1%	2%	8%	13%	<30	<30	35	141

a Rabbit was immunized IV with 10^9 SRBC 7 days prior to sacrifice.

b Splenic MNC were incubated with ITSF or NTS for 4 hours, washed once with culture medium and incubated for an additional 1, 8, 24 or 72 hours in culture medium. The cells were then assayed for IF and the cell culture supernatants were assayed for Ig.

c Determined by the ELISA assay.

d ITSF and NTS were assayed for the presence of rabbit Ig and were found to contain rabbit Ig at concentrations of 46 and 57 ng per ml, respectively.

5.8. The characterization of the suppressor factor (ITSF)

5.8.1. Rational and objective

Up to this point of the investigation only the functional role of ITSF as an immunosuppressive agent was investigated. No experiments were conducted to define the nature of ITSF.

The OBJECTIVE of this last part of the investigation was to determine the temperature and pH stability, chemical properties and the heterogeneous nature of ITSF.

5.8.2. Experimental protocol

The immunization of the rabbits, the preparation of the thymocytes and splenic MNC from the immunized rabbits, the preparation of ITSF, the absorption of ITSF with SRBC and HRBC, and the hemolytic PFC assay were carried out as described previously. The isoelectric focusing was carried out as described in Chapters 4.2.28 and 4.2.29.

5.8.3. Experimental results

As can be seen in Table 34, incubation of ITSF for up to 24 hrs at $4^{o}C$, $24^{o}C$, $37^{o}C$ or $56^{o}C$ at pH 7, or at $24^{o}C$ at pH 4 or pH 10 did not result in significant changes in its suppressor activity. However, ITSF exposed to $100^{o}C$ for only 10 minutes at pH 7 lost all of its suppressor activity. ITSF in 10 ml did not pass through the dialysis membrane even following dialysis for 24 hrs at $4^{o}C$, pH 7 against 2 litres of medium (RPMI 1640).

The immune thymocyte culture supernatants containing the ITSF and the culture supernatants of thymocytes of unimmunized rabbits (NTS) were examined by isoelectric focusing (Figure 1). Both supernatants were highly heterogeneous as they were each composed of more than 15 distinct protein bands. The patterns obtained with ITSF and NTS were similar but

not identical (Figure 1). It should be noted that the medium control gave no protein bands.

The isoelectric focusing patterns of ITSF (SRBC) (ITSF obtained from the thymocytes of SRBC immunized rabbit), ITSF (SRBC) absorbed with SRBC and ITSF (SRBC) absorbed with HRBC are presented in Figure 2. The result suggest that ITSF (SRBC) absorbed with SRBC but not HRBC resulted in the partial removal of protein constituents since the isoelectric focusing pattern of the ITSF (SRBC) absorbed with SRBC differed markedly from the isoelectric focusing pattern of the ITSF (SRBC) absorbed with HRBC or the unabsorbed ITSF (SRBC) (Figure 2). The primary difference is the decrease in staining intensity of a number of protein bands.

5.0.4. Commentary

ITSF appears to be a very stable entity since it can withstand exposure to pH 4 and 10 for 24 hrs, and is non-dialyzable through a dialysis membrane with an exclusion M.W. of 20,000 daltons. Since suppressor activity is totally lost following exposure of ITSF to 100°C for only 10 minutes it may be presumed that the suppressor factor is a protein. The results of the treatment of ITSF with protease could not be interpreted since the protease treated immune thymocyte culture supernatant containing ITSF caused clumping of the target AFC in the PFC assay (results not presented in Tables). On the other hand, treatment of ITSF with RNase and DNase resulted in the loss of 100 percent and 50 percent of the suppressor activity, respectively (2 experiments, results not presented in Tables). These data if confirmed in future experiments suggest that the suppressor factor is composed of at least DNA, RNA and protein.

It was not expected that the immune thymocyte culture supernatant containing the ITSF would present with at least 15 protein bands, since

the thymocytes were incubated in serum-free medium it must be assumed that these proteins were either passively shed from the thymocyte surface membrane and/or actively secreted by the thymocytes. The present results do not permit one to distinguish between these two possibilities. Never the less the results indicate that the concentration of certain constituents in the ITSF (SRBC) is decreased following absorption with SRBC but not HRBC. These results however are very preliminary and must be repeated before proper conclusions can be drawn.

TABLE 34

THE PHYSICOCHEMICAL PROPERTIES AND THE STABILITY TO TEMPERATURE AND pH OF THE IMMUNE THYMUS SUPPRESSOR FACTOR (ITSF)

ITSF[a] subjected to the following treatment	Percent suppression of the number of PFC per 10^6 splenic MNC following 4 hours of incubation of the 7 day immune spleen MNC with the treated ITSF in the following dilutions.	
	1:10	1:20
Untreated (Control)	100	100
Incubated for 4 Hr at 4°C, pH 7.0	92	100
Incubated for 24 hr at 4°C, pH 7.0	92	80
Incubated for 72 hr at 4°C, pH 7.0	88	90
Incubated for 4 hr at 24°C, pH 7.0	88	80
Incubated for 24 hr at 24°C, pH 7.0	70	60
Incubated for 4 hr at 37°C, pH 7.0	100	100
Incubated for 24 hr at 37°C, pH 7.0	72	63
Incubated for 4 hr at 56°C, pH 7.0	85	70
Incubated for 24 hr at 56°C, pH 7.0	66	63
Incubated for 10 min at 100°C, pH 7.0	0	0
Incubated for 4 hr at 24°C, pH 4.0	72	78
Incubated for 24 hr at 24°C, pH 4.0	70	55
Incubated for 4 hr at 24°C, pH 10.0	85	80
Incubated for 24 hr at 24°C, pH 10.0	70	55
Frozen and thawed 1 time	92	83
Frozen and thawed 2 times	73	70
Dialyzed for 24 hr (pH 7.0, 4°C)	100	80

a Rabbits were immunized IV with 10^9 SRBC. They were sacrificed 7 days later and the thymocytes were incubated in culture medium (5×10^7 cells per ml) at 37°C for 24 hrs. The cell suspention was centrifuged and the supernatant collected and tested for its suppressive capacity.

FIGURE 1. THE ISOELECTRIC FOCUSING PATTERN OF ITSF AND NTS

Lane	1.	NTS
	2.,3.,4.	ITSF (SRBC)
	5.	Medium (RPMI 1640)

