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THE THYMIC ORIGIN OF THE SPLENIC SUPPRESSOR CELLS
IN BOTH THE PRIMARY AND SECONDARY IMMUNE RESPONSES
IN THE OUTBRED RABBIT.

by
ISABELLE TRUDEL

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

University of Ottawa.



Isabelle Trudel, Ottawa, Canada, 1988.

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ABSTRACT

Previous findings from this laboratory established that the spleen in the immunized rabbit is the organ in which antibody synthesis takes place and the thymus is the organ in which antigen-specific suppressor cells are first detected following primary intravenous (IV) immunization with a T dependent antigen, SRBC. It was also demonstrated that the thymic suppressor cells were T cells capable of secreting an antigen-specific suppressor factor in culture. The objective of this research was to investigate the functional role for the thymic suppressor cells in the cessation of the immune response in the spleen.

It was demonstrated that antigen-specific suppressor cells can be detected in the spleen within a few days (day 14) following the detection of maximum number of antibody synthesizing cells (day 7-8) following IV immunization. The detection of the suppressor cells coincided with the dramatic loss in the capacity to generate a secondary immune response in in vitro culture. The splenic suppressor cells were also shown to be T cells capable of secreting an antigen-specific suppressor factor in culture. Thymectomy either before immunization or within 7 days following immunization resulted in the subsequent failure to detect suppressor cells in the spleen and a heightened capacity of the splenic memory cells to generate a secondary immune response in vitro. These results indicate that the splenic suppressor cells originate in the thymus.

It was also demonstrated that suppressor cells are present in the spleen following secondary immunization IV (60 days after primary immunization) and that thymectomy prior to secondary immunization results in failure to detect the suppressor cells in the spleen. These results indicate that the suppressor cells in the spleen following primary immunization do not enter a dormant state from which they can be reactivated into overt suppressor cells by contact with the antigen. Rather, it appears that the splenic suppressor cells die following primary immunization and are recruited from the thymus during the secondary immunization.

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LIST OF ABBREVIATIONS

Ab	Antibody
AFC	Antibody forming cells
APP	Appendix
ARC	Antigen receptor bearing cell(s)
BM	Bone marrow
C'	Complement
C'3	Third component of complement
E	Erythrocytes
E rosette	Formation of rosettes by T cells - SRBC
EAG	IgG complexed Ox RBC
Fc	Crystallizable fragment of Ig
FCS	Fetal calf serum
FcG	Fc of IgG
FcM	Fc of IgM
g	Gravity
HARBMS	Horse anti-rabbit bone marrow serum
HARTS	Horse anti-rabbit thymocyte serum
HBSS	Hank's Balanced Salt Solution
HI	Humoral immunity
hrs	Hours
HRBC	Horse red blood cells
ISLSF	Immune splenic lymphocyte(-derived) suppressor factor
Ig	Immunoglobulin
IgA	Immunoglobulin A
IgG	Immunoglobulin G
IgM	Immunoglobulin M

1°IM	Primary immune response
1°IMM	Primary immunization
2°IM	Secondary immune response
2°IMM	Secondary immunization
ITSF	Immune thymus suppressor factor
IV	Intravenous
IU	International units
MO	Macrophages
MW	Molecular weight
NSE	Non-specific esterase
PB	Peripheral blood
PFC	Plaque forming cell(s)
PLN	Popliteal lymph nodes
PP	Peyer's patches
RBC	Red blood cells (erythrocytes)
RRBC	Rabbit red blood cells
SC	Subcutaneous
SG	Specific gravity
SPL	Spleen
SR	Sacculus rotundus
SRBC	Sheep red blood cells
Tar ⁺	T autologous rosetting cells
Tar ⁻	T autologous non-rosetting cells
O	Theta
T _α	T cells with receptor for FcG
T _H	T helper cells
THY	Thymus

T _M	T cells with receptor for FcM
T _s	T suppressor cells
T _{se}	Suppressor effector T cell(s)
T _{si}	Suppressor inducer T cell(s)
T _{sp}	Suppressor precursor T cell(s)
WBC	White blood cells

1. INTRODUCTION

Resistance induced toward specific infectious microorganisms has been recognized since antiquity. The ancients knew from experience that anyone who survived certain diseases would not be likely to get them again. Alexander the Great, in his conquest of Asia Minor, employed the veterans of previous campaigns as army nurses and physicians. He knew that the survivors of previous campaigns, who had also survived the myriad infections experienced during those military campaigns, were resistant to these infections and would therefore be able to nurse the new recruits who would surely come down with these infections. It is known that as early as the 11th century, and probably earlier, the Chinese had learned the art of immunization against smallpox by inhaling pulverized scabs from smallpox survivors. Lady Montague, upon her return to England in 1785, spoke publicly about the Chinese immunization program for smallpox but she was not listened to. In 1796, Edward Jenner introduced an approach still used today to immunize against smallpox. He vaccinated people with cowpox which, because it carries antigenic determinants that cross-react with smallpox, immunized very effectively to smallpox. However, in retrospect, Jenner's spectacular discovery must be seen as anecdotal since it relied solely on intuition and results. There was no known scientific basis to it. Nevertheless, from a temporal perspective, his discovery is recognized as the one which ushered in the modern approach of immunization to disease.

The subsequent discovery of the bacteria by Louis Pasteur and his disciples, of bacterial toxins and their conversion into potent non-toxic vaccines by von Behring and his followers, the discovery of phagocytic cells by Metchnikoff and the elucidation of the mechanism of antibody synthesis by Paul Erlich (Erlich and Metchnikoff shared the first Nobel prize in Immunology) set the stage for the flowering of immunology in the 20th century.

The major contribution of immunology to the health of the population, both in terms of increasing the life-span and the quality of life, has been the introduction of relatively harmless bacterial and viral prophylactic vaccines which induce specific primary immune responses at times when the hosts are well and not in danger of morbidity or death from infection. As a result, the subsequent encounter with the live infectious agent induces a secondary immune response which is much more rapid than the primary response and therefore provides much better protection sooner to the infected host. The successful use and exploitation of vaccines is today the principal weapon in the continuous battle with infectious microorganisms.

A question which was addressed over 30 years ago is- "what is the mechanism whereby contact with the antigen induces the synthesis of specific antibodies?" The large number of investigations carried out between 1965 and 1970, sparked by the milestone discovery by Claman and his associates that cells of the thymus and of the bone marrow

must cooperate to facilitate antibody synthesis in the mouse, disclosed the existence of two lineages of immunocompetent lymphocytes, the T (or thymus derived) and the B (or bursal derived in birds or bone marrow derived in mammals) cells. It was demonstrated that the role of the T cell is to react with the antigenic determinants on the antigen, via precommitted receptors, and subsequently transmit information to the B cell precursor of the antibody forming cell. The B cell then undergoes transformational changes, blastogenesis, dedifferentiation, mitosis and differentiation into the antibody synthesizing and secreting lymphoblasts and plasmablasts. During the transition of the plasmablast into the end stage moribund plasma cells (life span 3-5 days), the ability to secrete antibodies is lost faster than the capacity to synthesize antibodies. Therefore the plasma cell has antibodies in abundance in its cytoplasm. With respect to T dependent antigens, both T and B lymphocytes are required. From a functional point of view, these cells are referred to as ARC (antigen-receptor bearing cells, antigen-reacting cells or antigen-responsive cells) and AFC (antibody forming cells).

It was also shown that a number of the antibody synthesizing lymphoblasts and plasmablasts revert back to small lymphocytes which have a very long life span (greater than 20-30 years) and which can be stimulated quickly to antibody synthesis by contact with the specific antigen.

These cells are therefore called memory cells and are responsible for the rapidly induced secondary immune response.

A question which was not addressed until 1970 was "how is the immune response turned off once it is no longer required?" Once antibodies have been secreted into the circulation in more than sufficient numbers to neutralize the toxin secreted by the infectious microorganisms and/or facilitate the killing and elimination of the infectious agent by activating the complement system, what is the mechanism which downregulates and terminates antibody synthesis? Jerne and Henry, in 1967, showed that antibodies themselves regulate antibody synthesis, that IgM antibodies stimulate IgG antibody synthesis and that IgG antibodies inhibit IgM antibody synthesis which, of course, results in diminished synthesis of IgG antibodies. This is an example of feedback suppression. However, doubts were expressed as to whether this antibody regulatory mechanism was the primary regulator of antibody synthesis. In 1969, Gershon demonstrated the existence of antigen specific suppressor T cells which are activated by the encounter with the antigen and which actively suppress the synthesis of antibodies by the AFC and the further generation of new AFC. The vast majority of the hundreds of investigations carried out since 1970 on the mechanism of immunoregulation have confirmed beyond doubt the existence of antigen-specific suppressor

cells. With few exceptions, these investigations have revealed the presence of suppressor cells in the spleen of the immunized mouse. However, no temporal studies have been conducted to indicate when, following immunization, the suppressor T cells can be detected in the spleen; nor was it demonstrated whether the suppressor T cells detected in the spleen originate in the thymus or in situ from precursor T cells (parenchymal T cells after birth) in the spleen.

The discovery of suppressor cells begged the question as to whether there exists cells which act antagonistically to the suppressor cells to enhance or facilitate antibody synthesis. To complete the picture, Moretta and his associates, in 1975, discovered the T helper cell which functions to facilitate and enhance immunoglobulin and antibody synthesis. They demonstrated that the T helper cell exhibits surface membrane receptors for the Fc of IgM and that the T suppressor cell exhibits surface membrane receptors for the Fc of IgG. Thus, the cellular regulatory mechanism for antibody synthesis consist of the antigen-specific T helper and T suppressor cells.

In 1986, Richter and Talor, in this laboratory, demonstrated that antigen-specific suppressor cells in the rabbit can be detected in the thymus within 4 to 5 days following immunization with a T dependent antigen, SRBC and that these cells secrete in culture an immune thymocyte suppressor factor (ITSF) capable of inhibiting the secretion of antibodies by the AFC. These investigators also

demonstrated that ITSF injected into host rabbits simultaneous with the original antigen inhibits the synthesis of antibodies toward this antigen but not towards other non-cross-reacting antigens. The suppressor T cell was shown to have receptors for both the antigen, SRBC, and the FcG on IgG antibody-sensitized erythrocytes (the EAG indicator cells).

The subject of this investigation was to establish a functional immunoregulatory role for the suppressor cells detected in the thymus early following immunization.

References have been omitted in this section as the pertinent subject matter discussed is elaborated upon in the following chapter.

2. Rationale, Hypothesis and Objective.

The RATIONALE of this investigation is that antigen-specific suppressor cells are detected in the thymus by 4 to 5 days following intravenous immunization of the adult outbred rabbit with a T dependent antigen, SRBC. Antibody forming cells on the other hand are detected primarily in the spleen by day 5 to 6 following immunization. The HYPOTHESIS to be tested is that the thymic suppressor cells migrate to the spleen where they act on the antibody forming cells and terminate the immune response. The OBJECTIVE is to demonstrate that the antigen-specific suppressor cells first detected in the thymus following intravenous immunization leave the thymus and infiltrate the peripheral lymphoid organs such as the spleen and the lymph nodes where they secrete an antigen-specific suppressor factor which acts locally on antibody forming cells to inhibit further antibody synthesis and brings about the cessation of the immune response.

3. HISTORICAL REVIEW

3.1 Introductory statement

The subject matter of this thesis is the demonstration of suppressor cells in the spleen and their role in bringing about the cessation of antibody synthesis. However, in order to appreciate the role of the suppressor cells, it is first necessary to define the organ sites of antibody synthesis and the cells which participate in this immune response.

It is common practice today, when discussing the cells involved in antibody synthesis, to first introduce the participating B and T lymphocytes in terms of their specific markers and then to define their roles in antibody synthesis. In the course of the discussion, it may or may not be revealed that the primary organ in which antibody formation takes place is the spleen especially following intravenous immunization with a T dependent antigen. It is almost an afterthought. The reason for the benign neglect of the organ sites of antibody synthesis may be due to the fact that we have known that the spleen is the primary site of antibody synthesis for more than 40 years and it is therefore taken for granted today. Historically, knowledge of the spleen as the organ site of antibody formation following intravenous immunization preceded by 5 years the demonstration of the lymphocyte-plasmablast - plasma cell series of cells as the antibody forming cells and by about 20 years the identification of the participating lymphocytes as T and B cells.

The material in this chapter will therefore be presented in its historical context, that is in the chronological order in which the findings appeared in the literature, beginning with the identification of the organ sites of antibody synthesis.

3.2 The organ sites of antibody synthesis

3.2.1 The spleen is the primary organ in which antibody synthesis takes place following intravenous immunization.

The evidence is so overwhelming today that antibody synthesis takes place in the spleen especially following intravenous immunization that investigators today would not seriously consider questioning this fact. However, it is worthy of note that, as recently as 1953, McMaster (1) presented rather uncertain and conflicting views concerning the site(s) of antibody synthesis. He concluded his review article with the statement "the sites of actual antibody formation still remain unknown, although much circumstantial evidence is at hand"(1). Nevertheless, McMaster also stated in the text that "there is so much well-known older work indicating antibody formation by the spleen - especially if antigen is injected intravenously - that lack of space forbids a full review here". He cited about 30 references in favour of the spleen as the site of antibody synthesis. Numerous studies in the 1950's and early 1960's substantiated this view (2-10).

With respect to the rabbit, Richter and Berry (11) demonstrated that antibody forming cells (AFC) are detected

first in the spleen following intravenous immunization with SRBC or HRBC antigen. At the peak of the immune response, AFC can be transiently detected in the circulation and in the bone marrow (11). They are not detected in the lymph nodes, thymus or any other lymphoid organ (11).

3.2.2 The lymph node is the site of antibody synthesis following immunization locally.

Although the spleen is the only organ in which antibody forming cells are detected following intravenous immunization, antibody forming cells are also detected in the draining lymph node, especially the popliteal lymph node, following local injection of the antigen subcutaneously, intradermally or intramuscularly either in saline or emulsified with adjuvant. In 1949, Susanna and Ted Harris (12) injected serologically distinct strains of influenza virus separately into the right and left hind-foot-pads of rabbits. They then cannulated the efferent lymphatics of the popliteal lymph nodes and intermittently tested samples of the efferent lymph for antibodies. They observed that antibodies to each strain of influenza virus were detected earlier in the respective efferent lymph than in the blood. These results strongly indicate that the draining popliteal lymph node cells can respond with antibody synthesis following local immunization and that the spleen may not be involved in antibody synthesis if the amount of antigen administered locally is too small to stimulate the spleen even if it gets into the circulation.

Following local immunization and using the then newly-described immunofluorescent technique, White et al (13) demonstrated antibodies within cells in the draining popliteal lymph nodes. Numerous investigations, of which only a few are cited (14-18), subsequently confirmed the participation of the popliteal lymph nodes in antibody synthesis following local immunization.

