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THE ONTOGENY OF ANTIBODY PRODUCTION IN C57BL/6 MICE

by

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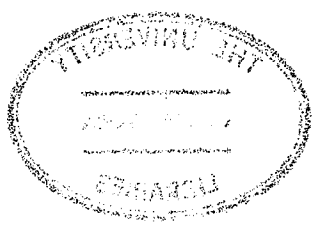

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EDWARD STANLEY RECTOR

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

DOCTOR OF PHILOSOPHY

to

Mom and Dad

The childhood shows the man,

As morning shows the day.

MILTON - *Paradise Regained*

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ABSTRACT

The development of immunocompetence in C57BL/6 mice was investigated during the first two weeks following birth by determining the ability to mount IgM and IgG plaque-forming cell responses using sheep red cells (SRC) as antigen. Responsiveness at a given age was determined in two systems: (1) by direct immunization of intact mice and (2) by stimulation of neonatal spleen cells following transfer to adult irradiated recipients. Spleen cells from 1- to 3-day-old mice gave only IgM PFC responses in irradiated recipients; when thymus cells from adult mice were injected together with such spleen cells, IgG PFC responses were also obtained. The rate of increase of IgM responsiveness shortly following birth was markedly different from that of IgG responsiveness, however, after four days of age, both responses increased with the age of the donor in a parallel manner. In contrast, the IgM and IgG responses of intact mice were barely detectable until age 9-10 days, at which time both responses increased dramatically; this increase was attributed to a maturational change in the antigen-processing system.

The maturation of antibody responsiveness was further investigated by comparing the sensitivities of 12-day-old and 12-week-old mice to SRC. Sensitivity was established by constructing antigen dose-antibody response curves for the IgM and IgG responses. Twelve-day-old mice yielded parabolic dose-response curves with well-defined maxima and relatively narrow bandwidths (the bandwidth being defined as the ratio of the two antigen doses which elicited a response equal to 50% of the maximum). The optimal antigen doses for the two antibody classes studied were 1.2×10^9 SRC for the IgM response and 3.4×10^9 SRC for the IgG response. Twelve-

week-old mice yielded dose-response curves with similar but not identical characteristics. The optimal antigen doses were lower in the adult group (2.9×10^8 for the IgM and 1.0×10^9 for the IgG response), indicating an increased sensitivity to antigen. However, these differences in antigen sensitivity were not apparent when spleen cells from 12-day-old and 12-week-old mice were challenged with varying doses of SRC in irradiated recipients. Therefore, it was concluded that the optimal antigen dose was a parameter of the antigen-processing system and that this system was relatively inefficient in 12-day-old C57BL/6 mice.

The bandwidths of dose-response curves derived from neonatal mice were narrower than those exhibited by their adult counterparts. Moreover, these differences were maintained when spleen cells from the respective age groups were challenged in adult irradiated recipients. It was concluded that the bandwidth was not a parameter of the antigen-processing system, but was a reflection of an intrinsic property of the immunocompetent cell population.

The antigen-processing systems of neonatal and adult mice were assessed in terms of accessory cell (A cell) frequencies. It was observed that when IgG responses were compared, a lower frequency of accessory cells was demonstrated in neonatal compared with adult spleens. The functional properties of the antigen-processing system were further investigated by assessing the relative abilities of neonatal and adult mice to retain passively administered suppressive anti-SRC antibody for prolonged periods of time. It was observed that mice, at birth, lacked the ability to retain passively administered suppressive antibody, but began to acquire this ability shortly after birth. It was concluded that the antigen-processing system was involved in the long-term retention of

passively administered antibody and this system was not functional in newborn mice.

The rates of clearance from circulation and the localization in various organs of intravenously injected radioiodinated SRC were determined and compared in 12-day-old and adult mice. Within the two-hour time period following injection of radioiodinated SRC, no basic differences were observed between the two age groups. It was concluded that the relative functional deficiency observed in the antigen-processing systems of neonatal mice was not reflected in the activation of their reticuloendothelial systems. A heterogeneity in antigen-handling cells was proposed, of which A cells constituted a subpopulation.

Studies undertaken to determine the possible crossreactivity between SRC and "environmental" antigens demonstrated that SRC crossreacted with the lipopolysaccharide antigens isolated from at least one commonly occurring enteric bacterium. The possible relation of this finding and the increase in dose-response curve bandwidths observed in older mice is discussed.

Finally, the delayed onset of responsiveness observed in neonatal C57BL/6 mice to SRC is discussed in terms of a possible defect in T cells, B cells and A cells. It is concluded that the primary cause for such unresponsiveness is a relatively immature antigen-processing system in the spleens of neonatal mice. It is proposed that the anatomic microenvironments necessary for the appropriate accommodation of A cells and their subsequent interaction with immunocompetent cells is lacking in the spleens of neonatal mice.