FIGURE 1

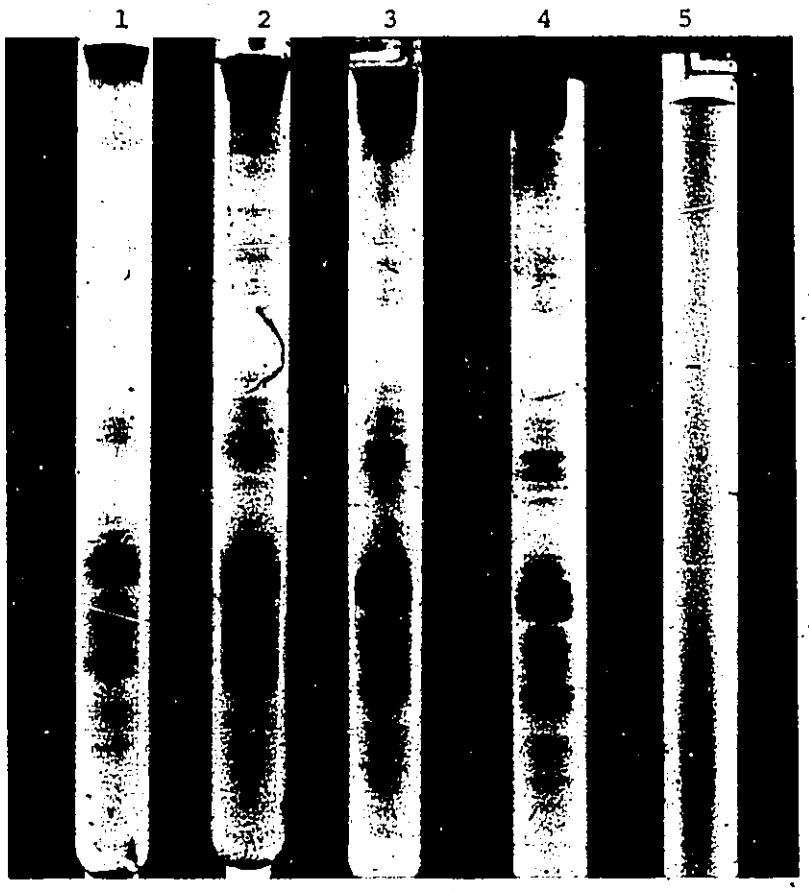


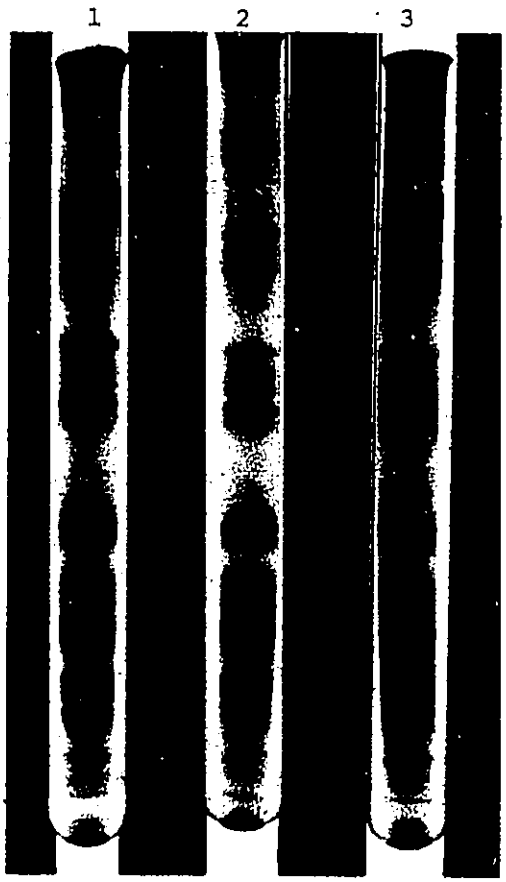
FIGURE 2. THE ISOELECTRIC FOCUSING PATTERN OF ITSF (SRBC) ABSORBED WITH SRBC AND HRBC

Lane 1. ITSF (SRBC) unabsorbed

2. ITSF (SRBC) absorbed with 10^9 SRBC

3. ITSF (SRBC) absorbed with 10^9 HRBC

FIGURE 2



6. DISCUSSION

The initial objective of this research was to determine why overt, antibody secreting AFC are not detected in the rabbit thymus during the primary immune response following IV immunization or during the secondary immune response in vivo carried out 3 to 12 months later. Large numbers of AFC can be detected by the PFC assay in the spleen, and lesser numbers in the circulation and bone marrow at the height of the primary immune response (Richter et al, 1986). During the secondary immune response in vivo, AFC are detected in the spleen, PLN, bone marrow and the circulation but not in the thymus (Richter et al, 1986). Memory cells are detected only in the spleen up to 3 to 4 months post-1^{o}IMM IV. However, by 4 to 6 months post-1^{o}IMM, memory cells can be detected not only in the spleen but also in the PLN and in the thymus (Richter et al, 1986). In fact, they increase in numbers in the thymus so that by 9 months post-1^{o}IMM, there are more memory cells in the thymus than in the spleen (Richter et al, 1986). At no time are memory cells detected in any of the other lymphoid organs of the rabbit (sacculus rotundus, appendix, Peyer's patches, bone marrow) and the circulation. One might expect that the AFC spilling out from the spleen into the circulation at the height of the primary immune response following IV immunization would infiltrate the thymus as they do the PLN and the bone marrow and be detected by the PFC assay. However, the consistent absence of detectable AFC in the thymus and PLN at these times suggests that (i) these organs are not conducive for the survival of AFC, (ii) there exist blood-thymus and blood-PLN barriers, barring activation of endogenous AFC or the infiltration of circulating AFC, (iii) the circulating AFC are dying AFC and cannot survive within the lymphoid organs they infiltrate other than the bone marrow where they survive for only a few days, or (iv) there exist suppressor mechanisms within the thymus and

PLN which mitigate against the detection of infiltrating or endogenously-generated (parenchymal) AFC by the PFC assay.

Evidence presented previously from this laboratory does not support the existence of a blood-PLN barrier since IV immunization of splenectomized rabbits results in the detection of large numbers of AFC in the PLN by day 7 to 8 post-1°IMM (Barron et al, 1986). However, no PFC are detected in the thymus of the IV immunized splenectomized rabbit (Barron et al, 1986).

Previous finding of memory cells to SRBC in the thymus beginning 4 months post-1°IMM IV with SRBC suggested that suppressor cells might be present in the thymus during the active antibody-synthetic phase and the early post-1°IMM memory period capable of inhibiting any infiltrating AFC. The results presented in this investigation unequivocally demonstrate the presence of antigen-specific suppressor cells (immune thymus suppressor cells or ITSC) in the thymus of the IV immunized rabbit at the height of the primary antibody immune response (day 7 to 8 post-1°IMM IV). The ITSC totally inhibited the generation of hemolytic plaques by splenic AFC if the splenic AFC were first coincubated with the autologous thymus cells for 4 hrs. Even a 2 hr coincubation of the splenic AFC with the autologous thymocytes was sufficient to suppress the splenic PFC by 70 to 80 percent.

It has been reported that the injection of antigen directly into the guinea pig thymus results in the formation of germinal centers, lymphoid follicles and plasma cells (Marshall et al, 1961), and antibody formation (Marshall et al, 1961; Blau and Waksman, 1964) within the thymus. These findings suggest that a strong antigenic stimulus in the thymus acting directly on thymocytes and/or infiltrating cells can overcome whatever immunosuppressive mechanisms exist in the thymus. Furthermore, cells in the rabbit thymus have been shown to be capable of synthesizing and secreting immunoglobulins in vitro (Putman et al, 1980; Jentz et al, 1979).

In fact, the cells in the thymus which secrete Ig were shown to be contaminating B cells; the Ig synthetic capacity of the "thymic" B cells was 34 times greater than the Ig synthetic capacity of the splenic B cells (Jentz et al, 1979). It must therefore be concluded that when the antigenic stimulus is provided in such a way as to stimulate antibody-forming cells only in the spleen following IV immunization, immunosuppressor mechanisms activated in the thymus can presumably gain the upper hand over any limited or feeble attempt at antibody synthesis in the thymus by either actively generated or passively infiltrating AFC.