Berry and Richter (19) demonstrated that the vast majority of antibody forming cells are localized to the spleen following intravenous immunization of the rabbits. Some antibody forming cells are also detected only transiently in peripheral blood and bone marrow at the height of the immune response. However, if the rabbits were splenectomized several weeks prior to intravenous immunization, then antibody forming cells were detected in the popliteal lymph nodes. These results indicate that the involvement of the popliteal lymph nodes in antibody synthesis is not strictly dependent upon the mode of immunization, that is local as opposed to intravenous, since intravenous immunization of the splenectomized rabbit resulted in the recruitment of popliteal lymph node cells into antibody synthesizing cells.

3.3 The antibody forming cell is a lymphocyte

That antibody synthesis is carried out by lymphocytes is today considered as the absolute truth. The results of many thousands of investigations lend testament to this role of the lymphocyte (a very few of which are ref.20-23).

and it is not in the purview of this thesis to attempt to fan a flame of controversy on this matter. The contentious issue which is still to be resolved to the total satisfaction of everyone is whether it is the lymphocyte, the differentiated lymphoblast or plasmablast or the mature plasma cell which synthesizes and secretes antibodies. Fagraeus, in her Doctoral thesis published in 1950 (24,25) used the then pioneering tool of in vitro cell culture to demonstrate that the concentration of antibodies secreted into the culture medium by the immune cells is related to the number of lymphoblasts and plasmablasts in the culture, not to the number of mature lymphocytes or plasma cells. In fact, no antibodies were detected in the culture supernatants when the majority of the cultured cells were small, medium and/or large lymphocytes, or mature plasma cells. Her conclusion was that the lymphoblasts and plasmablasts secrete antibodies, and not the lymphocytes and plasma cells. A large number of investigators (27-37) have confirmed Fagraeus' original observations and it is difficult to avoid the conclusion that it is the lymphoblasts and plasmablasts which synthesize and secrete antibodies. In contradistinction, investigators utilizing the immunofluorescent technique to resolve this question have uniformly concluded that it is the plasma cells which synthesize and secrete antibodies since only these cells contain large amounts of antibodies in the cytoplasm. It is generally accepted today that the plasma cell is a cell with

a life-span of 3 to 5 days, that it is the transformed product of the plasmablast, and that during the transition from plasmablast to plasma cell morphology, the cell loses the capacity to secrete antibodies before it loses the capacity to synthesize antibodies. The balance of synthesis and secretion which allows the lymphoblasts and plasmablasts to secrete the antibodies as quickly as they are synthesized, and therefore appears to make these cells "rein" or naked of antibodies, is distorted in the plasma cell with secretion initially adversely affected, thus allowing a build-up in concentration of antibodies in the cytoplasm (25-37). It is, however, universally recognized today that all of the morphologically-identified cells referred to above are cells of a single lineage - the small mature lymphocyte. The mature lymphocyte can undergo transformation into medium and large lymphocyte which can transform, under the stimulation by antigen, into an undifferentiated blast cell. The latter can transform into the antibody synthesizing and secreting lymphoblast and plasmablast. All of these transformational and proliferative changes are reversible, so that the plasmablast can revert to the small lymphocyte memory cell. The only step which is irreversible is that from the plasmablasts to the plasma cell. This latter constitutes a safety valve utilized by the immune system to eliminate excessive numbers of antibody forming cells.

3.4 The obligatory participation of at least two distinct lymphocytes and the macrophage in the primary immune response.

3.4.1 B and T cell cooperation in antibody synthesis

Prior to 1966, the prevailing thinking was that a single lymphocyte was activated by antigen and transforms into an antibody-forming cell. However, in a milestone discovery in 1966, Claman et al (38) reported that two ontogenically distinct lymphocytes had to interact in the mouse to facilitate antibody synthesis. They transferred bone marrow or thymic cells into irradiated, immunosuppressed mice of the same inbred strain (syngeneic cells) along with the antigen and detected no immune responses by these recipients. However, when they transferred bone marrow and thymic lymphoid cells into the syngeneic irradiated recipients along with the antigen, they observed very good, almost normal immune responses in these otherwise immunosuppressed recipients. These results demonstrated that at least two distinct lymphoid cells, thymus-derived (T) cells and other bone marrow-derived (B) cells had to interact to facilitate antibody synthesis (38). It was subsequently shown by Claman et al (37-41) and other investigators (42-47) that the T cell possesses receptors for the antigenic determinants on the antigen and thereby interacts with the antigen. Activation of the T cell leads to a series of events which culminate in the stimulation of the B cell precursor of the antibody forming

cell to undergo the transformational, proliferative and differentiative changes into the antibody-forming lymphoblast/plasmablast. The T cells were referred to as ARC (antigen receptor-bearing cells) and were shown to be precommitted and clonally selected. The B cells, referred to as AFC (antibody forming cells) were shown to synthesize the antibodies. Furthermore, the T and B cells are of distinct lineages, and do not transform one into the other. In the rabbit, it has been demonstrated that the primary immune response results from the initial interaction of the antigen with precommitted, unipotent, antigen-receptor bearing cells (ARC) present in the bone marrow. This is followed by the activation, transformation and proliferation of the virgin (naive) uncommitted pluripotent precursors of the antibody forming cell (AFC), also present in the bone marrow, into overt antibody forming cells (AFC) (48-54). By cell transfer experiments, it was shown that these two functionally-distinct cells, ARC and AFC, do not transform into one another (55). In both the mouse and the rabbit the antibody forming cell is detected primarily, if not only, in the spleen following primary intravenous immunization. The progeny of these cells, the memory cells, are also localized to the spleen.

3.4.2 Surface markers which distinguish the B and T cells.

The results described above prompted investigations into ways to distinguish the T and B cells on the basis of surface markers. Tallberg et al (56) working with human

thymocytes and Reif and Allen (57) working with rodent thymocytes were the first to publish results of experiments which demonstrated that murine thymocytes carry organ-specific antigens on their cell membranes. Reif and Allen (58) were also the first to refer to the mouse thymocyte specific surface membrane antigen as the theta (θ) antigen. Raff and his associates (59-61) carried out systematic studies of the θ -associated cells in the different lymphoid and non-lymphoid organs of the mouse and demonstrated that cells in the central nervous system also possess the θ antigen. They established the presence of θ bearing cells in the spleen and lymph nodes of the mouse. Simultaneous studies by numerous investigators in the human confirmed that human thymocytes also possess an organ specific surface membrane antigen similar to the theta antigen in the mouse (62-64).

de la Noue, Koperstych and Richter (65) were the first to demonstrate thymocyte-specific antigens in the rabbit. This finding was later verified by Fradelizi et al (66).

The B cells were also found to possess specific surface-membrane antigens both in the mouse (67) and in the rabbit (68). In addition, B cells were observed to possess surface membrane immunoglobulins and receptors for C'3b which could also be used to distinguish them from the T cells (67-72).

Thus by the early-1970's, it had been firmly established that the B and T cells which are obligatory

participants in the primary antibody immune response are of distinct cell lineages and can be distinguished from each other, and separated from each other, on the basis of specific cell-surface markers.

3.4.3 The role of the macrophage in antibody formation.

Up to now, we have been developing the argument that there is cooperation between the T ARC and the B cell in the generation of the antibody response. In this section, we will introduce a third cell, the macrophage. Although it does not express antigenic specificity, it is nevertheless required for interaction with the antigen-specific lymphocytes in order to generate an antibody response.

It has been proposed, on the basis of in vitro and in vivo, experiments that the macrophage plays a key role if the immunogen is cellular, microbial or a macromolecular aggregate. Gorczynski and his associates (73), using irradiated mice, concluded that bone marrow and thymus cells cannot cooperate or that this cooperation does not lead to an immune response when macrophages are absent from the spleen of the irradiated recipients. Furthermore, many investigations (only a few are ref. here 74-82). have shown that the macrophage is also required for the generation of the antibody response in vitro.

There exists an enormous literature on macrophage function. In terms of their role in the induction of the specific antibody response which is our concern here, there are only a few points on which there is general agreement,

such as the necessity of their presence in generating the antibody response and the fact that they interact with the antigen in some antigen-nonspecific manner. Most cellular immunologists would probably agree with the notion that the macrophage functions by presenting antigen to lymphocytes. The basis for this proposal is that the function of the macrophage is to process the antigen into suitable immunogenic forms. Part of the experimental justification of this notion comes from the fact that antigen which is associated with macrophages is more immunogenic than antigen not associated with macrophages (83-86).

There are however some data from which it could be argued that the role of the macrophage is to provide "nutritional" factors or to participate in information transfer (87).

3.4.4 Possible mechanisms of cooperation between the B lymphocyte, the T lymphocyte and the macrophage in the antibody response.

In the previous sections, evidence suggesting the participation of the T ARC cell, the B cell and the macrophage in the antibody response were presented.

The initial theory to explain the mechanism of T cell help in antibody formation is by a way of antigen presentation or cell contact. In this model, the carrier portion of the immunogen is thought to react with the antigen receptor on the T ARC cell. The hapten portion of the immunogen is then presented to the receptor on the B

cell, causing the B cell to proliferate and differentiate. In this model, Ia antigens^e (molecules coded for in the I region of the major histocompatibility gene complex) play an important role.

On the other hand, other models (88-92) hypothesize B cell-Tcell cooperation via soluble factors elaborated by the activated T cell. In these models, the carrier portion of the immunogen reacts with the T cell and as a result of this interaction the T cell secretes a soluble factor which reacts with the B cell. In order to proliferate and differentiate, the B cell must react with this T cell factor and the hapten portion of the immunogen.

In another model (93), the carrier part of the immunogen reacts with a specific T cell through IgT, an antibody-like molecule on the surface of the T cell. The antigen-IgT complex is shed from the T cell surface and reacts with the macrophage, which then presents the hapten portion of the complex to the B cell.

A final model (94) suggests that the T cell reacts with self MHC and the carrier portion of the antigen on the macrophage. This results in activation and secretion of an antigen soluble factor which reacts with the resting B cells inducing them to proliferate and differentiate.

3.5. Suppressor cells and suppressor factors

3.5.1 Antigen-specific suppressor cells

3.5.1.1 Demonstration of the presence of antigen-specific suppressor cells.

In this section references will only be made with respect to the generation and regulation of the protective IgG and IgM. References to the synthesis and regulation of IgA, IgD and IgE are intentionally omitted due to constraint of space and to the fact that these immunoglobulins do not relate to the subject matter of this thesis.

Antibody synthesis takes place in addition to the normal ongoing synthesis of the "normal", non-antibody immunoglobulins IgG, IgM and IgA. The antibody concentration in the circulation reaches a peak between days 7 and 10 after immunization in the rabbit and falls very rapidly over the following 2 to 4 weeks. During this time, the concentration of the total immunoglobulins in the circulation remains constant. Following the discovery of suppressor cells by Gershon and Kondo in 1970 (95), it becomes necessary to assume the existence of two different regulatory mechanisms - one for specific antibody synthesis and one for general non-antibody immunoglobulin synthesis. In point of fact, evidence has been presented in favor of two immunoregulatory systems composed of helper and suppressor cells - the antigen-specific system which regulates the synthesis of antibodies (see section 3.5.1 and section 3.5.2) and the non-specific system which regulates

the synthesis of non-antibody immunoglobulins (95-100). The antigen specific system is only activated following stimulation with the antigen, whereas the cells in the latter system are in continuous state of activity and do not require exogenous stimuli in order to be activated.

Since the subject matter of this thesis is concerned only with the antigen-specific regulatory mechanism, only this regulatory mechanism will be elaborated upon in the discussion which follows.

Antigen-specific suppressor cells were first identified by Gershon and Kondo in 1970 (95). Their discovery was subsequently corroborated by numerous investigators (101-108). They may be defined as cells which abort, inhibit, curtail, or terminate the immune response and they are invariably found to be T cells. Since the normal, immunologically mature animal is fully capable of giving an immune response, the function of the suppressor cells must be to dampen the already initiated immune response and to terminate it.

Antigen-specific suppressor cells, capable of inhibiting antibody synthesis, include thymus cells in the South African clawed toad (109-111), concanavalin-A activated mouse splenic T cells (112), bone marrow cells in the unimmunized mouse (113-115), normal human bone marrow cells (116) circulating human T cells (100,117-120), and rabbit thymocytes between days 5 and 12 post-immunization (121). All of the suppressor cells except for the latter

were assayed for suppressor activity in an in vitro system. However, the rabbit thymocytes, obtained from rabbits immunized 7 days prior to sacrifice, have been shown to be capable of inhibiting the synthesis of antibodies in vivo when injected IV along with the antigen (122).

3.5.1.2 The induction of suppressor cells

Ballieux and his associates (100,117-121) demonstrated the existence of cells within the T_H helper cell population which could activate the T_S suppressor effector cells following their incubation with the antigen. These cells were referred to as suppressor inducer cells (100,121). It was demonstrated that the suppressor inducer T cell (T_{Si}) act on the suppressor precursor T cells (T_{Sp}) which then transform into the T suppressor effector cells (T_{Se}). The T_{Sp} cells were shown to be capable of rosetting with autologous erythrocytes and were referred to as T autologous rosetting cells or T_{ar+} cells. However, the T_{Si} and T_{Se} cells did not exhibit this property and were therefore referred to as T_{ar-} cells. On the basis of differential rosetting and staining with specific monoclonal antibodies by the immunofluorescence technique, the T_{Si} , T_{Sp} and T_{Se} cells were identified as $TM+G-4+8-ar-$, $TM-G-4-8+ar+$, and $TM-G+4-8+ar-$ respectively. Thomas et al (123) and Davidsen and Kristensen (124) have confirmed these findings of Ballieux et al.

3.5.2 Antigen-specific suppressor factors

Literally hundreds of non-specific suppressor factors have been obtained from normal human plasma, tissues, and cells following various forms of activation and treatment (for review of these factors, please see ref 125-127). However, none of these factors can be considered to have a physiological role in the induction and cessation of the specific immune response due to the non-specific suppressive activities of these factors (121, 125-130). These factors have been prepared from human circulating T cells (129), concanavalin-A activated mouse spleen cells (131-133), cultured rodent bone marrow cells (132) and supernatants of cultured spleen cells (133-134).

On other hand, there are only a few reports of antigen specific suppressor factors. Uytdehaag et al (135) and Heijnen et al (136) isolated antigen-specific suppressor factors from human T cells following in vitro immunization of these cells. Richter and Talor obtained antigen-specific suppressor factor by culturing the thymocytes of rabbits 5 to 12 days following intravenous immunization (121). The cell-free culture supernatant of the thymocytes cultured for only 4 hours at 37°C contained a suppressor factor which could inhibit specifically the induction of PFC in vitro with respect to the immunizing antigen, SRBC, but not with respect to a non-cross-reacting antigen (121). Furthermore, it could also inhibit the synthesis of antibodies to the specific antigen in vivo following its administration IV along with the antigen (122).

3.6 Summary

Antibody synthesis by the B antibody forming cell takes place only following non-random interactions between the antigen and the T ARC cell and probably also the macrophage. Antibody synthesis following intravenous immunization takes place essentially in the spleen whereas it takes place in both the spleen and the popliteal lymph nodes following local immunization within the drainage area of the lymph node. Antibody synthesis is regulated by the T helper and T suppressor cells, with the T helper cell stimulating antibody synthesis and the T suppressor cell inhibiting antibody synthesis. Normally, the T helper cells are activated first and thereby can stimulate antibody synthesis before the inhibitory influence of the T suppressor cells effectively terminate the immune response.

Richter and Talor, in this laboratory, have demonstrated that antigen-specific suppressor cells can be detected in the thymus of the adult outbred rabbit within 5 days after intravenous immunization (137). These cells attain peak suppressor activity by day 7 to 8 post-immunization and demonstrate markedly decreased immunosuppressive activity by days 12 to 15 post-immunization. Their suppressive activity is negligible after day 25 post-immunization (137). Since antibody synthesis takes place principally in the spleen, it may logically be asked what the role of the thymic suppressor cell is. The possibility was raised that the thymic suppressor cells

vacate the thymus and infiltrate the spleen where they act directly on the antibody-forming cells to suppress them. This thesis is concerned with the demonstration of the role of the thymus in the regulation of antibody synthesis in the rabbit.

4. MATERIALS AND METHODS

4.1 Materials

4.1.1 Reagents

Antiserum - the chromatographically pure rabbit IgM immunoglobulin fraction containing IgM antibodies directed to 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 complement was fresh guinea pig serum stored at -20°C.

Culture medium - the culture medium was RPMI 1640 fortified with fetal calf serum (FCS) (final concentration 10%), penicillin (100 units/ml), streptomycin (100 ug/ml), gentamicin (50 ug/ml) and 1M HEPES (final concentration 2%).

Ethanol (95% ethyl alcohol) - purchased from Les Alcools Commerce Ltd., Montreal, Quebec.

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

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

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

Hank's Balanced Salt Solution (HBSS) (500 ml) - obtained from Mediatec, Herndon, Virginia, and stored until use at 4°C.

Hematal (LA-Hgb reagent) - obtained from Fisher Scientific, Orangeburg, New York.

Heparinized Sheep Blood and Horse Blood - purchased fresh weekly from Frappier Diagnostics Inc., Laval, Quebec, and stored at 4°C until required.

Hepes buffer - obtained from Whittaker M.A. Bioproducts Inc., Walkersville, MD, and stored until use at 18°C.

Hypaque, sodium (diatrizoate) (50% w/v pH 6.5-7.7)- obtained from Winthrop Laboratories, Aurora, Ontario, and stored in the dark at 18°C.

Methyl cellulose (high substitution) - obtained from BDH Chemicals Ltd., Poole, England, and stored at 18°C.

Penicillin G, potassium (5000 units/ml) and streptomycin sulphate (5000 ug/ml) - obtained pre-mixed in 100 ml quantities from Whittaker Bioproducts Inc., Walkersville, MD, and stored until use at -20°C.

Saline solution (0.9% NaCl) in pyrogen free sterile water- obtained in 1 liter quantities from Abbott Laboratories Ltd., Montreal, Quebec, and stored at 18°C.

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

Sterile water (for irrigation USP) - obtained from Abbott Laboratories Ltd, Montreal, Quebec, and stored at 18°C.

Tissue culture medium RPMI 1640 (500 ml) - obtained from Mediatec, Herndon, Virginia, and stored at 4°C.

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

4.1.2 Supplies

Ayercillin (penicillin G, procaine suspension USP) (300,000IU) - obtained from Ayerst Laboratories, Montreal, Quebec, and stored at 4°C.

Betadine (surgical scrub containing 7.5% povidone iodine USP) - obtained from Frederick Inc., Toronto, Ontario.

Conical glass centrifuge tubes (50 ml, round bottom) - obtained from Fisher Scientific, Orangeburg, New York.

Culture tubes - 15 ml (17 x 100 mm) Falcon 2001 plastic sterile disposable tubes were obtained from Becton Dickinson Co., Lincoln Park, New Jersey.

Disposable polypropylene conical tubes (50 ml) 2048 Falcon - obtained from Fisher Scientific, Orangeburg, New York.

Double coated tissue tape (12 mm x 33 m) - obtained from 3M Canada Inc., London, Ontario.

Gauze sponges, sterile (5.1 x 5.1 cm and 10.2 x 10.2 cm) purchased from Kendal Canada, Toronto, Ontario.

Gloves (surgical) - obtained from Smith and Nephew Inc., Lachine, Quebec.

Halothane (2 bromo 2 chloro 1,1,1 trifluoroethane containing 0.01% thymol) - obtained from Halocarbon Laboratories Inc., distributed by Superham, St-Lambert, Quebec.

Microscope slides, plain (75 x 25 mm) - obtained from Fisher Scientific, Orangeburg, New York.

MLA tips (0 - 200 ul, sterile) - obtained from Biorad, Mississauga, Ontario.

MSI syringe adaptable filters, sterile (0.22 um) - obtained from MSI, Westborough, MA.

Needles - 18G (1"), 22G (1") and 25G (5/8") were obtained from Becton Dickinson Labware, Rutherford, New Jersey.

Nalgene filters (0.22 um) - obtained from Nalgene Labware, Rochester, New York.

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

Surgical blades (sterile carbon steel blades #10) - obtained from Becton Dickinson Co., Rutherford, New Jersey.

Sutures - chromic gut 3.0, 2.0 silk braided, 2.0 silk cuticular and 0 silk general closure were purchased from Ethicon Ltd, Peterborough, Ontario.

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

4.1.3 Equipment

Co₂ Incubator - cultures were maintained in a National incubator NAPCO, Portland, Oregon. This incubator is fully automated with respect to temperature (37.5°C + 0.5) and pCO₂ (5.0% + 0.1)

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

Fluorotec 3 - purchased from Cyprane North America Inc., Lancaster, New York.

Laboratory counter - obtained from Clay Adams, Parsippany, New Jersey.

Microscope - purchased from American Optical Corp., Buffalo, New York.

MLA pipettes (10, 20, 25, 50, 100, and 200 ul) - purchased from Medical Laboratories Automated Inc., Pleasantville, New York.

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

Pipette aid (Automatic Pipette) - obtained from Nalgene Labware, Rochester, New York.

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

Surgical and dissecting instruments - haemostatic forceps (straight and curved), Mayo scissors (straight and curved), dressing scissors, dissecting forceps, Poirier tissue forceps, " B.P". scapel handle, Littauer suture scissors, Metzenbaum scissors, Mixter artery forceps and O'Shaughressy artery forceps were all purchased from Blackhawk Medical Specialities Inc., Toronto, Ontario.

4.1.4 Animals

4.1.4.1 Rabbits - immunologically mature outbred normal New Zealand White rabbits (3 months of age, 4 to 5 lbs), were

obtained from Riemans Fur Ranches, Ste-Agathe, Ontario, and were 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, and kept under the same conditions as the rabbits above.

4.2 Methods

4.2.1 Preparation of erythrocytes - heparinized sheep and horse bloods were centrifuged at 500g for 10 min at 18°C following which the plasma was removed. The RBC were then washed three times in HBSS or saline at 500g for 10 min at 18°C and resuspended in RPMI 1640 or saline to the desired cell concentration. The washed cells are considered to be SRBC or HRBC although they are contaminated with WBC to an extent of 0.1%.

4.2.2 Complement - the source of complement was normal guinea pig serum. A 1 ml volume of this serum was absorbed with 0.1 ml volume of packed SRBC or HRBC at 4°C for 1 hour, centrifuged at 500g for 10 min at 18°C and absorbed once more with the RBC at 37°C for 1 hour. The absorbed serum did not agglutinate the respective RBC even in the undiluted state.

4.2.3 Preparation of Ficoll-Hypaque (SG 1.10 at 18°C)- Ficoll-hypaque was prepared by adding 96g of Ficoll and 1.36 grams of methyl cellulose to 1 liter of distilled water. The mixture was stirred at 4°C overnight, and the specific

gravity adjusted at 18°C by adding 8-9 vials of sodium hypaque. The Ficoll-hypaque solution was then stored at 4°C in the dark until required.

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

4.2.5 Non-specific esterase staining (NSE) - the macrophages and lymphocytes were identified by staining for NSE according to the method described by Yam et al (138).

4.2.6 Thymectomy - the rabbit was anesthetized with halothane. The area between the sternal notch and the xyphisternum was shaved and scrubbed with betadine. A 2 cm incision was made proximal to the sternal notch and the skin separated. A deeper incision (2 cm) was then made through the sternohyoideus muscles, exposing the thymus overlying the heart. First one lobe and then the second lobe of the rabbit bilobed thymus were teased out slowly. Minimal bleeding was encountered. A negative pressure was applied to the thoracic cavity prior to the application of the final closing suture. Within 2 hours, the rabbit had fully recovered and was eating and drinking. The mortality rate was less than 10% and no morbidity was observed in those rabbits that did survive. Following sacrifice of these rabbits, no residual or ectopic thymic tissue was detected.

4.2.7 Immunization of rabbits and preparation of Mononuclear cells (MNC) - immunologically mature outbred New Zealand White rabbits were immunized IV with 10^8 SRBC or HRBC. At

predetermined intervals of time thereafter, the rabbits were sacrificed by the IV injection of sodium pentothal. The abdominal and chest cavities were immediately entered, blood was withdrawn from the heart into heparinized syringes (50 units heparin per ml of drawn blood), and the lymphoid organs (SPL, THY, BM, APP, SR, PP and PLN) were excised and placed into sterile specimen containers containing medium RPMI 1640. 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 the cells were collected in medium in sterile plastic tubes. The tubes were centrifuged at 500g for 10 min at 18°C, the cells were suspended in medium, washed, and then resuspended to the desired cell concentration in medium. The spleen, bone marrow and circulating cells were centrifuged through Ficoll-hypaque (SG 1.10 at 18°C) (139) in the manner as originally described by Boyum for human circulating MNC (140). The MNC at the interface were collected, washed twice with medium and resuspended to the desired cell concentration.

4.2.8 Macrophage-depleted mononuclear cells (MNC-MO₂ and MNC-MO₂) suspensions - MNC were depleted of macrophages by incubating 10⁷ MNC in 5 ml of RPMI diluted with autologous serum (50% v/v) and 10 mg of carbonyl iron. The tubes were incubated on a tissue culture rotator for 45 min at 37°C. The tube contents were then layered onto a Ficoll-hypaque discontinuous gradient (SG 1.10 at 18°C) and centrifuged at

1200g for 45 min at 18°C. The non-phagocytic cells isolated from the interface were washed twice in HBSS at 500g for 10 min at 18°C and were referred to as once depleted mononuclear cells or MNC-MO₁. The MNC-MO₁ cells were cultured overnight in culture medium and subjected to macrophage depletion with carbonyl iron once more as described above. These twice macrophage-depleted cells are referred to as MNC-MO₂. About 96-99% of the MNC-MO₁ were lymphocytes as identified by differential staining of the cells for non-specific esterase (NSE), whereas >99% of the MNC-MO₂ were identified as lymphocytes.

4.2.9 Preparation of cell-free culture supernatants - immune or normal SPL, PB or PLN MNC from untreated, sham THYX or THYX rabbits were incubated in medium RPMI 1640 (10⁶ cells/ml) at 37°C in 5% CO₂ in air for 24 hours. The tubes were centrifuged at 500g for 10 min. The cell-free supernatants were filtered through millipore filters (0.22um) and frozen at -20°C until used. These cell culture supernatants were fortified with FCS to a final concentration of 10% when used as culture media for immune memory splenic MNC obtained from a rabbit immunized with SRBC and sacrificed 7 days later.

4.2.10 Preparation of the immune splenic lymphocyte(-derived) suppressor factor (ISLSF) - ISLSF was prepared by incubating splenic cells, obtained from rabbits sacrificed 25 days post-1°IM, in culture medium (10⁶ cells/ml) at 37°C in 5% CO₂ in air for 24 hours in the absence of the

immunizing antigen. The tubes were centrifuged at 500g for 10 min and the cell-free supernatants were filtered through millipore filters (0.22 μ m) and frozen at -20°C until used.

4.2.11 The hemolytic plaque-forming cell (PFC) assay to detect AFC - the PFC assay was carried out in liquid medium as described by Cunningham and Szenberg (141) and modified by Richter et al (142). Anti-SRBC antibodies (rabbit) were added to the assay mixture in a subhemolytic concentration, thereby increasing the sensitivity of the assay about 10 fold. The PFC assay used is a modification of the PFC assay in gel originally described by Jerne and Nordin (143).

4.2.12 The secondary immune response (2° IR) in vitro - the in vitro culture for the induction of the 2° IR carried out as described previously (121). Briefly, the responder immune splenic MNC (4×10^6 cells) were cultured with the antigen, SRBC or HRBC (4×10^6 cells) in 4 ml of culture medium in sterile, capped Falcon 2001 polystyrene tubes. The tubes were maintained in an upright position at 37°C in 5% CO_2 in air for 5 days, centrifuged at 500g for 10 min and the cells were washed and assayed for PFC.

4.2.13 Inhibition the 2° IR in vitro by ISLSE - ISLSE was used as culture medium for the induction of the 2° IR in vitro by immune splenic MNC obtained from a rabbit 7 days post- 1° IM, as described in section 4.2.12. The culture media were replaced on days 2 and 4 of the 5 day culture with previously frozen cell-free supernatant. At the end of the

culture, the MNC were assayed for PFC as described in section 4.2.11.

4.2.14 Identification of the splenic cells which secrete (ISLSF) -splenic MNC obtained from the rabbits sacrificed 25 days following immunization with SRBC were incubated for 1 hour at 37°C with horse anti-rabbit thymocyte (T cell) serum (HARTS) and complement (fresh guinea pig serum diluted 5 fold), or with horse anti-rabbit bone marrow (B cell) serum (HARBMS) and complement, in order to lyse the T or B cells, respectively. The HARTS antiserum absorbed with rabbit erythrocytes is capable of specifically lysing 90 to 100% of the rabbit T cells but not rabbit bone marrow MNC (121) and the HARBMS antiserum absorbed with rabbit thymocytes and rabbit erythrocytes is capable of specifically lysing 90 to 95% of the rabbit bone marrow MNC but not rabbit thymocytes (121). The splenic MNC were also depleted of macrophages as described in section 4.2.8. The T cell depleted, the B cell depleted, and the macrophage depleted splenic MNC were cultured for 24 hours to prepare cell-free supernatants as described in section 4.2.9. These cell-free culture supernatants were used as culture media for the induction of the 2°IR in vitro by the splenic MNC obtained from rabbit immunized with SRBC and sacrificed 7 days later as described in section 4.2.12 and 4.2.13.