The capacity of the ITSC to inhibit the PFC generated by autologous splenic AFC is not MHC restricted as the ITSC also inhibited formation of hemolytic plaques by allogeneic immune splenic AFC. These results support those of Ruben et al (1980, 1983, 1984) who demonstrated that thymus cells obtained from the South African clawed toad, Xenopus laevis, 4 days post-immunization with SRBC inhibited antibody formation by autologous or allogeneic spleen cells in culture. However, Ruben et al (1980, 1983, 1984) did not establish the antigenic specificity of the suppressor cells nor their mechanism of action. It has been demonstrated in this investigation that the ITSC are antigen-specific since the thymocytes of rabbits immunized simultaneously with SRBC and HRBC, two non-cross reacting antigens, and depleted of cells which rosette with SRBC retain the capacity to inhibit the generation of HRBC PFC but lose the ability to inhibit the generation of SRBC PFC by autologous or allogeneic immune splenic MNC. Similarly, the thymocytes depleted of cells which rosette with HRBC retain the capacity to inhibit the generation of SRBC PFC but lose the ability to suppress the generation of HRBC PFC. The ability of cells in the immunized rabbit to rosette with SRBC and HRBC is immunologically determined since neither the thymocytes nor the cells of

any of the other lymphoid organs of the unimmunized rabbits rosette with either SRBC or HRBC.

The ITSC is a T cell with receptors for the immunizing antigen and for FcG. This conclusion is based on the following findings which were consistently obtained:

(i) Prior to immunization and during the first 4 days following immunization, there are few cells in the rabbit thymus capable of rosetting with the antigen, SRBC or HRBC, or with the EAG indicator cells. However, by day 5 following immunization with SRBC or HRBC, antigen-specific suppressor cells, capable of rosetting with the antigen (SRBC or HRBC) and the EAG indicator cells were detected in the rabbit thymus. The percentages of rosetting cells increased in concert with the suppressor cell activity of the thymocytes and declined with the fall in suppressor activity of these cells.

(ii) The ITSC induced by immunization with SRBC or HRBC can be removed by rosetting with only the immunizing antigen, SRBC or HRBC, respectively. Thus, the ITSC must have a surface receptor(s) for the immunizing antigen.

(iii) The ITSC can be removed by rosetting with the EAG indicator cells irrespective of the antigen which caused their induction. Thus, all ITSC possess surface receptors for FcG.

(iv) The ITSC do not rosette with the EAC indicator cells, thus demonstrating that they do not possess detectable receptors for C'3b or C'3d.

The ITSC incubated at 37°C in antigen- and serum-free medium secreted a factor, immune thymus suppressor factor or ITSF, into the supernatant. Incubation of ITSF with the immune splenic AFC for 4 hrs at 37°C resulted in total inhibition of the generation of hemolytic plaques by the AFC in the

PFC assay. ITSF, like its parent ITSC, is antigen specific since the ITSF secreted by thymocytes of rabbits immunized with SRBC could only be absorbed out following incubation with SRBC but not with the non-cross reacting HRBC, HuRBC and RRBC. The ITSF secreted by the thymocytes of the HRBC-immunized rabbits was similarly demonstrated to be HRBC specific.

Like the ITSC, the ITSF suppressive activity is not MHC-restricted since it can inhibit the secretion of antibodies from autologous and allogeneic immune splenic MNC to the same degree.

A question which was addressed is whether ITSF acts directly on the immune splenic B lymphocyte to inhibit the secretion of antibodies or whether it acts on another cell, i.e. T cell, Null cell or macrophage, which then suppresses the B lymphocyte. To resolve this question, immune splenic MNC were depleted of T cells and/or Null cells and/or macrophages and the remaining cells were incubated with ITSF for 2 hrs before assaying for plaque-forming cells. The results leave no doubt but that ITSF acts directly on the antibody-forming B cell since elimination of any or all of the other cells did not have any effect on the suppression induced by the ITSF on the AFC. Furthermore, incubation of ITSF with the pure AFC/memory cells resulted in subsequent failure of these cells to generate hemolytic plaques in the PFC assay. NTS did not exhibit suppressor activity at any time. Identical results to those obtained with ITSF and NTS were obtained with ITSC and NTC, respectively.

The ITSC were not detected during the first 4 days post-1^{o}IMM IV. By day 5 post-1^{o}IMM the thymus possessed ITSC capable of totally suppressing the PFC by allogeneic splenic MNC but not as yet capable of secreting detectable amounts of ITSF. By day 7 post-1^{o}IMM, the ITSC were capable of secreting ITSF. The ITSC were detected in the thymus in their maximal suppressive

state until about day 11 post-1°IMM.

Over the following 4 weeks, their capacity to suppress the PFC gradually diminished so that by day 40 post-1°IMM the ITSC could only suppress the splenic PFC by 50 percent. The ITSC could no longer be detected by day 60 post-1°IMM. Furthermore, the ITSC began to lose the capacity to secrete ITSF beginning about day 12 post-1°IMM; by day 40 post-1°IMM the ITSC could no longer secrete detectable amounts of ITSF.

It must be stressed that all of the lymphoid organs (thymus, spleen, PLN, bone marrow, appendix, Peyer's patches, sacculus rotundus) and the circulation of the unimmunized and immunized rabbit were investigated for suppressor cells and only the thymus of the immunized rabbit possessed cells capable of suppressing the PFC generated by autologous and allogeneic immune splenic MNC. It was consistently observed that the secondary immune (memory) response induced in vitro by the immune splenic MNC is very much lower between days 14 and 22 following 1°IMM IV with SRBC than it is between days 7 and 14 and beyond day 30 (Table 1 and Figure 3). These data strongly indicate that there are overt suppressor cells in the spleen between days 14 and 22 post-1°-IMM in addition to their presence in the thymus. Work is currently in progress to identify the splenic suppressor cells and to determine whether they originate from splenic parenchymal cells or whether they are the progeny of thymus-derived cells which have migrated to the spleen.

The objectives in the second part of this investigation were to determine (i) whether ITSC and ITSF could inhibit the primary immune response in vivo, and (ii) whether ITSC and ITSF could inhibit the secondary immune response by rabbit memory cells in vitro.

Splenic MNC containing memory cells were incubated with the antigen, SRBC or HRBC, for 5 days in culture medium at 37°C in 5 percent CO_2 in air.

The maximum 2^{o}IM, as defined by the number of PFC in the PFC assay. was attained after 5 days in culture. However, when ITSF was added to the cultures consisting of splenic MNC and the antigen (SRBC) at the beginning (day 0) of the 5 day culture, no inhibition of the 2^{o}IM was observed. It was reasoned that this was due to the absorption of ITSF by the SRBC, thereby nullifying its suppressive effect. Sonicates of the SRBC and HRBC stroma (SSSF and HSSF, respectively) are as effective as the particular SRBC and HRBC in inducing the 2^{o}IM in vitro and they do not neutralize the suppressive activity of ITSF in vitro (results not presented in Tables). The SRBC and HRBC were therefore substituted by the SSSF and HSSF in the cultures of splenic memory cells.