4.2.15 Preparation of ultrasonicates - Splenic MNC obtained from rabbits 25 days post-primary immunization were prepared at a cell concentration of 4×10^6 cells/ml. These cell

suspensions were subjected to ultrasonic desintegration on ice at 60,000 cycles/sec for 1 minute. The sonicates were then filtered through millipore filters (0.22 μ m) and frozen at -20°C until required. The sonicates were used as culture media for allogeneic splenic MNC as described in section 4.2.13.

4.2.16 Dialysis -

Rabbits were immunized IV with 10⁸ SRBC. They were sacrificed 25 days later, the spleens were excised and splenic MNC suspensions were prepared as described in section 4.2.7. The splenic MNC were cultured for 24 hours to obtain ISLSF as described in section 4.2.10. ISLSF was dialysed for 24 hours through dialysis membranes of exclusion m. wt. of 14,000 and 50,000. The ISLSF was assayed for its capacity to inhibit the 2°IR induced in vitro by allogenic splenic memory cells as described in section 4.2.13.

5. RESULTS

5.1 Evidence in favour of suppressor cells in the spleen following primary immunization of the normal outbred rabbit.

5.1.1 Rationale and objectives

It has been shown that antigen-specific suppressor cells are detected in the thymus of the immunized rabbit by the fifth day post-immunization IV (137). However, antibody synthesis takes place principally in the spleen of the IV immunized rabbit (11,121). If one assumes a functional physiological role for the thymic suppressor cell, one might anticipate that it must migrate to the spleen where it would act directly on the AFC and suppress antibody synthesis. The objective here is to present evidence in favour of the presence of suppressor cells in the spleen of the immunized rabbit following the peak of antibody synthesis.

5.1.2 Experimental protocol

Rabbits were immunized with 10^8 SRBC IV. They were sacrificed in groups of 3 on days 5,7,10,14,18,22,30,40 and 60 post-immunization. The rabbits were bled from the heart into heparinized syringes and the circulating MNC (PB MNC) were prepared as described in section 4.2.7. The lymphoid organs were excised and suspensions of MNC were prepared as described in section 4.2.7. The splenic AFC were assayed immediately for PFC as described in section 4.2.11 and they were also cultured with the antigen, SRBC, for 5 days to generate a secondary immune response as described in section 4.2.12. The cultured cells were then assayed for PFC.

5.1.3 Results

As can be seen from Table 1A, immediate PFC were detected in maximum numbers (as a percentage of the cells assayed) in the spleen on day 7 post-immunization. They decreased in numbers markedly through days 10 to 14 and could not be detected by day 40 post-primary immunization.

When the MNC of the different lymphoid organs were cultured for 5 days with the antigen were assayed for PFC, (it was observed that only the splenic MNC, with the exception of the PB MNC on day 7 post-immunization, generated PFC. There was a consistent peak with the cells obtained on day 7 post-immunization, a sharp decrease with the cells obtained on day 10 post-immunization, and a negligible PFC response with the cultured cells obtained between days 18 and 30 post-immunization (Figure 1). The cells obtained after 34 days post-immunization generated large numbers of PFC in in vitro culture with the antigen.

As can be seen in Figure 2, the splenic MNC obtained from rabbits 18, 25 or 28 days post-immunization with SRBC but only 7 days post-immunization with HRBC generated insignificant numbers of PFC to SRBC but normal numbers of PFC to HRBC.

5.1.4 Discussion

The splenic MNC of rabbits 7 to 14 days and 30 to 48 days following immunization with SRBC generate marked secondary immune responses, characterized by large number of PFC, in in vitro culture with the antigen, SRBC, for 5 days.

However, between days 14 and 30 post-immunization, the splenic MNC gave very poor secondary immune responses in vitro. This period of limited, or absent, immune responsiveness is referred to as the trough. The failure to give significant secondary immune responses in vitro during the trough period is antigen-specific since the secondary immune responses to a second non-cross-reacting antigen, HRBC, injected IV 7 days prior to sacrifice and culture, were normal. These results clearly suggest that suppressor cells are present in the spleen 18 to 30 days following immunization and that they are capable of inhibiting both ongoing antibody synthesis and the induction of a secondary immune response by the parenchymal antibody forming cells or memory cells.

TABLE 1A .

THE NUMBER OF PFC (AFC) GENERATED THE BY CELLS OF THE DIFFERENT LYMPHOID ORGANS OF THE RABBIT IS A FUNCTION OF THE TIME FOLLOWING PRIMARY IV IMMUNIZATION WITH 10^8 SRBC.

Cells of organs assayed	The no. of PFC per 10^6 MNC on the following days after primary IV immunization with 10^8 SRBC								
	4	7	10	14	18	22	30	40	60
SPL	80	250	90	28	20	14	10	5	0
PB	0	105	18	0	0	0	0	0	0
BH	0	52	14	0	0	0	0	0	0
THY	0	0	0	0	0	0	0	0	0
PLN	0	0	0	0	0	0	0	0	0
APP	0	0	0	0	0	0	0	0	0
PP	0	0	0	0	0	0	0	0	0
SR	0	0	0	0	0	0	0	0	0

- Each value represents the mean obtained with the cells of 3 rabbits.

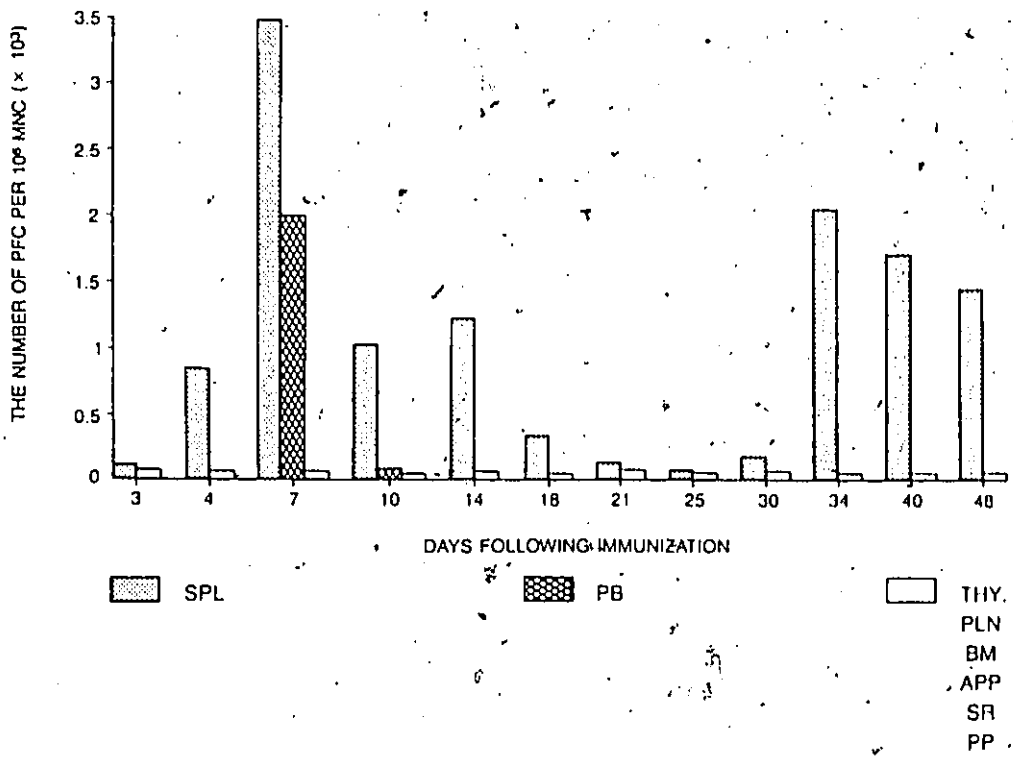


Figure 1. The in vitro secondary immune responses by cells of the lymphoid organs of the rabbit following primary immunization with 10⁹ SRBC IV. The results are presented as the number of PFC per 10⁶ MNC recovered after 5 days in culture with the antigen, SRBC.

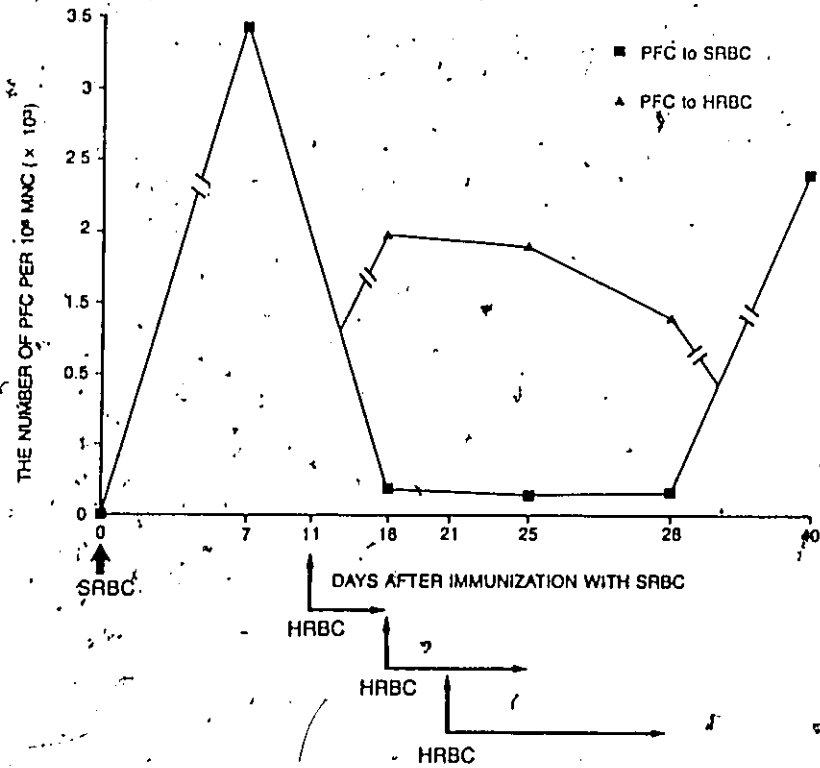


Figure 2. The presence in the spleen of suppressor cells to SRBC which inhibit the in vitro secondary response to SRBC but not to HRBC.

5.2 The demonstration that the suppressor cells detected in the spleen during the trough following the primary antibody response originate in the thymus.

5.2.1 Rationale and objectives.

In the previous section, it was demonstrated that, during a specific period of time post-primary immunization IV with SRBC referred to as the trough, that is the interval of time between days 18 to 30 post-immunization, the splenic MNC can only mount a barely significant secondary immune response in in vitro culture with the immunizing antigen, the SRBC. Between days 7 and 14 and after day 30 post-immunization, the splenic MNC gave very good secondary immune responses in vitro. Furthermore, the failure by the splenic MNC to give a secondary immune response to the immunizing antigen during the trough period was shown to be antigen specific. These data strongly indicate the existence of antigen-specific suppressor cells in the spleen during the trough period.

The objective of the following experiments is to demonstrate that the splenic suppressor cells originate in the thymus.

5.2.2 Experimental protocol

The rabbits were either thymectomized (thyx) or sham-thyx in the manner described in section 4.2.6. They were immunized with 10^8 SRBC IV at varying times either before or after the thyx or sham-thyx. The rabbits were sacrificed on various days following immunization, the

spleen was excised and splenic MNC suspensions were prepared as described in section 4.2.7. The MNC were cultured with the antigen for 5 days following which the cells were assayed for PFC as described in section 4.2.11.

5.2.3 Results

Rabbits were thyx 14 days prior to immunization. Groups of 3 rabbits were sacrificed on different days post-immunization, and their splenic MNC were cultured with the immunizing antigen and assayed for PFC. As can be seen in Figure 3, the number of PFC generated per 10^6 MNC in culture by splenic MNC obtained from rabbits sacrificed between days 10 and 30 post-immunization were not significantly different from each other. Only the splenic MNC obtained 7 days post-immunization generated obviously greater numbers of PFC per 10^6 splenic MNC in culture. No trough was therefore observed with the splenic MNC of the thyx rabbits (Figure 3)

The results presented with the non-thyx rabbits in Table 1 and the thyx rabbits in Figure 3 are presented together in Figure 4 in order to facilitate a comparison of the data obtained with these two populations of rabbits. It is obvious that the trough observed with the splenic MNC of the non-thyx rabbits does not exist in the case of the splenic MNC of the thyx rabbits.

In order to rule out the possibility that the stress of the surgery, and not necessarily the thyx, could result in alterations in the immunological responsiveness of the splenic MNC, groups of rabbits were thyx or sham-thyx 14

days prior to immunization. The rabbits were sacrificed 25 days post-immunization, which is the approximate mid-point of the trough with splenic MNC of non-thyx rabbits. The splenic MNC of the sham-thyx rabbits generated low numbers of PFC in culture with the antigen, similar to the immune responses given by the splenic MNC of untreated immunized rabbits (Table 1). However, the splenic MNC of the thyx rabbits gave strong secondary immune responses in in vitro culture with the antigen. They generated on average almost 10 times as many PFC in culture as did the splenic MNC of the untreated and sham-thyx rabbits.

Experiments were then carried out to ascertain the relationship between the time of thyx relative to the day of immunization and the immunological responses of the splenic MNC of rabbits sacrificed 25 days post-immunization. It can be seen from the data presented in Table 2 that no trough was exhibited by the splenic MNC obtained from rabbits thyx 14 or 7 days prior to immunization or 3 days following immunization. However, the splenic MNC of rabbits thyx 7 or 14 days post-immunization exhibited the trough, that is they generated very few PFC in culture. The splenic MNC of rabbits which had been sham-thyx either 14 days prior to immunization or 3 days after immunization generated far fewer PFC than did the splenic MNC of their thyx counterpart.

5

5.2.4 Discussion

The objective of these experiments was to demonstrate whether the thymus or thymic cells are involved in the markedly diminished immune response in vitro by splenic MNC obtained from rabbits sacrificed 25 days after immunization. It was observed that splenic MNC of rabbits thyx 14 or 7 days prior to immunization, or 3 days after immunization, generated large numbers of PFC in in vitro culture with the antigen, that is they failed to exhibit the trough. On other hand, the splenic MNC of rabbits thyx 7 or 14 days after immunization generated low numbers of PFC in in vitro culture with the antigen. The splenic MNC of sham-thyx rabbits behaved as did the cells of untreated immunized rabbits. These results strongly suggest that the suppressor cells initially detected in the thymus on day 5 post-immunization vacate the thymus between day 3 and day 7 post-immunization and infiltrate the spleen (and probably the other lymphoid organs as well) where they act directly on the antibody forming cells to suppress them.

It may be argued that the thymus need not necessarily provide the suppressor cells detected in the spleen but may indirectly affect their function and thereby distort the temporal relationship normally observed between the day of immunization and the day of detection of suppressor cells in the spleen. However, such does not appear to be the case since suppressor cells were not detected in the spleen of immunized rabbits thyx 14 days prior to immunization

although the thymic rabbits were assayed for splenic suppressor cells on various days between days 7 and 30 post-immunization.

The results strongly suggest that suppressor cells, induced initially in the thymus by day 5 following immunization, vacate the thymus between days 3 and 7 following immunization and infiltrate the spleen where they probably interact with the overt antibody forming cells and suppress their capacity to synthesize and secrete antibodies.

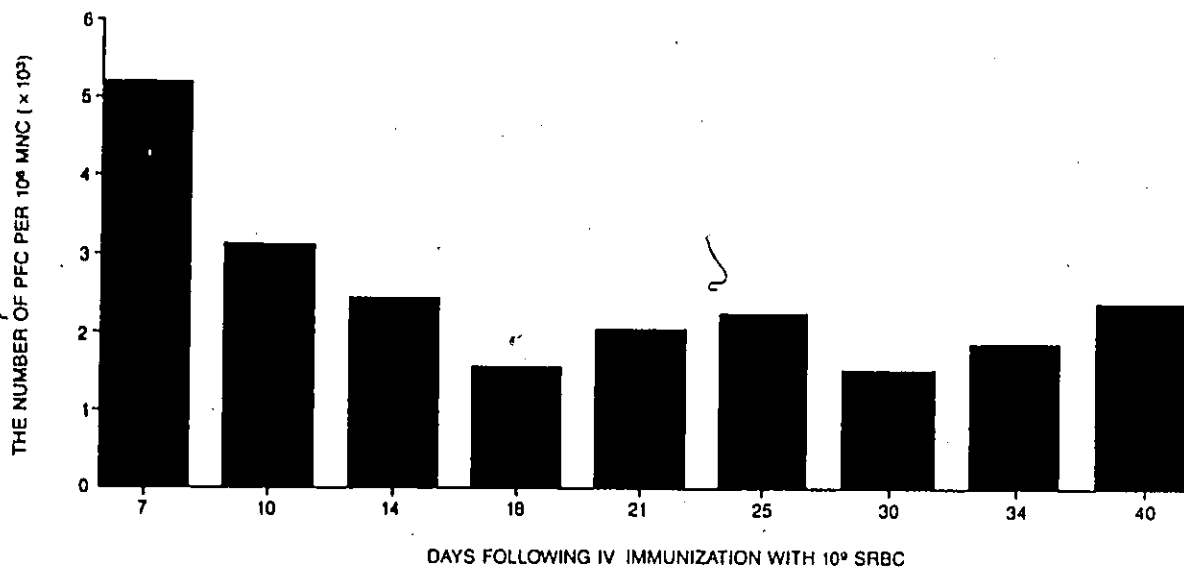


Figure 3. The secondary immune response induced in in vitro culture of splenic MNC of rabbits thymectomized 14 days prior to IV immunization and sacrificed at varying times post-primary immunization.

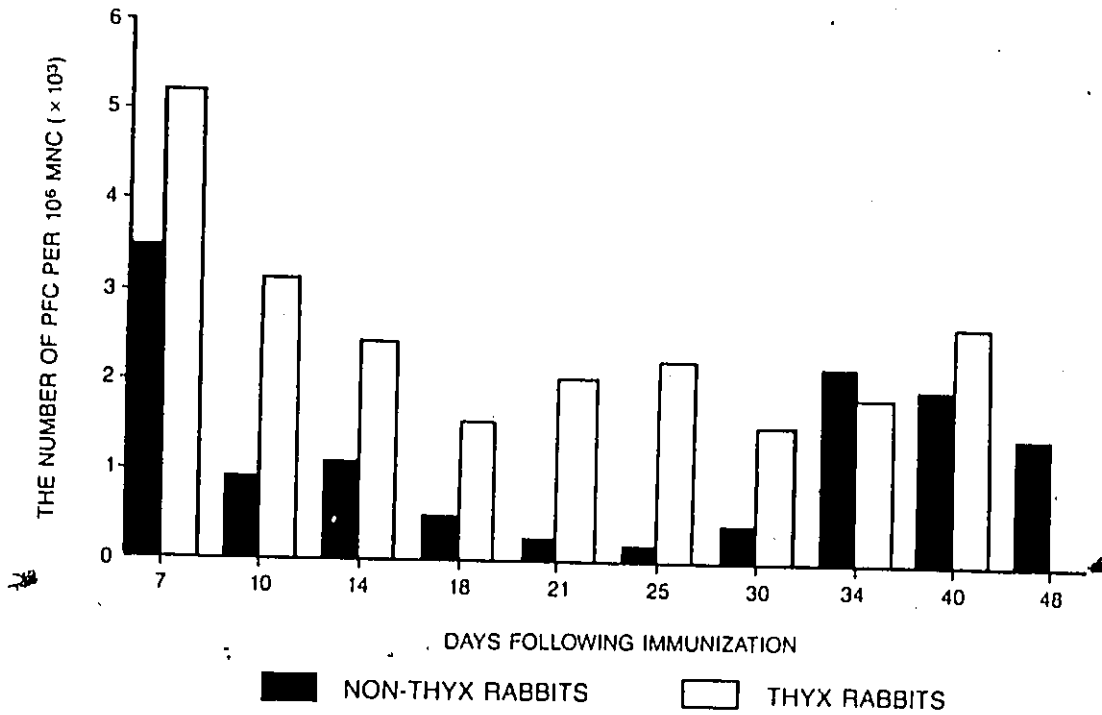


Figure 4. A comparison of the secondary immune responses induced in in vitro culture of splenic MNC of thymectomized rabbits as a function of time following immunization. Thymectomy was carried out 14 days prior to immunization.

TABLE 1

THE SECONDARY IMMUNE RESPONSES INDUCED IN IN VITRO CULTURE OF SPLENIC MNC OF THYMECTOMIZED, SHAM THYMECTOMIZED OR UNTREATED IMMUNIZED RABBITS.

Treatment of Rabbits	The no. of PFC per 10 ⁶ splenic MNC, following culture for 5 days, of rabbits sacrificed 25 days post-immunization.										x ^a
	1	2	3	4	5	6	7	8	9	10	
NIL ^b	82	136	5	213	269	488	172	164	5	498	202
Sham thyx ^c	280	50	379	355	370	136	-	-	-	-	265
Thyx ^d	2298	1600	5224	1772	1714	1892	1715	2074	-	-	2286

^a NIL = Untreated controls. These rabbits were immunized IV with 10⁶ SRBC and sacrificed 25 days later.

^b These rabbits were sham-thymectomized (sham-thyx) 14 days prior to immunization IV with 10⁶ SRBC. They were sacrificed 25 days post-immunization.

^c These rabbits were thymectomized (thyx) 14 days prior to immunization IV with 10⁶ SRBC. They were sacrificed 25 days post-immunization.

^d x = Average

TABLE 2

THE SECONDARY IMMUNE RESPONSES IN VITRO OF SPLENIC MNC OF RABBITS THYMECTOMIZED AT DIFFERENT TIMES PRIOR TO OR AFTER IV IMMUNIZATION WITH 10⁸ SRBC.

Rabbits thymectomized (sham-thymectomized) on following days relative to IV immunization on day 0	The no. of PFC per 10 ⁶ MNC, following culture for 5 days, of rabbits sacrificed 25 days post-immunization IV. (rabbit no.)							average
	1	2	3	4	5	6	7	
-14 (-14)	2298 (875)	1600 (928)	5224 (513)	1772	1714	-	-	2522 (772)
-7	2312	1726	1642	5290	5990	-	-	3392
+3 (+3)	5904 (307)	1428 (164)	1522 (280)	1600	2128	1335	1482	2200 (250)
+7	0	1396	5	84	484	490		410
+14	172	149	5	5	351	473		200

* The results of sham thymectomized rabbits are presented in the brackets.

5.3 Evidence for the secretion of a suppressor factor, Immune splenic lymphocyte(-derived) suppressor factor or ISLSF, by the splenic suppressor cells which inhibits the induction of the secondary immune response by immune splenic mononuclear cells in vitro.

5.3.1 Rationale and objectives

In the previous section, evidence was presented that antigen-specific suppressor cells are detected in the spleen of the immunized rabbit between days 18 and 30 post-immunization, with a peak of suppressive activity around day 25 post-immunization. The results with the thymic rabbits suggested that the suppressor cells originated in the thymus.

The objective in the following experiments is to demonstrate that the suppressor cells in the spleen secrete a factor which is capable of inhibiting antibody synthesis by allogeneic immune rabbit splenic MNC in in vitro culture.

5.3.2 Experimental protocol

The rabbits were immunized with 10^8 SRBC or HRBC IV and sacrificed at predetermined intervals of time post-immunization. The spleen, thymus and PLN were excised and MNC suspensions were prepared as described in section 4.2.7. The MNC were subjected to ultrasonic desintegration as described in section 4.2.16. The MNC were also cultured for 24 hours at 37°C and the cell-free culture supernatants (containing ISLSF) were filtered as described in sections 4.2.9 and 4.2.10.

The splenic MNC were cultured for 5 days with the antigen for the induction of the secondary immune response as described in section 4.2.12. The suppression of the secondary immune response in vitro by ISLSF or by the sonicates was carried out as described in section 4.2.13.

5.3.3 Results

As can be seen in table 3, filtered ultrasonicates of splenic MNC obtained from rabbits 25 days post-primary immunization failed to suppress the 2°IR in vitro by allogeneic immune splenic MNC.

As can be seen from the data presented in Figure 5, the cell-free supernatants of cultures of splenic MNC of rabbits immunized 14, 20, 25 or 30 days previously with SRBC were able to suppress the in vitro 2°IR by allogeneic immune splenic MNC. Maximum suppression took place with the supernatants of cultures of splenic MNC of rabbits immunized 20, 25 or 30 days previously. The percent suppression by these supernatants ranged from 60 to almost 100 percent. Culture supernatants of splenic MNC of unimmunized rabbits or of rabbits immunized with SRBC only 7 days prior to sacrifice failed to inhibit the 2°IR to SRBC. Furthermore, culture supernatants of splenic MNC of rabbits immunized 25 days prior to sacrifice with a non-cross-reacting antigen, HRBC, failed to inhibit the 2°IR by allogeneic splenic MNC to SRBC (results not presented). The suppressor factor in the cell-free culture supernatants is referred to as immune splenic lymphocyte(-derived) suppressor factor or ISLSF.

As can be seen from Figure 6, the supernatants of cultures of splenic MNC of thyx rabbits immunized 25 days prior to sacrifice failed to suppress the 2°IR by allogeneic immune splenic MNC in vitro whereas supernatants of cultures of splenic MNC of untreated or sham-thyx rabbits immunized 25 days prior to sacrifice markedly inhibited the 2°IR.

The cell-free supernatants of immune splenic MNC, obtained from rabbits sacrificed 25 days following immunization and cultured for various periods of time at 37°C, were assayed for ISLSF activity. As can be seen in Table 4, maximum capacity to suppress the 2°IR was exhibited by the cell-free supernatants of 20 to 24 hour cultures of the cells. Potent ISLSF was also secreted between 24 and 48 hours of culture. However, much less ISLSF was secreted by the cells between 48 and 72 hours of culture and no ISLSF was detected following culture of the cells for longer than 72 hours (Table 4),

A final question which was addressed in this series of experiments is whether the cells of other lymphoid organs, specifically the thymus and the PLN, also secrete ISLSF in cultures. It has previously been shown in this laboratory that the thymic cells obtained from rabbits sacrificed 7 days post-immunization secrete a potent suppressor factor after culture for 4 hours in the absence of the antigen, capable of inhibiting PFC in vitro (121) and antibody synthesis in vivo (122). This suppressor factor, referred to as immune thymocyte suppressor factor or ITSF,

is secreted maximally between days 7 and 12 following immunization IV with SRBC (137). Its secretion by the thymus cell diminishes markedly but is detectable in culture supernatants of cells obtained up to day 30 post-immunization. As can be seen from Figure 7, no suppressor activity is detected in culture supernatants of spleen, thymus and PLN MNC of unimmunized rabbits (day 0 on Figure 7). Suppressor activity is very high in the 24 hour cell-free supernatants of thymus and PLN cell cultures but cannot be detected in splenic MNC cultures of rabbits sacrificed 7 days post-immunization. Whereas the suppressor activity of cell-free supernatants of 24 hour thymus cell cultures declines through days 14 and 25 post-immunization, the suppressor activity of the cell-free supernatants of cultures of PLN cells stays high through days 14 and 25 post-immunization. The suppressor activity of the cell-free supernatants of cultures of spleen MNC, although negligible on day 7 post-immunization, increases sharply by day 14 post-immunization and attains suppressive activity equal to that expressed by the supernatants of PLN cell cultured on day 25 post-immunization (Figure 7).

5.3.4 Discussion

The objective of this series of experiments was to demonstrate whether the suppressor cells in the spleen act on the antibody forming cell to suppress its synthesis of antibodies via the secretion of an antigen-specific suppressor factor.

The evidence presented unequivocally supports the secretion of an antigen-specific suppressor factor as the vehicle whereby the suppressor cells inhibit the induction of a 2°IR in vitro by splenic memory cells. This conclusion is based on the findings that sonicates of immune splenic MNC do not suppress the 2°IR in vitro by allogeneic MNC whereas cell-free supernatants of cultures of splenic MNC obtained from rabbits 14 to 30 days post-immunization inhibited the induction of a 2°IR by allogeneic splenic memory cells. On the other hand, the cell-free supernatants of splenic MNC of rabbits thyx 14 days prior to immunization and sacrificed 25 days later failed to inhibit the 2°IR by allogeneic memory splenic MNC, indicating the absence of thymus-derived suppressor cells.

It should be noted that supernatants of cultures of splenic MNC obtained from rabbits 7 days post-immunization exhibited no suppressor activity whereas the supernatants of cultures of thymocytes and PLN MNC were very suppressive. Most interestingly, the supernatants of cultures of the splenic MNC obtained 14 days post-immunization was significantly suppressive and the suppressive activity of the cultures of splenic MNC obtained 25 days post-immunization was even more dramatic. Simultaneous to the rise in suppressive activity of the supernatants of the splenic MNC obtained from rabbits 14 and 25 days post-immunization is the precipitous drop in suppressive activity of the supernatants of cultures of thymocytes obtained on

days 14 and 25 post-immunization. Most impressive was the observation that the supernatants of cultures of PLN cells obtained from rabbits 7, 14 or 25 days post-immunization were almost equally suppressive. These results suggest that the suppressor cells are present in the thymus early in the immune response following which they vacate the thymus (and/or become inactive in the thymus?) and infiltrate the spleen and the lymph nodes. The consistent failure of the supernatants of the splenic MNC obtained from rabbits 7 days post-immunization to suppress the 2°IR in vitro whereas the supernatants of cultures of the PLN cells of the same rabbits suppressed the 2°IR very well may be explained by the fact that the spleen on day 7 contains many helper cells which act on the antibody forming cells to drive them to actively synthesize and secrete antibodies. The helper cells probably carry out this stimulating function via the secretion of a helper factor. The supernatants of cultures of the splenic MNC obtained from rabbits 7 days post-immunization probably consists of both helper factors and suppressor factors (secreted by the newly arrived suppressor cells); however, the balance is in favour of the helper factor at this time and therefore no suppression of the 2°IR is seen.