It was demonstrated that the addition of ITSC or ITSF, derived from rabbits immunized with SRBC IV 7 days previously, to the cultures of splenic memory cells and SSSF on days 0 and 3 totally suppressed the induction of a 2^{o}IM in vitro normally detected on day 5 of culture. The reason for the addition of ITSF on day 3 of the 5 day culture is that the functional life span of ITSF at 37^{o}C is approximately 3 days (results not presented in Tables). Both the ITSC and ITSF were antigenically specific as the ITSC and ITSF from SRBC-immunized rabbits suppressed the in vitro 2^{o}IM to SRBC but not to HRBC by allogeneic splenic MNC obtained from rabbits immunized with both SRBC and HRBC 7 days previously, whereas the ITSC and ITSF from HRBC-immunized rabbits suppressed the in vitro 2^{o}IM to HRBC and not to SRBC. Furthermore, when the ITSC or ITSF were obtained from rabbits immunized with both SRBC and HRBC, the removal of ITSC directed to the SRBC (by rosetting the ITSC with SRBC) or the removal of ITSF directed to the SRBC (by absorbing the ITSF with SRBC) resulted in the subsequent failure to suppress the in vitro response to SRBC but not to HRBC. Similarly, the removal of the ITSC and ITSF directed toward HRBC resulted in the loss of ability to suppress the

2^{o}IM in vitro to HRBC but not to SRBC. The removal of ITSC and ITSF directed toward both SRBC and HRBC resulted in failure to suppress the in vitro 2^{o}IM to both SRBC and HRBC. It may therefore be concluded that the ITSC, and the ITSF which they generate, are antigen-specific and can inhibit the secondary immune response in vitro.

ITSC and ITSF inhibited the primary immune response in vivo when injected on days 0, 3, and 5 (ITSC) or daily for 5 days (ITSF) following IV immunization with 4×10^5 SRBC and/or 4×10^5 HRBC. Immunization with these doses of SRBC and HRBC results in a consistently significant immune response but not the maximum response which is generated following IV immunization with 10^8 or 10^9 SRBC or HRBC. The rationale for immunizing the rabbits with the lower doses of SRBC and HRBC is that the immune responses would be more physiological and would be more susceptible to suppression by ITSC or ITSF. The administration of ITSC IV on days 0 and 3 following the IV immunization with the SRBC resulted in a markedly reduced immune response (circulating antibody titer and splenic PFC) at day 7 post-immunization. Moreover, the IV injection of ITSC on days 0, 3 and 5 following immunization resulted in a total suppression of the immune response on day 7. It must be stressed that NTC administered in the same numbers and at the same times did not induce any discernible suppressive effect on the immune response. The fact that allogeneic ITSC but not allogeneic NTC suppressed the 1^{o}IM in vivo indicates that no host-versus-graft or graft-versus-host reaction (in vivo MLR) took place in the rabbits. This conclusion is supported by the data presented in Table 24 which clearly shows that rabbit thymocytes do not stimulate or respond in the one-way MLR. It has been previously demonstrated in this laboratory that rabbit thymocytes do not stimulate allogeneic rabbit responder cells nor do they respond significantly in the rabbit one-way MLR

(Milthorp and Richter, 1979). Thus, the injected allogeneic thymocytes (ITSC and NTC) may survive and proliferate in the host and may possibly be rejected slowly over a long period of time (chronic rejection).

It is important to note that ITSF, but not NTS, administered IV daily on the first 5 days following IV immunization was also able to totally suppress the immune response. Like the suppression of the 1^{o}IM in vivo by ITSC, the suppression by ITSF is also antigen specific. Thus, the antigen-specific suppressor cells or ITSC, and the antigen-specific suppressor factor or ITSF secreted by the ITSC, are both capable of suppressing the primary immune response in vivo and the secondary immune response in vitro.

Experiments were carried out to determine whether the ITSF suppressed rabbits can, in fact, recover the capacity to generate antibodies towards the original immunizing antigen. In these experiments, SSSF and HSSF were injected instead of SRBC and HRBC in order to avoid neutralization of the injected ITSF by the antigen. The results indicate that the immunized rabbits injected with ITSF daily for 5 days beginning with the primary immunization IV maintained their suppressed state for at least 50 days following primary IV immunization. Tertiary immunization IV on day 80 post-primary immunization resulted in a normal antibody response to the original immunizing antigen. Thus, the rabbits can, in time, overcome the specific immunosuppression induced by the ITSF without any obvious long-term deleterious effects. It is important to stress that the immune response to a non-cross-reacting antigen was at no time affected by the administered antigen-specific ITSF. Furthermore, there were no signs of morbidity in the rabbits for at least 80 days following the injection of ITSF for five

consecutive days.

Histological examination of the lymphoid organs (thymus, spleen and PLN) and the kidney and liver of rabbits sacrificed two days after the fifth daily injection of ITSF did not reveal any significant alterations from the normal rabbits or from rabbits injected with NTS in the same manner as was ITSF. A slight to mild hyperplasia was observed in the outer marginal zones of the follicles in the spleen and the PLN (results not presented).

A third objective of the research was to determine whether the synthesis by rabbit B lymphocytes of only antibodies, or both antibodies and non-specific Ig, are inhibited by ITSF. It was observed that the percentage of IF positive cells and the quantity of Ig secreted by the rabbit B lymphocytes cultured in vitro in the presence of ITSF did not differ from the results obtained with rabbit cells incubated in the presence of NTS or just medium. These results were anticipated since only the PFC to the specific antigen and not those to a non-cross-reacting antigen were suppressed by ITSF. Interestingly, when ITSF was added to cultures of human circulating T_M cells, non-T cells and PWM phytomitogen, the ITSF totally inhibited the synthesis and secretion of non-specific IgG and IgM whereas NTS displayed no inhibitory effects. Furthermore, ITSF was not cytotoxic to the human circulating or the rabbit splenic MNC (results not presented in Tables). These data suggest that ITSF may have potential application as an immunosuppressive agent in patients with autoimmune disease (i.e. autoimmune hemolytic anemia, autoimmune neutropenia, autoimmune thrombocytopenia purupura, myasthenia gravis, systemic lupus erythematosus, Goodpasture syndrome, acute progressive rheumatoid arthritis, etc... .) and in recipients of allografts in conjunction with Cyclosporin-A and/or Immuran and/or ATG (anti-human thymocyte globulin). ITSF might also be used along with plasmaphoresis; usually there is a rebound of autoantibody synthesis following plasmaphoresis

treatment which would be suppressed by ITSF.

It is obvious that ITSF must be purified, and its composition must be defined, before it can be used in clinical trials in patients.

A final objective was to define ITSF in terms of its physicochemical properties and composition. By isoelectric focusing, ITSF was found to consist of at least 15 distinct proteins (Figure 1). Therefore, if ITSF is a protein, it will be necessary to batch-fractionate the rabbit immune thymocyte culture supernatant containing the ITSF, elute each of the protein bands and assay each for its capacity to suppress the PFC response of rabbit immune splenic AFC. However, it is possible that ITSF is not a protein. Exposure of ITSF to a number of enzymes - DNAase, RNAase and Protease K, disclosed that ITSF treated with RNAase lost all suppressive activity whereas ITSF treated with DNAase lost 45 to 50 percent of its suppressive activity (results not presented in Tables). It was not possible to assess the effect of Protease K on ITSF due to the fact that Protease K consistently clumped the target immune splenic AFC in the PFC assay. It must be emphasized that these are only preliminary experiments as they were carried out only two times. Nevertheless, it would appear that ITSF is either RNA, an RNA-DNA complex, or an RNA-DNA-protein conjugate. Of course, there may be multiple ITSFs with different compositions.