The splenic suppressor cells began to lose the ability to secrete ISLSF after 2 days in culture and ceased to secrete ISLSF after 3 days in culture. In this respect, the splenic suppressor cell is very similar to the thymic

suppressor cell which also ceases to secrete the suppressor factor ITSF after 3 days in culture (122). This finding explains why it is necessary to add fresh ISLSF to the cultures on days 2 and 4 of the 5 day culture in order to obtain maximum suppression of the 2°IR by the ISLSF.

TABLE 3

ISLSF IS ACTIVELY SYNTHESIZED AND SECRETED BY THE SPLENIC MNC OBTAINED FROM RABBITS 25 DAYS POST-PRIMARY IMMUNIZATION AND IS NOT SIMPLY RELEASED FOLLOWING LYSIS OF THE FRESHLY ISOLATED CELLS.

Medium used in culture	The number of PFC per 10 ⁶ MNC following culture of splenic MNC of an allogeneic day 7 rabbit ^a for 5 days in medium		
	Exp. 1	Exp. 2	Exp. 3
sonicates ^b	5109 (2) ^c	4952 (5)	5109 (2)
ISLSF ^b	1668 (68)	1355 (74)	1460 (72)

^a These splenic MNC generated 5213 PFC following culture in regular culture medium with the SRBC for 5 days.

^b These sonicates were prepared by ultrasonication of the splenic MNC obtained from a rabbit immunized IV with SRBC 25 days prior to sacrifice.

^c ISLSF is the cell-free supernatant of a 24 hour culture of splenic MNC obtained from a rabbit immunized IV with SRBC 25 days prior to sacrifice.

^d The figures outside and inside the brackets represent the no. of PFC observed and the percentage inhibition of PFC generated in the culture in the presence of the sonicates or ISLSF, respectively.

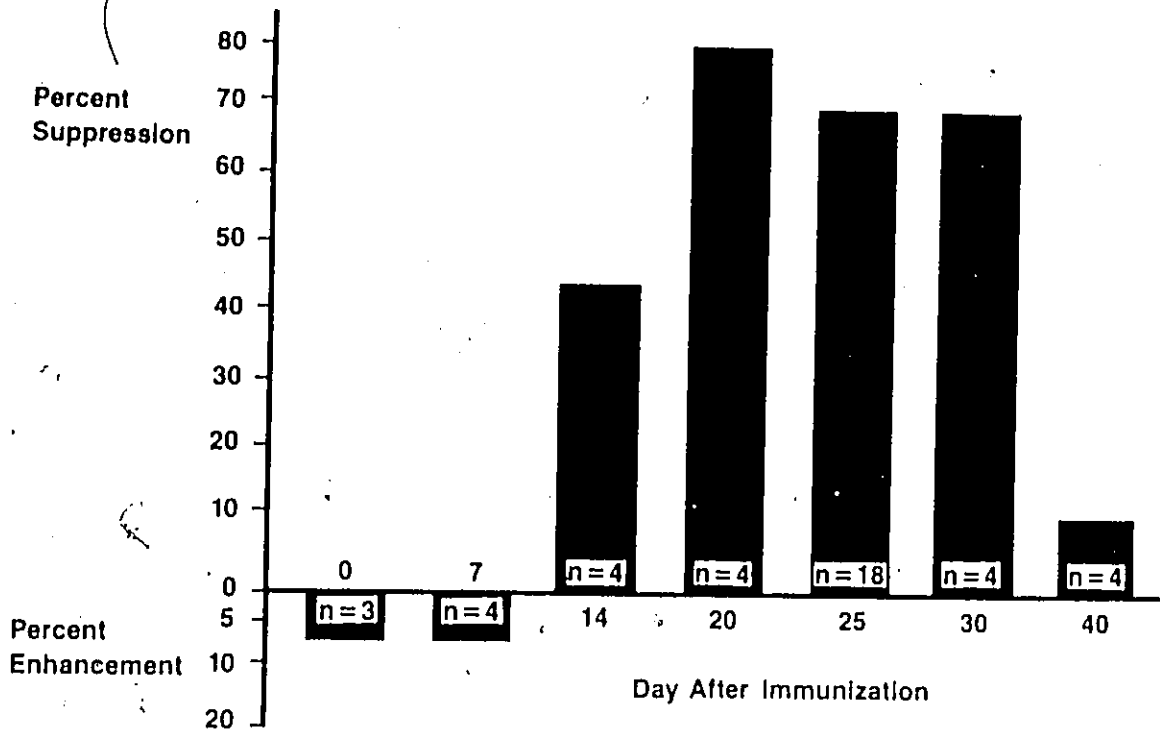


Figure 5. The splenic MNC 14 to 30 days after IV immunization secrete a factor, immune spleen lymphocyte suppressor factor or ISLSF, which suppresses the secondary immune PFC responses by cultured allogenic memory spleen MNC in vitro.

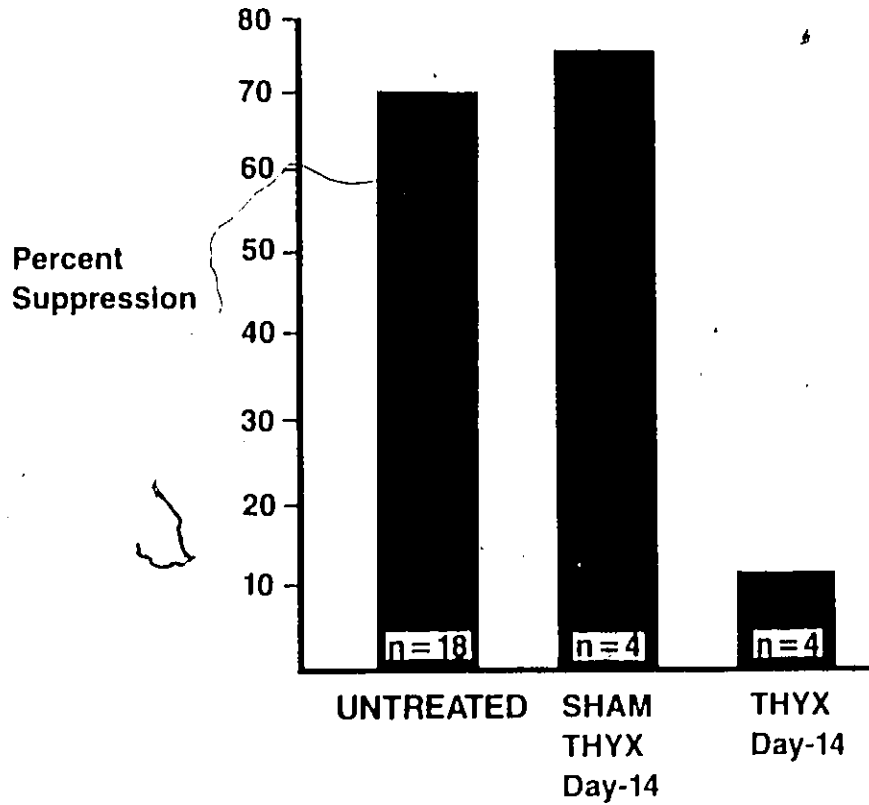


Figure 6. The splenic MNC 25 days after IV immunization of non-thymectomized or sham-thyx, but not of thyx rabbits secrete ISLSF which suppresses the secondary immune PFC response by cultured allogeneic memory spleen MNC in vitro.

TABLE 4

THE SECRETION OF ISLSF IS A FUNCTION OF THE TIME IN CULTURE OF THE SPLENIC MNC 24 DAYS OBTAINED AFTER IMMUNIZATION IV WITH 10⁸ SRBC.

cell-free supernatants of immune splenic MNC which were cultured at 37°C for the following periods of time (hr)	The percent suppression of the secondary immune response in vitro by memory splenic MNC cultured for 5 days in the cell-free supernatants	
	Exp. 1	Exp. 2
2	0	0
4	12	5
8	12	54
12	48	67
20	50	74
24	68	67
24 to 48	58	48
48 to 72	25	27
72 to 96	<1	<1
96 to 120	<1	<1

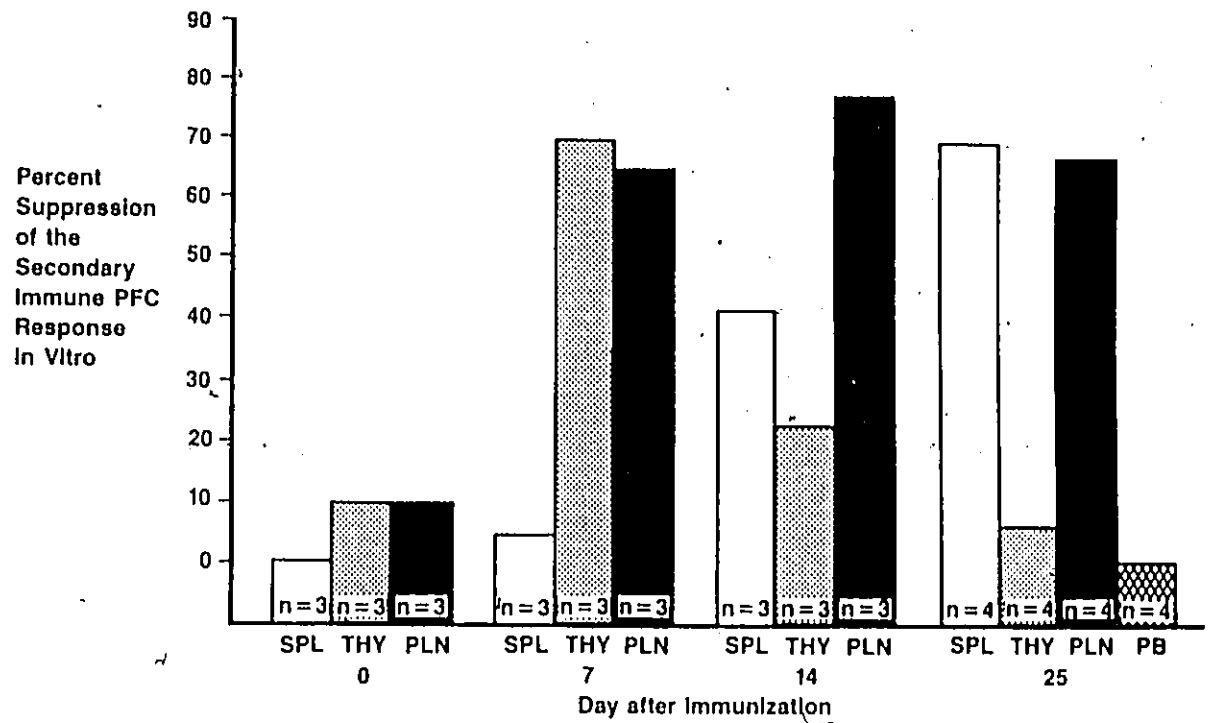


Figure 7. The capacity of splenic, thymic, PLN and PB MNC to secrete ISLSF in in vitro culture for 24 hours. The suppression by ISLSF of the secondary immune PFC response by cultured allogeneic memory splenic MNC in vitro.

Peripheral blood obtained from rabbits immunized 7 and 14 days following immunization do not secrete ISLSF. These results are therefore not presented in the figure.

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5.4 The identification of the splenic suppressor cell as a T cell and the demonstration of the antigenic specificity of the immunosuppressor factor, ISLSF, secreted by the splenic T suppressor cell.

5.4.1 Rationale and objectives

The results of the experiments presented in the previous sections of this Chapter demonstrated the dependence of the detection of suppressor cells in the spleen on an intact thymus. These findings indicate that the splenic suppressor cell is derived from the thymus and that it is therefore a T cell. The objectives of the following experiments are (i) to prove that the splenic suppressor cell is a T cell, and (ii) to demonstrate the antigenic specificity of ISLSF.

5.4.2 Experimental protocol

The rabbits were immunized with 10^8 SRBC or HRBC IV and sacrificed 25 days later. The spleen was removed and splenic MNC suspensions were prepared as described in section 4.2.7. The splenic MNC were depleted of T cells, B cells, or macrophages as described in sections 4.2.8 and 4.2.14. The cells were cultured for 24 hours at 37°C and the cell-free culture supernatants were filtered and stored as described in section 4.2.9. The cultures of immune splenic MNC were stimulated with antigen to generate PFC in a 2°IR as described in section 4.2.12.

5.4.3 Results

The cell-free supernatants of cultures of splenic MNC obtained from rabbits immunized with SRBC, containing ISLSF absorbed with the original immunizing antigen, SRBC, lost their capacity to suppress the 2°IR to SRBC. On the other hand, the supernatants absorbed with a non-cross-reacting antigen, HRBC, retained their suppressive activity (Table 5).

The cellular origin of ISLSF is the T cell since the supernatants of cultures of splenic MNC from which T cells had been eliminated prior to preparation of ISLSF failed to suppress the 2°IR to SRBC (Table 6). On other hand, removal of B cells or macrophages from the splenic MNC did not prevent the secretion of ISLSF by the remaining cells into the culture supernatant (Table 6).

5.4.4 Discussion

It has previously been demonstrated by Richter and Talor (121) that the thymus suppressor cells in the rabbit detected early in the immune response secrete a factor in vitro which is capable of inhibiting, in an antigen-specific manner, the secretion of antibodies by the autologous or allogeneic immune splenic MNC. Since it has been demonstrated in this investigation that suppressor cells capable of secreting a suppressor factor, ISLSF, are also detected in the spleen following the peak of antibody synthesis and that they are T cells, it was felt that it would be prudent to determine whether ISLSF also functions

in an antigen-specific manner. To resolve this question, ISLSF was absorbed with the immunizing antigen (SRBC) or with a non-cross-reacting antigen (HRBC) and the absorbed ISLSF was assayed for its ability to suppress the 2°IR in vitro to SRBC. It was demonstrated that only the absorption of ISLSF with SRBC, but not with HRBC, resulted in the elimination of suppressive activity, thus demonstrating the antigenic specificity of ISLSF. Furthermore, the ISLSF prepared from splenic MNC obtained from rabbits 25 days after immunization with HRBC failed to suppress the 2°IR to SRBC.

It was demonstrated that the cells which secrete ISLSF are T cells. Elimination of T cells from the splenic MNC obtained from rabbits 25 days post-immunization, at the peak of the trough, resulted in failure by the residual cells to secrete ISLSF whereas elimination of B cells or macrophages from the splenic MNC did not affect the ability of the remaining cells to secrete ISLSF. These results permit the conclusion that antigen-specific T suppressor cells, which are present in the spleen of the immunized rabbit between days 14 to 20 post-immunization, secrete an antigen-specific suppressor factor capable of inhibiting a 2°IR by allogenic memory splenic MNC in vitro.

TABLE 5

THE ANTIGENIC SPECIFICITY OF THE ISLSF. INCUBATION WITH THE IMMUNIZING ANTIGEN, SRBC, RESULTS IN ALMOST TOTAL ABSORPTION OF THE ISLSF.

Treatment of ISLSF	The percent inhibition of the generation of PFC to SRBC by memory splenic MNC cultured in the ISLSF with SRBC.			
	Exp. 1	Exp. 2	Exp. 3	Exp. 4
ISLSF (SRBC) ²				
Unabsorbed	73	74	63	60
Absorbed with SRBC ²	8	16	6	2
Absorbed with HRBC ³	70	78	61	57
ISLSF (HRBC) ⁴	2	2	-	-

² ISLSF (SRBC) is the cell-free supernatant of a 24 hour culture of splenic MNC obtained from a rabbit immunized IV with SRBC 25 days prior to sacrifice.

³ 3 ml of ISLSF were incubated with 1 ml of packed, previously washed, SRBC for 1 hour at 4°C.

³ 3 ml of ISLSF were incubated with 1 ml of packed, previously washed, HRBC for 1 hour at 4°C.

⁴ ISLSF (HRBC) is the cell-free supernatant of a 24 hour culture of splenic MNC obtained from a rabbit immunized IV with HRBC 25 days prior to sacrifice.

TABLE 6

THE SUPPRESSOR CELL IN THE SPLEEN OF THE IMMUNIZED RABBIT CAPABLE OF SECRETING THE ANTIGEN-SPECIFIC SUPPRESSOR FACTOR ISLSF IS A T CELL AND NOT A B CELL OR A MACROPHAGE

24 HOUR CULTURE SUPERNATANTS OF SPLENIC MNC OF A DAY 25 RABBIT ¹ depleted of	The number of PFC per 10 ⁶ MNC following culture of splenic MNC of an allogeneic day 7 rabbit for 5 days in the culture supernatants ²			
	Exp. 1 ³	Exp. 2 ³	Exp. 3 ⁴	Exp. 4 ⁴
Nil	3330 (68) ⁵	2327 (77)	1620 (70)	1735 (68)
T cells	8733 (15)	8940 (12)	5212 (4)	4802 (12)
B cells	4266 (50)	3258 (60)	2355 (57)	1944 (64)
Macrophages	4870 (52)	7305 (29)	2381 (56)	2180 (60)

- 1 The rabbit was sacrificed 25 days following IV immunization with SRBC
- 2 The splenic MNC of rabbits sacrificed 7 days after immunization were cultured with the antigen, SRBC, for 5 days. The culture media were the supernatants of the 24 hour cultures of the treated and untreated day 25 splenic MNC. The culture medium was changed on days 2 and 4 of the 5 day culture.
- 3 These splenic MNC generated 10,260 PFC following culture in regular culture medium with the SRBC for 5 days.
- 4 These splenic MNC generated 5540 PFC following culture in regular culture medium with the SRBC for 5 days.
- 5 The figures outside and inside the brackets represent the no. of PFC observed and the percentage inhibition of PFC generated in the culture in the presence of ISLSF, respectively.

5.5 The origin of suppressor cells in the spleen of the rabbit following secondary immunization IV

5.5.1 Rationale and objectives

The investigation to this point dealt with the regulatory mechanism governing the immune response following primary immunization. It was demonstrated that T suppressor cells are detected in the spleen of the immunized rabbit 14 to 30 days post-immunization. These results strongly suggest that the T suppressor cells originate in the thymus following immunization since suppressor cells are detected in the thymus by day 5 post-immunization and are subsequently detected in the spleen and popliteal lymph nodes. Furthermore, thyx prior to or within 7 days following primary immunization results in the failure to detect suppressor cells in the spleen.

The suppressor cells in the spleen of the non-thyx rabbit appear to disappear by day 30 post-primary immunization. One explanation is that the suppressor cells in the spleen die; another explanation is that the suppressor cells in the spleen enter a dormant state and can be reactivated following secondary immunization in vivo with the same antigen. If the former explanation is correct, then thyx prior to reimmunization or secondary immunization will result in failure to detect suppressor cells in the spleen following secondary immunization with the original antigen. However, if the second explanation is correct, then thyx prior to secondary immunization IV should not affect the subsequent detection of suppressor cells in the spleen.

The objective of this series of experiments is to determine whether the thymus is relevant in the generation of suppressor cells in the spleen following secondary immunization IV.

5.5.2 Experimental protocol

Rabbits were initially immunized IV with 10^8 SRBC. Forty five days later, half of the immunized rabbits were thyx. All of the rabbits were reimmunized IV with the original antigen 60 days after the primary immunization. They were sacrificed on the specified days following secondary immunization. The thymus and spleen were excised and thymocyte and splenic MNC suspensions were prepared as described in section 4.2.7. The secondary immune responses with the splenic MNC were carried out as described in section 4.2.12. Following culture with the immunizing antigen for 5 days, the cells were assayed for PFC as described in section 4.2.11.

5.3 Results

Rabbits were immunized with 10^8 SRBC IV and reimmunized with 10^8 SRBC IV (secondary immunization) 60 days later. The thymocytes were assayed for suppressor cell activity on predetermined days post-immunization by their ability to suppress the secretion of antibodies by allogeneic immune splenic MNC following co-incubation of the cells for 4 hours (121). As can be seen in Table 7, significant suppressor activity was expressed by the thymocytes

beginning on day 4 post-secondary-immunization. The thymocytes were able to totally suppress antibody secretion by the allogeneic splenic antibody forming cells by day 5 post-secondary-immunization (Table 7).

Rabbits were then immunized with 10^8 SRBC IV on days 0 (primary immunization) and day 60 (secondary immunization) and groups of 3 rabbits were sacrificed 5, 10, 15, 20, 25, 30 and 40 days post-secondary immunization. The splenic MNC of these rabbits were cultured for 5 days in vitro with the antigen and then assayed for PFC in order to determine whether the splenic MNC after secondary immunization exhibit the trough as do the splenic MNC following primary immunization. As can be seen in Figure 8, the splenic MNC of rabbits sacrificed 15, 20 and 25 days post-secondary immunization IV generated far fewer PFC in culture than did the splenic MNC obtained from rabbits sacrificed 5 or 10 days post-secondary immunization. The minimum in vitro PFC responses were given by the splenic MNC of rabbits sacrificed 25 days post-secondary immunization.

Experiments were then conducted to rule out the possibility that the trauma of the operative procedure itself contributes toward the trough on days 15 to 30 post-secondary immunization. As can be seen in Figure 9, the splenic MNC of rabbits sham-thy 14 days prior to secondary immunization IV and sacrificed 25 days later gave much weaker immunological responses in vitro (16%) as compared to the responses of the splenic MNC of rabbits which had been thyx. The latter generated large numbers of PFC in the in

vitro culture.

Rabbits were sacrificed on various days following secondary immunization IV and their splenic MNC were cultured for 24 hours at 37°C and the cell-free culture supernatants were assayed for ISLSF suppressor activity. As can be seen in Table 8, the splenic MNC even 5 days post-secondary immunization secreted detectable quantities of ISLSF. Maximum ISLSF was secreted by the splenic MNC 15, 20, 25 and 30 days post-secondary immunization.

On other hand, the splenic MNC of rabbits thyx 14 days prior to secondary immunization and sacrificed 25 days post-secondary immunization, the mid-point of the trough in the non-thyx rabbits, failed to secrete detectable amounts of ISLSF (Table 9), suggesting that these splenic MNC were devoid of suppressor cells.

5.5.4 Discussion

The results presented above with the splenic MNC of rabbits following secondary immunization indicate that suppressor cells are present in the spleen at the same times following secondary immunization as after primary immunization. As was demonstrated previously with the thymocytes of rabbits following primary immunization (125), suppressor cells are detected in the thymus by day 5 following secondary immunization.

The most interesting finding was that the suppressor cells could not be detected in the spleen if the rabbits had been thyx 14 days prior to secondary immunization IV. These results imply that the suppressor cells detected in the

spleen following the primary immune response do not survive in the spleen beyond the time of their detection (day 30 post-primary immunization) and that the suppressor cells detected in the spleen following secondary immunization are also derived from the thymus.

TABLE 7

SUPPRESSOR CELLS ARE DETECTED IN THE THYMUS
FOLLOWING SECONDARY IMMUNIZATION IV WITH SRBC

Thymocytes obtained from rabbits sacrificed on the following days after secondary immunization	The no. of PFC per 10 ⁶ immune splenic MNC (I, II and III)* following incubation of the MNC with the allogeneic thymocytes for 4 hours prior to assay for PFC		
	I	II	III
-1	220	274	298
2	235	260	315
3	220	240	295
4	140	120	155
5	20	0	10
6	0	0	0
7	0	0	0
8	0	0	0
10	0	0	0

* The splenic MNC were obtained from rabbits I, II and III
sacrificed 7 days following IV immunization with 10⁸ SRBC.

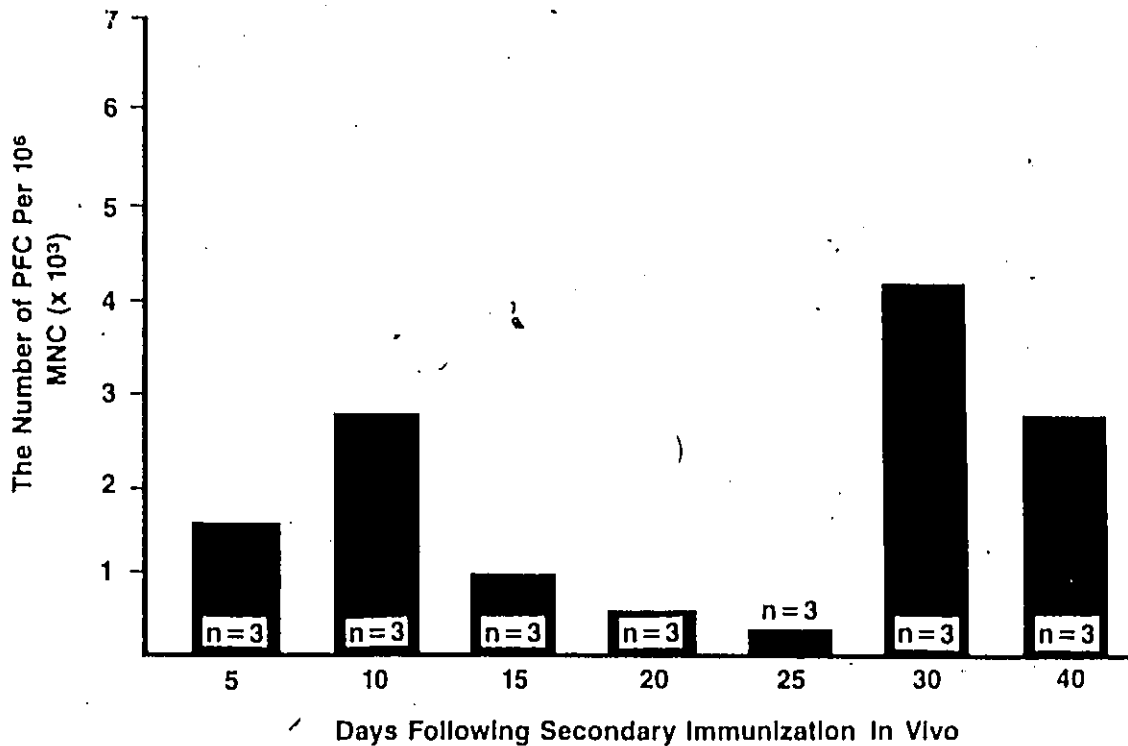


Figure 8. The in vitro immune response by splenic MNC of rabbits sacrificed at varying times following secondary immunization IV with SRBC.

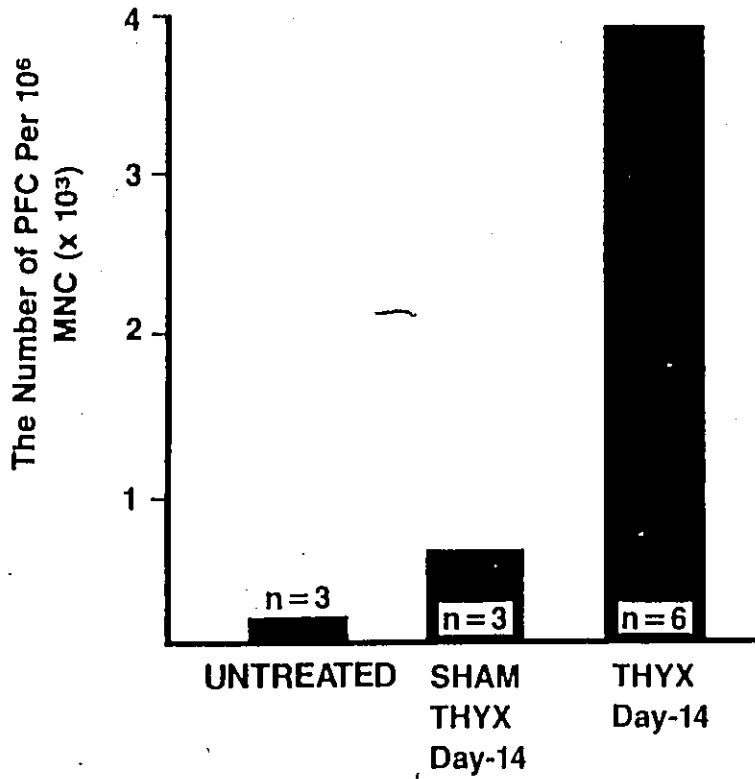


Figure 9. The immune response induced in in vitro cultures of splenic MNC of rabbits thymectomized 14 days prior to secondary immunization IV and sacrificed on day 25 post-secondary immunization.

TABLE 8

THE SPLENIC MNC OF NON-THYMECTOMIZED RABBITS FOLLOWING SECONDARY IMMUNIZATION IV SECRETE ISLSF IN CULTURE CAPABLE OF SUPPRESSING THE SECONDARY IMMUNE PFC RESPONSE BY ALLOGENEIC SPLENIC MEMORY MNC IN VITRO.

Cell-free supernatants of 24 hour cultures of splenic MNC ¹ obtained from rabbits (I and II) sacrificed on each of the following days after secondary immunization IV with 10 ⁸ SRBC ²	The number of PFC per 10 ⁶ splenic ³ memory MNC ³ following culture of the MNC with the antigen, SRBC, for 5 days in the cell-free supernatants of cultures of rabbits I and II ⁴	
	I	II
5	5410 (31) ⁵	5766 (26)
10	4175 (47)	4112 (40)
15	3632 (54)	3154 (60)
20	3229 (59)	3323 (50)
25	3208 (60)	3271 (59)
30	3822 (51)	3905 (50)
40	5950 (24)	4855 (18)

¹ Splenic MNC (10⁸ per ml) were cultured in culture media for 24 hours at 37°C in 5% CO₂ in air.