A question which may be asked is whether ITSF might be a type of antibody, or a molecule with antibody-like activity, directed to the antigen since it reacts specifically with the immunizing antigen (SRBC and/or HRBC). ITSF (SRBC), obtained from immune thymocytes (rabbits immunized 7 days previously with SRBC IV) depleted of B cells, was absorbed with the antigen, SRBC. These SRBC-ITSF conjugates were then analyzed by the conventional immuno-fluorescence (IF) assay in the direct manner, using the FITC-conjugated $F(ab)_2$ fragment of goat anti-rabbit Ig, and in the indirect manner using

the $F(ab)_2$ fragment of goat anti-rabbit Ig followed by the FITC-conjugated $F(ab)_2$ fragment of rabbit anti-goat Ig. Since no fluorescence was observed at any time with the SRBC-ITSF conjugate preparation, whereas control rabbit spleen cells were consistently IF positive, the results strongly suggest that ITSF does not have the antigeneic composition of an antibody molecule (results not presented in Tables).

It would appear that ITSF is a high molecular weight (>20,000 daltons) entity as it is not dialyzable even after 24 hrs of dialysis at $4^{\circ}C$. Freezing and thawing twice also does not effect the suppressor activity of ITSF to any appreciable degree. Furthermore, ITSF exposed to $56^{\circ}C$ for 24 hrs retained more than 75 percent of its suppressive activity. However, exposure to $100^{\circ}C$ for 10 minutes resulted in the loss of all suppressive activity. ITSF is stable at low and high pH. These data suggest that ITSF is a relatively stable molecule.

Antigen-specific and non-specific suppressor cells and suppressor factors have been described by many investigators over the past 15 years (see Chapter 3). It would be impossible to comment on even all the relevant publications or the many reviews which have been published on suppressor cells and suppressor factors. Suffice it to say that the evidence to date strongly favours the generation of suppressor cells first described by Gershon and Kondo (Gershon and Kondo, 1970) in 1970, and the secretion of soluble suppressor factors by the suppressor cells as the mediator(s) which abrogates the antibody immune response.

Suppressor cells capable of inhibiting antibody synthesis in vitro include (i) thymus cells in the South African clawed toad 4 days post-1°IMM, (ii) Con A activated mouse splenic T cells, (iii) bone marrow cells in the unimmunized mouse and the normal human, and (iv) circulating human T cells.

Suppressor factors have been prepared from (i) cultured circulating human T cells (Heijnen et al, 1979; Uytdehaag et al, 1981) (ii) human bone marrow cells (Singhal, 1985), (iii) Con A and MLR (Gisler and Fridman, 1975, 1976; Neauport-Sautes et al, 1975; Fridman et al, 1977, 1981, 1984; Yagello et al, 1979) activated normal mouse spleen cells, (iv) cultured bone marrow cells of normal mice (McGarry et al, 1982), (v) spleen, thymus and lymph node cells of GAT-primed nonresponder mice (Kaap et al, 1976, 1981), and (vi) supernatants of cultured spleen cells of mice 4 weeks following immunization with SRBC or HRBC (Yamauchi et al, 1981 a,b).

Rich and Pierce (Rich and Pierce, 1973, 1974) demonstrated that Con A-activated mouse splenic T cells could suppress the primary PFC response by mouse spleen cells in vitro. Duwe and Singhal demonstrated the presence of suppressor cells in the bone marrow of unimmunized mice (Duwe and Singhal, 1979 a,b) and humans (McGarry and Singhal, 1982) capable of suppressing the in vitro antigen-induced PFC response by isologous or allogeneic cells. Heijnen et al (1979) demonstrated antigen-specific human circulating T suppressor cells capable of inhibiting the antigen induced (primary?) immune response by human circulating B cells in vitro. This group of investigators described an antigen-specific suppressor factor, TsF-120, secreted by circulating human suppressor T cells obtained from unimmunized humans capable of inhibiting the in vitro immune response by human circulating B cells (Utdehaag et al, 1981; Ballieux et al 1982). Pierce and his colleagues (Rich and Pierce, 1973, 1974; Tadakuma and Pierce, 1976, 1978; Aune and Pierce, 1981 a,b, 1982, 1983; Irons et al, 1984) described a factor isolated from Con A stimulated spleen cells of unimmunized mice capable of inhibiting the PFC response of mouse spleen cells to SRBC. This factor was referred to as soluble immune response suppressor or SIRS. The target for SIRS was shown to be the

macrophage, not the B cell (Aune and Pierce, 1981 a,b, 1982). Following short-term incubation with SIRS, the macrophages release a second factor which was shown to be directly responsible for the suppression of the immune response in vitro. This factor was referred to as a macrophage-derived suppressor factor of MØ-SF. This factor was shown to be a modified form of SIRS since MØ-SF could be obtained by reacting SIRS with H_2O_2 in the absence of macrophages (Aune and Pierce, 1982, 1983). The authors suggested that the macrophages serve only as a source of H_2O_2 which oxidizes SIRS and converts it to SIRSox (or MØ-SF). Both SIRS and SIRSox appear to function via oxidative processes since suppressor activity is rapidly lost following treatment of MØ-SF with reducing agents such as 2-mercaptoethanol, cysteine, $NaBH_4$ and a variety of peroxidase substrates (Aune and Pierce, 1983; Irons et al, 1984). SIRS must be added at the beginning of the in vitro 5-day culture in order for the PFC response to be inhibited (Rich and Pierce, 1974; Tadakuma and Pierce, 1976, 1978). On the other hand, the in vitro PFC response by mouse spleen cells could be inhibited by the addition of MØ-SF up to day 4 of the 5-day culture. The addition of MØ-SF even 2 hrs prior to assay resulted in marked inhibition of the generation of PFC (Aune and Pierce, 1983), indicating that MØ-SF can inhibit the secretion of antibodies from the antibody-synthesizing cells.

Fridman and his colleagues described a factor secreted by MLR activated spleen T cells obtained from unimmunized mice (Gisler and Fridman, 1976; Fridman et al, 1977, 1981; Yagello et al, 1979) and by circulating MNC of normal unimmunized humans (Fridman et al, 1984) in culture, which could suppress the antibody response by mouse spleen cells and human MNC in vitro. This factor was found to bind to immunoglobulins and was referred to as immunoglobulin-binding factor or IBF. Fridman et al (1977, 1981, 1984) demonstrated that IBF is the FcG receptor, present on the surface of the FcG

receptor-bearing T suppressor cells, which is shed into the culture medium upon incubation of these cells. Singhal et al reported the secretion of a suppressor factor, BSF, following incubation of mouse (Duwe and Singhal, 1979 a,b) or human (McGarry and Singhal, 1982; McGarry et al, 1982) bone marrow cells for 24 hrs in serum-free medium, capable of suppressing the in vitro antigen (SRBC)-induced PFC response by murine spleen cells and human circulating cells, respectively.