² The interval between primary and secondary immunization IV with 10⁸ SRBC was 2 months.

³ The splenic memory MNC generated 7840 PFC per 10⁶ MNC after culture for 5 days with the antigen, SRBC.

⁴ The media were replaced on days 2 and 4 of the 5 day culture period.

⁵ The figures outside and inside the brackets represent the number of PFC observed and the percent suppression of PFC generation, respectively.

TABLE 9

THYMECTOMY (THYX) 14 DAYS PRIOR TO SECONDARY IMMUNIZATION IV RESULTS IN THE FAILURE OF THE SPLENIC MNC TO SECRETE ISLSF 25 DAYS POST-SECONDARY IMMUNIZATION.

cell-free supernatants of 24 hr cultures of splenic MNC of rabbits thyx 14 days prior to secondary immunization IV with 10 ⁸ SRBC and sacrificed 25 days post-secondary immunization ¹	The no. of PFC per 10 ⁶ immune splenic MNC ² cultured for 5 days with SRBC in the 24 hr culture supernatants ³	Percent inhibition from control ³
thyx rabbit	10,220	0
thyx rabbit	9,763	0
thyx rabbit	11,574	0
thyx rabbit	10,775	0
thyx rabbit	9,725	0
thyx rabbit	9,029	10
sham thyx rabbit	4,750	52
sham thyx rabbit	3,798	62
untreated rabbit	4,086	51
untreated rabbit	4,186	58

¹ The interval between the primary and secondary immunization IV with SRBC was 2 months.

² The splenic MNC were obtained from a rabbit 7 days following immunization IV with 10⁸ SRBC. The no. of PFC generated in in vitro culture by these splenic MNC was 9,966 per 10⁶ MNC (control).

³ The culture medium was replaced on days 2 and 4 of the 5 day culture period.

5.6 Some physico-chemical properties of ISLSF: Temperature stability and molecular weight.

5.6.1 Rationale and objectives

The results presented in the previous sections of this thesis demonstrate the presence of suppressor cells in the spleen of the immunized rabbit, between days 14 and 30 following primary or secondary immunization, and the secretion by these cells of an antigen-specific suppressor factor, ISLSF, capable of inhibiting the secondary immune response in vitro by allogeneic splenic memory cells.

The objectives of this part of the investigation were to define the stability of ISLSF to temperature and to determine its approximate molecular weight.

5.6.2 Experimental protocol

Rabbits were immunized IV with 10^8 SRBC. They were sacrificed 25 days later, the spleens were excised and splenic MNC suspensions were prepared as described in section 4.2.7. The splenic MNC were cultured for 24 hours at 37°C to obtain ISLSF as described in section 4.2.10. ISLSF was dialysed for 24 hours through dialysis membranes of molecular weight exclusion of 14,000 and 50,000 as described in section 4.2.16. The ISLSF was assayed for its capacity to inhibit the 2°IR induced in vitro by allogeneic splenic memory cells as described in section 4.2.13.

ISLSF was also incubated either at 4°C or 37°C for 1, 2 or 3 days and then assayed for its capacity to inhibit the 2°IR induced in vitro by allogeneic splenic memory cells as described in section 4.2.13.

5.6.3. Results

The splenic MNC of rabbits sacrificed 25 days post-immunization were cultured for 24 hours at either 4°C or 37°C and their cell-free culture supernatants were assayed for suppressor activity. As can be seen in Table 10, much more ISLSF was secreted by the cells at 37°C than at 4°C. The ISLSF secreted at 37°C was able to inhibit the in vitro 2°IR by about 55 percent whereas the ISLSF secreted at 4°C was only able to inhibit the 2°IR by 23 percent.

The stability of ISLSF to temperature was then investigated. Cell-free culture supernatants containing ISLSF were maintained at either 4°C or 37°C for up to 3 days and then assayed for suppressor activity. As can be seen in Table 11, ISLSF appears to be more stable at 37°C than at 4°C, at least for 2 days, with respect to 2 of the 4 specimens of ISLSF. The other 2 specimens of ISLSF behave equally well at 37°C and at 4°C. ISLSF lost essentially all suppressive activity if kept at either 37°C or 4°C for 3 days prior to assay.

ISLSF appears to have a molecular weight between 14,000 and 50,000 since ISLSF dialysed through the 14,000 mol. wt. exclusion tubing for 24 hours retained suppressor activity whereas ISLSF dialysed through the 50,000 mol. wt. exclusion tubing for 24 hours lost all suppressor activity. However, these results were obtained in only 1 experiment and it must be repeated (Table 12).

5.6.4 Discussion

Not surprisingly, the suppressor cells secreted more ISLSF when cultured for 24 hours at 37°C than at 4°C. This is due to active secretion of ISLSF by the suppressor cells and not simply the release of ISLSF by dying cells since sonicates of the splenic MNC are not suppressive (results presented in Table 3) and the viability of the cells in culture at either 37°C or 4°C was very high (more than 80 percent).

ISLSF is relatively unstable at either 37°C or 4°C since it lost suppressor activity if it was kept at either of these two temperatures for more than 2 days. Obviously, the structure of ISLSF is such as to be easily denatured.

The results of a single experiment disclosed that the molecular weight of ISLSF is greater than 14,000 but less than 50,000. However, it must be emphasized once again that this experiment was carried out only once and must be repeated at least 2 more times.

TABLE 10

ISLSF IS SECRETED IN GREATER QUANTITY BY THE
SPLENIC SUPPRESSOR CELLS AT 37°C THAN AT 4°C

Cell-free culture supernatants containing ISLSF were incubated for 24 hours at the following temperature ¹	Percent suppression by the culture supernatants ² of the number of PFC generated by allogeneic memory MNC cultured with the immunizing antigen, SRBC, for 5 days ³
37°C (rabbit A)	53
(rabbit B)	56
4°C (rabbit A)	24
(rabbit B)	24

¹ Splenic MNC were obtained from rabbits sacrificed 25 days following the immunization IV with 10⁸ SRBC. These cells were cultured for 24 hours at 37°C (10⁶ cells per ml).

² The culture supernatants containing ISLSF were used as culture medium for the induction of the 2-IR in vitro by allogeneic memory splenic MNC. The culture medium was replaced on days 2 and 4 of the 5 day culture.

³ The memory splenic MNC generated 7254 PFC in the secondary immune response induced in vitro in culture medium.

TABLE 12

THE EFFECT OF DIALYSIS FOR 24 HOURS ON THE SUPPRESSIVE ACTIVITY OF ISLSF (RESULTS OF A SINGLE EXPERIMENT)

ISLSF ^a dialysed for 24 hours at 4°C in dialysis tubing with exclusion molecular weight of	Percent suppression by the dialysed ISLSF of the no. of PFC generated in the 2°IR in vitro by allogeneic memory splenic MNC cultured for 5 days with the immunizing antigen, SRBC ^b .	
	I	II
14,000	21	ND ^c
50,000	ND	0
Not dialysed	41	28

^a ISLSF is the cell-free culture supernatant obtained from 24 hour cultures at 37°C of splenic MNC obtained from a rabbit sacrificed 25 days after immunization IV with SRBC. It was used as culture medium for the induction of the 2°IR by allogeneic splenic memory cells.

^b The culture medium was replaced on days 2 and 4 of the 5 day culture.

^c ND = Not done.

6. GENERAL DISCUSSION

It has been known since the 1940's that the spleen is the primary, and often the only, organ in which antibody synthesis takes place (see Chapter 3). The splenic antibody forming cells have been isolated, cultured, identified by immunofluorescent techniques, and demonstrated to be B cells on the basis of numerous cell-surface markers. However, for many years an understanding of the mechanism(s) which initiates antibody synthesis and which later down-regulates antibody synthesis following specific antigenic stimulation thwarted investigators. The discovery of the need for T cells to interact with B cells by Claman et al (38-40), and the demonstration that those T cells function by virtue of cell-surface receptors precommitted to interact with the antigenic determinants on antigens following initial contact with the antigen, increased our appreciation of the complexity of the immune response but not our understanding of the immune response. The milestone discoveries of T suppressor cells by Gershon and Kondo in 1970 and the T helper cells by Moretta and his colleagues in 1975 (144) did much to provide an understanding of the cellular regulatory mechanism for antibody synthesis. Coupled with the discovery of the regulation of antibody synthesis by IgM and IgG antibodies by Jerne and Henry in 1968 (145), and confirmed by many other investigators (146-149), investigators felt confident in explaining the regulation of antibody synthesis, that is the very rapid synthesis and

secretion of large quantities of antibodies early in the immune response (when they are needed by the host) and the cessation of antibody synthesis and secretion later in the immune response (when they are no longer needed by the host). The problem which has existed since the discovery of the suppressor cells is that they are rarely detected in the spleen at a time during the immune response which would impart to them the role of inhibitors of antibody synthesis. The majority of investigators have dealt with non-specific suppressor cells, activated by a myriad of stimuli unrelated to antigens. Antigen-specific suppressor cells have only infrequently been demonstrated to be present in the spleen during or after the immune response (see discussion in ref. 121). Indeed, these few investigations may be considered to be anecdotal as they constitute single paper reports and were not further pursued. In order to state convincingly that the antibody response in the spleen is terminated by the actions of suppressor cells, it is necessary to demonstrate the physical presence and suppressive activities of splenic suppressor cells at the time of the down-turn of antibody synthesis and secretion (i.e. days 10 to 20 post-immunization in the rabbit).

Previous investigations in this laboratory by Richter and Talor (121) disclosed the presence of antigen-specific suppressor cells in the thymus by day 5 following immunization of the normal outbred rabbit. It was subsequently demonstrated that the thymic suppressor cells

secrete an antigen-specific factor in short-term (24 hours) culture (121) and that this factor, referred to as ITSF (121) (see Chapter 3) was capable of inhibiting specific antibody synthesis when injected IV into a naive host rabbit along with the original immunizing antigen (122). The question which was raised by these investigators (121,122,137) is what role the thymic suppressor cells have in dampening the antibody response in the spleen. It was speculated that the thymic suppressor cells must vacate the thymus and infiltrate the spleen where they act on the antibody synthesizing B cells to down-regulate antibody synthesis and terminate the immune response.

The objectives of this investigation were (i) to demonstrate the presence of antigen-specific suppressor cells in the spleen at or not too long after the the peak of the immune response is attained, and (ii) to demonstrate whether these splenic suppressor cells, if they can be identified, act by secreting a soluble factor which can replace the suppressor cells. A longer term objective was to characterize the suppressor factor.

It was demonstrated in this investigation that T suppressor cells capable of secreting a potent antigen-specific suppressor factor referred to as immune splenic lymphocyte suppressor factor or ISLSF, can be detected in the spleen of the immunized rabbit beginning on day 14 following immunization. They secrete maximum amounts of ISLSF, as detected in the in vitro assay, between days 18.

and 30 post-immunization. Since the in vitro assay cannot be as sensitive in the detection of suppressor cells as the physiological in vivo situation where suppressor cells are probably in close apposition to the antibody forming cells, it may be presumed that overt suppressor cells are probably present in the spleen by day 10 or so post-immunization, at just the right time to shut off the the immune response. Since suppressor cells would not be required following the cessation of the immune response, it is not surprising that nature dictates that they either die or enter a dormant phase.

The relationship of the splenic T suppressor cells with the thymus was then investigated. It was demonstrated that the extirpation of the thymus prior to or within 7 days of primary immunization results in the failure to subsequently detect suppressor cells in the spleen. Thymectomy performed more than one week post-immunization did not affect the appearance of suppressor cells in the spleen, thus indicating that the suppressor cells vacate the thymus, where they are originally activated or generated, after day 7 post-immunization and infiltrate the spleen. These results strongly suggest that the splenic suppressor cells originate in the thymus where they can be detected by day 4 post-immunization.

The splenic suppressor cells cannot be detected by the in vitro assay beyond 30 days post-primary immunization, suggesting that the suppressor cells either enter a dormant

period or die. If the former is correct, then these cells should be reactivated following a second contact with the antigen. If the latter is correct, then the suppressor cells detected in the spleen following the secondary immunization would once again have to originate outside the spleen, presumably in the thymus. Rabbits were therefore subjected to a secondary immunization 60 days following primary immunization. They were thymectomized 14 days prior to secondary immunization and sacrificed 25 days post-immunization when the suppressor cells normally express peak activity in the spleen. However, suppressor cells were not detected in the spleens of the thymectomized rabbits, indicating that the suppressor cells detected in the spleen after secondary immunization originate in the thymus just as after primary immunization. Thus, the splenic suppressor cells after the primary immune response appear to die and must be replaced by suppressor cells from the thymus following secondary immunization.

In summary, it has been demonstrated that, T suppressor cells are present in the spleen following the primary and secondary immune responses. These T suppressor cells probably originate in the thymus since thymectomy prior to immunization results in the failure to subsequently detect suppressor cells in the spleen. The presumed role of the splenic suppressor cells is to act on the antibody forming cells in the spleen, via the secretion of an antigen-specific suppressor factor, to inhibit further synthesis of antibody and thereby terminating the immune response.

7. CONCLUSION

The mechanism whereby the immune response (antibody synthesis and secretion), which takes place in the spleen following the primary and secondary immunization, is terminated was investigated. It had been previously reported that antigen-specific suppressor cells are detected in the thymus by day 5 post-primary immunization. It was therefore postulated that the thymic suppressor cells vacate the thymus and infiltrate the spleen where they act on the antibody forming cells to inhibit further synthesis and secretion of antibodies.

It was demonstrated in this investigation that antigen-specific T suppressor cells are detected in the spleen between days 14 and 30 post-primary immunization. These suppressor cells can secrete a soluble factor referred to as immune splenic lymphocyte (-derived) suppressor factor or ISLSF which can inhibit the synthesis and secretion of specific antibodies by antigen-stimulated memory splenic MNC in culture in vitro.

Thymectomy of rabbits prior to or within the first week following primary immunization resulted in the subsequent failure to detect suppressor cells in the spleen. However, thymectomy later than one week following immunization did not affect the normal disposition of suppressor cells in the spleen. These results strongly indicate that the splenic T suppressor cells detected following the primary immune response originate in the thymus where suppressor cells are

detected by day 5 following immunization.

It was also demonstrated that thymectomy prior to secondary immunization also results in failure to subsequently detect suppressor cells in the spleen. This result suggests that the suppressor cells detected in the spleen following primary immunization do not go into a dormant state in the spleen and survive "to rise and fight another day". Rather, they appear to die in the spleen following the primary response. Thus, the antigen-specific suppressor cells detected in the spleen following secondary immunization must be recruited from the thymus. Following secondary immunization, they infiltrate the spleen and terminate the secondary antibody response.

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