Pierce and his colleagues (Kapp, 1978; Kapp et al, 1976, 1978, 1980, 1983) extracted an antigen-specific suppressor factor from spleen, thymus and lymph node cells of GAT (terpolymer of L-glutamic acid^{60}-L-alanine^{30}-L-tyrosine^{10}) primed nonresponder mice. The suppressor factor, which must be extracted from the cells, is refered to as GAT-induced suppressor factor (GISF) in this text. GISF suppressed the in vivo immune response to GAT when administered into the nonresponder mice along with an immunogenic form of GAT, GAT-MBSA (GAT coupled to methylated boving serum albumin). GISF also inhibited the in vitro immune response of spleen cells of nonresponder mice to GAT-MBSA when the suppressor factor was added to the cells at the initiation of culture (Kapp et al, 1980, 1983). GISF extracted from thymus cells was more active than that extracted from spleen and lymph node cells (Kapp et al, 1980, 1983). ITSF differs from GISF by a number of criteria: (i) ITSF is secreted into the supernatant by the suppressor cells whereas GISF must be extracted from the cells; (ii) ITSF is obtained only from thymus cells whereas GISF is obtained from thymus, spleen and lymph node cells, (iii) ITSF inhibits the in vitro response of autologous and allogeneic spleen PFC whereas GISF only inhibits the in vitro immune response to GAT in cell cultures of responder mice, and (iv) ITSF can be added to the antigen-stimulated cultures spleen AFC up to 2 hrs prior to termination of culture

and totally inhibit the PFC response whereas GISF must be added to the cultured cells at the initiation of culture in order to suppress the in vitro PFC response.

Cantor and his colleagues have described two suppressor factors obtained from 48 hrs culture supernatants of spleen cells of mice following intraperitoneal immunization with SRBC or HRBC for 4 weeks (Cantor et al, 1978; Eardley et al, 1979; Yamauchi et al, 1981a,b). One suppressor factor was obtained from $Ly1^{+}_{2}{}^{-}I\text{-}J^{+}$ splenic T cells, designated Ly-1 TsiF (Cantor et al, 1978), whereas the second factor was obtained from $Ly\text{-}1^{-}_{2}{}^{+}$ $I\text{-}J^{-}$ splenic T cells, designated Ly-2 TsF (Yamauchi et al, 1981a). The former could only inhibit the late but not the early phase of antibody synthesis when added to 5-day antigen-stimulated spleen cell cultures at the initiation of the culture (Cantor et al, 1978; Eardley et al, 1979) whereas the latter could inhibit both the early and the late phases of the in vitro immune response when added at the initiation of the culture (Yamauchi et al, 1981b). ITSF is different from both Ly-1 TsiF and Ly-2 TsF. ITSF is obtained from thymus cell 7 days post-immunization whereas both Ly-1 TsiF and Ly-2 TsF are obtained from splenic T cells 28 days following immunization. Furthermore, ITSF does not exhibit MHC restriction since it can consistently inhibit the in vitro PFC response of allogeneic spleen AFC, whereas both Ly-1 Tsif and Ly-2 TsF are MHC restricted in their suppressor activity (Flood et al, 1982a). Since both Ly-1 TsiF and Ly-2 TsF suppressor factors are obtained long after the immune response has terminated and GISF is only able to suppress the immune response in a selected strain of nonresponder mice, the physiological role which they may play in vivo as immunoregulator factors in immunized normal outbred animals must be questioned.

All of the suppressor factors referred to above and the ITSF described here can be distinguished from each other as they exhibit different properties

and mechanisms of action (Tables 35 and 36). IBF, which is secreted by cells of the unimmunized rodent or human, has no immunosuppressive activity if added very early or very late to the 5-day mouse spleen cell culture; it is most suppressive when added to the 5-day culture on day 3 which is compatible with the view that IBF interferes with the terminal differentiation of the AFC (Fridman et al, 1977, 1981, 1984; Teilland et al, 1986). On the other hand, SIRS, which is secreted by Con A stimulated cells of unimmunized animals, must be added early (first 24 hrs) to the 5-day culture in order for the in vitro immune response to be suppressed (Rich and Pierce, 1974; Tadakuma and Pierce, 1976, 1978). However, the macrophage-altered SIRS, SIRSox (or MØ-SF), can be added to the cultured cells up to 2 hrs prior to the termination of the 5-day culture and still result in suppression of the in vitro immune response (Aune and Pierce, 1983; Irons et al, 1984). The antigen-specific suppressor factor TsF-120 which is secreted by circulating human MNC incubated with a random antigen (Uytdehaag et al, 1981; Ballieux et al, 1982), and the suppressor factor BSF which is secreted by unstimulated bone marrow MNC of unimmunized mice and humans (Duwe and Singhal, 1979a,b); McGarry and Singhal, 1982), must also be added at the beginning of the 5-day in vitro culture to totally suppress the immune response. The antigen-specific Ly-1 TsiF and Ly-2 TsF suppressor factors which are secreted from immunized mouse spleen cells 4 weeks following immunization, long after the immune response has terminated, display MHC restriction (Yamauchi et al, 1981 a,b). GISF must be extracted from the lymphoid cells and displays strain specificity in its suppressive activity (Kapp et al, 1980, 1983).

The immunosuppressive factor described here, ITSF, is distinct from SIRS, SIRSox, TsF-120, BSF and IBF (Table 35) and Ly1-TsiF, Ly-2 TsF and GISF (Table 36). ITSF does not show MHC restriction as do Ly-1 TsiF and

Ly-2 TsF (Flood et al, 1982a; Noguchi et al, 1985). It is synthesized and secreted by the suppressor thymus cells and does not have to be extracted from these cells as does GISF (Kapp et al, 1980, 1983). ITSF can totally suppress the secretion of antibodies from cells actively engaged in antibody synthesis and secretion, and does not inhibit antibody secretion by inhibiting (i) activation of the immunocompetent B cells (BSF), (ii) terminal differentiation of the AFC into the antibody-forming B cell (IBF) or (iii) antibody synthesis (SIRS and TsF-120).

ITSF most closely corresponds to the factor to which one would attribute physiological antigen-specific immunoregulatory functions. In order for the suppressor cell and the suppressor factor to be considered as physiological immunoregulators, they should satisfy a number of criteria: (i) they should be detected only after the immune response has been initiated in the normal animal and not before, (ii) they should not be detected in the unimmunized animal, (iii) they should be antigen specific, and (iv) the suppressor factor should be secreted by the suppressor cell. ITSF described here is the only suppressor factor which satisfies all of these criteria and therefore is a primary candidate to assume the role of a physiological immunoregulator. ITSF is present in cell-free supernatants of incubated thymus suppressor cells of immunized rabbits, it is not secreted by any lymphoid cells of unimmunized rabbits, it is antigen-specific in its mode of action, it is not detected early following immunization (day 1 to 5) at a time when the immune response is being initiated, and it can effectively suppress the manifestation of the immune response, i.e. secretion of antibodies by the AFC.

The suppressor cells described by Singhal et al (Duwe and Singhal, 1979a,b; McGarry and Singhal, 1982) and Fridman et al (Fridman et al, 1977, 1981, 1984) are FcG receptor-bearing cells and the suppressor factor, IBF,

appears to be the shed FcG receptor (Fridman et al, 1981, 1984; Yagello et al, 1979). Experiments aimed at identifying the ITSC in the rabbit thymus indicate that these cells are FcG receptor-bearing T cells. Rosetting the immune thymus cells with EAG but not EAC indicator erythrocytes resulted in the depletion of ITSC. Only the EAG rosetted thymocytes and not the EAC rosetted thymocytes possessed ITSC activity. Furthermore, treatment of the immune rabbit thymocytes with HARTS and complement, which resulted in the loss of 90 to 95 percent of the T cells, resulted in the loss of suppressor activity by the remaining cells irrespective of their cell concentration whereas treatment of these thymocytes with HARBMS and complement, which results in the loss of 90 to 95 percent of the B cells, did not affect the suppressor activity of the remaining thymocytes. It may therefore be concluded that the suppressor cell in the rabbit thymus following IV immunization is an FcG receptor-bearing T cell.

Both ITSC and ITSF are capable of inhibiting the 1°IM, and the 2°M in vitro. It is not possible to state at this time, however, whether ITSC and ITSF inhibit the synthesis and secretion or only the secretion of antibodies by the rabbit splenic antibody forming cells.

The immune splenic AFC 7 days post-1°IMM are synthesizing and secreting antibodies most actively as they generate the maximum number of PFC at that time during the primary immune response. It is therefore concluded that both ITSC and ITSF inhibit the generation of hemolytic plaques by the splenic PFC in vitro by inhibiting the secretion of antibodies from the antibody-synthesizing cells.

FIGURE 3. THE PRESENCE IN THE SPLEEN OF SUPPRESSOR CELLS TO SRBC WHICH INHIBIT THE IN VITRO SECONDARY IMMUNE RESPONSE TO SRBC BUT NOT TO HRBC

$\overset{\uparrow}{\text{SRBC}}$ - Rabbits were immunized with 10^9 SRBC IV on day 0 and sacrificed 7, 16, 18, 20 or 40 days later.

$\overset{\uparrow}{\text{HRBC}}$ - Rabbits were immunized with 10^0 SRBC IV on day 0 and with 10^9 HRBC IV on days 7, 8 or 9 following IV immunization with SRBC. They were sacrificed on days 16, 18 or 20 following immunization with SRBC.

THE PRESENCE IN THE SPLEEN OF SUPPRESSOR CELLS TO SRBC WHICH INHIBIT THE IN VITRO SECONDARY IMMUNE RESPONSE TO SRBC BUT NOT TO HRBC.

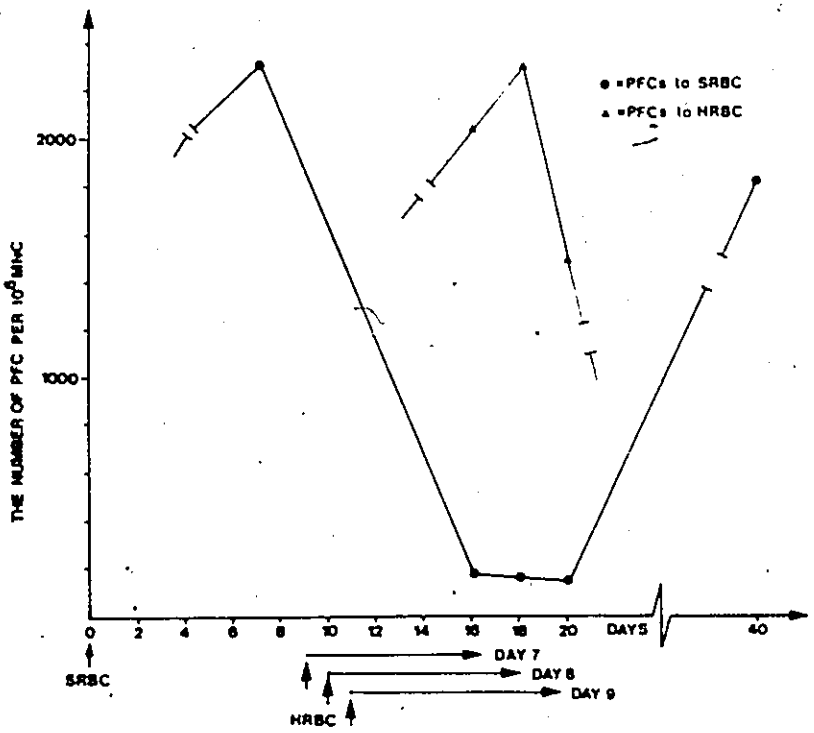


TABLE 35

A COMPARISON OF THE PROPERTIES OF ITSF, BSF, IBF, SIRS, MØ-SF AND TsF-120 SUPPRESSOR FACTORS

Properties of suppressor factors assessed	Suppressor factor described by: Richter and Talor ITSF[a]	Singhal et al BSF[b]	Fridman et al IBF[c]	Pierce et al SIRS[d] (MØ-SF)	Heijnen et al TsF-120[e]
1. Animal sources	Rabbit	Human Rodent	Human Rodent	Rodent	Human
2. Organ or cell source					
(i) bone marrow cells	No	Yes	No	No	No
(ii) thymus cells	Yes	No	No	No	No
(iii) circulating cells	No	No	Yes (human)	No	Yes
(iv) normal spleen cells	No	No	No	No	No
(v) activated T cells (spleen)	No	No	Yes (rodent)	Yes	No
3. Generated by in vivo activated suppressor cells.	Yes	No	No	No	No
4. Generated in vitro by cells of					
(i) previously unimmunized animal	No	Yes	Yes	Yes	Yes
(ii) previously immunized animal	Yes	No	No	No	No
5. Suppressor cell is an FcG-receptor positive cell.	Yes	Yes	Yes	ND[f]	Yes

TABLE 35 (Cont'd.)

Properties of suppressor factors assessed	Suppressor factor described by: Richter and Talor ITSF[a]	Singhal et al BSF[b]	Fridman et al IBF[c]	Pierce et al SIRS[d] (MØ-SF)	Heijnen et al TsF-120[e]
6. Generation in vitro by suppressor cells inquiries:					
(i) no stimulation	Yes	Yes	Yes	No	No
(ii) specific immunizing antigen	No	No	No	No	No
(iii) random antigen	No	No	No	No	Yes
(iv) phytomitogen (Con-A)	No	No	No	Yes	No
7. Factor is maximally immunosuppressive if added to antigen-stimulated cell culture (120 hr)					
(i) early (first 24-48 hr)	Yes	Yes	No	Yes (SIRS)	Yes
(ii) mid-culture (72 hr)	ND	No	Yes	Yes (MØ-SF)	No
(iii) late (last 24 hr)	Yes	No	No	Yes (MØ-SF)	No
(iv) only prior to assay (2 hr)	Yes	No	No	Yes (MØ-SF)	No

a ITSF = immune thymus suppressor factor.

b BSF = bone marrow suppressor factor.

c IBF = immunoglobulin binding factor.

d SIRS = soluble immune response suppressor factor.

e TsF120 = T cell derived suppressor factor following culture with antigen for 120 hrs.

f ND = Not done.

TABLE 36

A COMPARISON OF THE PROPERTIES OF ITSF, Ly-1, TsiF
Ly-2 TsF AND GISF SUPPRESSOR FACTORS

Properties of suppressor factors assessed	Suppressor factor described by: Richter and Talor ITSF[a]	Cantor et al (Ly-1 TsiF)[b]	Cantor et al (Ly-2 TsF)[c]	Pierce et al GISF[d]
1. Animal source	rabbit	mouse	mouse	mouse
2. Obtained from				
(i) splenic T cells	No	Yes	Yes	Yes
(ii) lymph node T cells	No	ND[e]	ND	Yes
(iii) thymus cells	Yes	ND	ND	Yes
3. Obtained from cells				
(i) early after immune response is initiated	Yes	No	No	Yes
(ii) following cessation of antibody synthesis (day 20-30)	No	Yes	Yes	ND
4. Secreted by suppressor cells	Yes	Yes	Yes	No
5. Must be extracted from suppressor cells.	No	No	No	Yes
6. To suppress in vitro immune response it can be added to cell cultures				
(i) early	Yes	Yes	Yes	Yes
(ii) late	Yes	No	No	No

TABLE 36 (Cont'd.)

Properties of suppressor factors assessed	Suppressor factor described by: Richter and Talor ITSF	Cantor et al (Ly-1 TsiF)	Cantor et al (Ly-2 TsF)	Pierce et al GISF
7. Suppressor factor displays				
(i) MHC restriction	No	Yes	Yes	No
(ii) non-MHC (strain) restriction	No	No	No	Yes
(iii) antigenic specificity	Yes	Yes	Yes	Yes

a ITSF = immune thymus suppressor factor.

b TsiF = T suppressor inducer factor.

c TsF = T suppressor factor.

d GISF = GAT-induced T suppressor factor.

e ND = Not done.

7. SUMMARY

The thymus of the immunized outbred New Zealand White Rabbit contains suppressor cells (ITSC) capable of inhibiting the secretion of the antibodies from autologous or allogeneic antibody forming cells (AFC) actively synthesizing antibodies, thereby preventing the AFC from generating plaques in the hemolytic plaque forming cell (PFC) assay. ITSC were detected in the thymus not earlier than 5 days post-primary IV immunization with 10^9 SRBC, 10^9 HRBC, or SSSF or HSSF equivalent to 10^9 SRBC or 10^9 HRBC, respectively. ITSC attained peak suppressor activity between days 7 and 15 post-immunization, and then began to decline and could no longer be detected by days 40 to 60 post-immunization. The ITSC were only detected in the thymus of the immunized rabbit and were not detected in any of the lymphoid organs of the unimmunized rabbit. The ITSC secrete a suppressor factor, referred to as immune thymus suppressor factor or ITSF, in a short term incubation (4 hrs) period which is capable, by itself, of inhibiting the generation of PFC by the immune splenic AFC. The suppressor activity of both ITSC and ITSF is strictly antigen specific. Both ITSC and ITSF exert their suppressive activity in a non-MHC restricted manner since both ITSC and ITSF can inhibit autologous and allogeneic immune splenic AFC to an equal extent. The ITSC and ITSF act directly on the AFC B cell to suppress the secretion of antibodies.

The ITSC are T cells with receptors for FcG since they can be eliminated from the immune thymus cells by incubation with anti-rabbit thymocyte serum and complement or by rosetting with EAG indicator erythrocytes.

ITSC and ITSF inhibited the secondary immune response in vitro when added to cultures of immune rabbit splenic MNC and the antigen. ITSF inhibited the secretion of specific antibodies by the immunized splenic mononuclear cells in vitro but it did not inhibit the synthesis or

secretion of non-specific immunoglobulins.

ITSC and ITSF inhibited the primary immune response in vivo when injected into the rabbit daily on 5 consecutive days beginning with the injection of the antigen. There was no evidence of cytotoxicity in vitro and no signs of morbidity in the injected rabbits. Furthermore, the rabbits injected with ITSF directed to SRBC and which failed to generate a primary immune response at the normal time (day 7 to 10 post-immunization) to SRBC responded normally to a non-cross-reacting antigen, HRBC, and recovered the ability to respond to the original immunizing antigen, SRBC, by day 87 post-primary immunization. Thus, the in vivo inhibitory effect(s) of ITSF is (are) not permanent.

On the basis of preliminary investigations as to the physicochemical properties and composition of ITSF, it would appear that ITSF is an RNA containing, and possibly DNA containing, macromolecule. It is non-dialyzable, withstands heating at 56°C for 24 hrs, but loses all suppressor activity following exposure to 100°C for 10 minutes. By isoelectric focusing, thymocyte culture supernatants containing the ITSF demonstrate more than 15 protein bands. Thus, if ITSF is a protein-RNA and/or DNA complex, it will have to be isolated from the other "contaminating" proteins before its composition can be determined.

8. ORIGINAL CONTRIBUTIONS TO KNOWLEDGE

8.1. The applicant has demonstrated that the thymus in the rabbit immunized intravenously with sheep erythrocytes, horse erythrocytes or soluble preparations of these erythrocytes possesses cells capable of inhibiting the secretion of antibodies from autologous splenic antibody forming cells (AFC), thereby preventing the generation of hemolytic plaques in the hemolytic plaque-forming cells (PFC) assay.

8.2. The applicant has demonstrated that the suppressor cells, referred to as immune thymus suppressor cells or ITSC, are detected in the thymus beginning 5 days post-primary immunization, peak in activity between days 7 and 15 post-immunization and can no longer be detected by days 40 to 50 post-immunization.

8.3. The applicant has demonstrated that the ITSC secrete a potent suppressor factor, referred to as immune thymic suppressor factor or ITSF, which is by itself capable of inhibiting the generation of hemolytic plaques by autologous immune splehic AFC.

8.4. The applicant has demonstrated that the ITSC are detected only in the thymus of the immunized rabbit and are not detected in any of the lymphoid organs of the unimmunized rabbit.

8.5. The applicant has demonstrated that both ITSC and ITSF are non-MHC restricted with respect to their suppressor activity since they can equally suppress autologous and allogeneic splenic AFC in the PFC assay.

8.6. The applicant has demonstrated that both ITSC and ITSF are antigen specific in their suppressor activity.

8.7. The applicant has demonstrated that ITSF acts directly on the rabbit splenic AFC, to inhibit antibody secretion and not on a different cell

(T cell, Null cell, macrophage) which then inhibits the secretion of antibodies from the splenic AFC in vitro.

8.8. The applicant has demonstrated that both ITSC and ITSF can inhibit the secondary immune response in in vitro culture of autologous or allogeneic splenic MNC to the specific immunizing antigen.

8.9. The applicant has demonstrated that both ITSC and ITSF can totally inhibit the primary immune response in vivo in the rabbit when administered on five consecutive days IV, beginning with the injection of the antigen to which they had been induced. This is again a demonstration of the antigenic specificity of the ITSC and ITSF.

8.10. The applicant has demonstrated that the ITSF-suppressed rabbits, which are unable to generate a primary antibody response in vivo, recover their capacity to synthesize and secrete normal levels of antibodies by 87 days post-primary immunization.

8.11. The applicant has demonstrated that the ITSC is a T cell with receptors for the Fc of antigen-complexed IgG(FcG). It is therefore a $T^{+}G^{+}$ cell.

8.12. The applicant has demonstrated that the ITSF may consist of RNA and DNA since 100 percent and 50 percent of the suppressive activity is lost following the treatment of the ITSF with RNAase and DNAase, respectively. The protein constituent of ITSF, if any, has not as yet been identified.

8.13. The applicant has demonstrated that the ITSF is non-dialyzable through conventional dialysis membranes which retain molecules above 20,000 M.W. and that it is reasonably heat stable since exposure to 56°C for 24 hrs affects only minimally its suppressor activity. It was destroyed following exposure to 100°C for 10 minutes.

8.14. The applicant has demonstrated that ITSF inhibits only the secretion of specific antibodies from cultured immune splenic MNC. It does not inhibit the synthesis and secretion of non-specific immunoglobulins by rabbit splenic MNC.

8.15. The applicant has demonstrated that under no conditions do thymocytes of normal unimmunized rabbits (normal thymus cells or NTC) or their culture supernatants (normal thymocyte culture supernatants or NTS) exhibit immunosuppressive activity.

8.16. The applicant has presented evidence in favor of ITSC and ITSF as the physiological regulators of the antibody response in the rabbit.

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