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ABBREVIATIONS

A cell - accessory cell

ATS - anti-thymocyte serum

B cell - bone marrow-derived cell

BGG - bovine gammaglobulin

BSA - bovine serum albumin

BW - bandwidth

Con A - concanavalin A

CFU-S - colony-forming units in the spleen

CR - complement receptor

D-GL - copolymer of D-glutamic acid and D-lysine

"direct" PFC - IgM PFC

DNA - deoxyribonucleic acid

DNP - dinitrophenyl

DTH - delayed-type hypersensitivity

E. coli - Escherichia coli

FCS - fetal calf serum

G_{IX} - Gross virus antigen; IX linkage group

GAT - copolymer of glutamic acid, alanine and tyrosine

GLØ - copolymer of glutamic acid, lysine and phenylalanine

GRF - genetically-restricted factor

GT - copolymer of glutamic acid and tyrosine

HEPES - N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid

(H,G)-A--L - branched copolymer of histidine, glutamic acid, alanine and lysine

HGG - human IgG

HRC - horse red cells

HT - HRC-treated

Ia - immune associated (antigen)

Ig - immunoglobulin

"indirect" PFC - IgG-2a PFC

Ir - immune response (gene)

Is - immune suppression (gene)

KLH - keyhole limpet hemocyanin

LPS - lipopolysaccharide

Ly - lymphocyte alloantigens

MBLA - mouse-specific lymphocyte antigen

MBSA - methylated BSA

MHC - major histocompatibility complex

MSLA - mouse-specific lymphocyte antigen

MSPCA - mouse-specific plasma cell antigen

NIP - 4-hydroxy-5-iodo-3-nitrophenacetyl

OA - ovalbumin

PBS - phosphate (0.01M)-buffered saline (isotonic)

PBS-G - PBS containing 10 mM glucose (isotonic)

PFC - plaque-forming cell

PHA - phytohemagglutinin

(Phe,G)-A--L - branched copolymer of phenylalanine, glutamic acid, alanine and lysine

PLL - poly-L-lysine

PWM - pokeweed mitogen

RNA - ribonucleic acid

S.D. - standard deviation

S.E. - standard error

SRC - sheep red cells

T cell - thymus-derived cell

(T,G)-A--L - branched copolymer of tyrosine, glutamic acid, alanine and lysine

TL - thymus leukemia (antigen)

TNP - trinitrophenyl

INTRODUCTION

I. GENERAL ASPECTS OF ANTIBODY FORMATION

a) Introduction

The basis of the study of immunology was founded on observations made in clinical medicine. The ancient Chinese practised a form of vaccination which was based on observations that following the recovery from a disease, an individual became less susceptible to that disease. The mechanisms by which the body is able to protect itself in this manner has been a subject of study ever since.

Paul Ehrlich (1) was one of the first in recent times to address himself to the problem of how the body recognizes foreign substances and, subsequently, responds to them with the production of protective substances found in the serum. He postulated that these serum substances were produced by cells which could recognize foreign organisms by virtue of having side chains on their surfaces. These side chains were, in fact, the serum substances and each cell displayed a full set of "side chains" - a reflection of all the responses the body could make. Ehrlich envisaged a foreign substance reacting with a specific cell-bound side chain, resulting in the disappearance of other side chains and the enhanced production of only the side chains with specificity for the foreign substance. These side chains were subsequently released and led to the elevation of the level of serum antibody. Almost eighty years later, the basic concept of a selective process in the initiation of an immune response is generally accepted as a fundamental tenet of modern immunology and stands as a testament to the insight of Paul Ehrlich.

Karl Landsteiner (2) produced evidence which lead to the abandoning of the side chain theory by demonstrating that specific antibody responses could be elicited by many different synthetic haptens, raising doubt that

single cells could express specific side chains for a seemingly endless array of compounds.

During the 1940's and 1950's, there was a drift away from the selective theories and mechanisms to instructive theories such as the direct template theory proposed by Linus Pauling (3). Antigen was envisaged to interact with the antibody molecule as it was being synthesized and in so doing provide a structural template for the acquisition of the specific secondary and tertiary structure. This theory was proposed somewhat before the mechanisms of protein synthesis were fully understood. However, when it became clear that secondary and tertiary structures were determined solely by the primary structure of the protein and, ultimately, the genetic code, such instructive theories had to be abandoned.

The latter 1950's saw a reaffirmation of the selective theories of antibody formation. In 1955, Niels Jerne (4) proposed the natural selection theory, which proposed that a single cell was programmed to make only one specificity of antibody and it did this even in the absence of a deliberate antigenic stimulus. Thus, the gammaglobulin molecules found in the sera of unimmunized animals represented antibodies directed to a vast number of structurally distinct antigens, with a given antibody specificity present in very small amounts. Following immunization, the antigen was envisaged to combine with the preformed "natural" antibody and this antibody-antigen complex attached to those cells which originally produced the specific "natural" antibody. This attachment provided an appropriate signal for cell division and the synthesis of more specific antibody.

Subsequently, F. M. Burnet (5), by modifying some of the concepts

found in Jerne's theory, proposed the clonal selection theory, which is generally accepted, in one form or another, by most immunologists today. Briefly, according to this theory, a cell is programmed in its DNA to produce one or at best a very few specificities of antibody. The expression of this specificity in the form of a receptor on the surface of the cell leads, following reaction with antigen, to the generation of a signal for the cell to divide. Following several divisions the numbers of such cells are increased as is the level of specific antibody.

During the period when Jerne and Burnet were advancing their respective theories on antibody formation, information was becoming available concerning the physicochemical properties of antibodies, it being already well established, chiefly from the work of Landsteiner (2), that antibodies were capable of recognizing extremely fine differences in antigenic structure. However, very little was known of the cells involved in antigen recognition or in antibody production; nor was there much known of the genetic requirements involved in immunity. Since the early sixties, these topics were addressed by a vast research effort in the general area of immunobiology; some of the basic findings largely restricted to humoral immunity will be reviewed in the following sections.

b) The Dichotomy of Immunocompetent Lymphocytes in Antibody Formation

The advent of modern cellular immunology was marked by a number of key observations made by several independent investigators. It soon became apparent that antigen recognition and antibody formation did not involve a unicellular mechanism. At least two distinct classes of lymphocytes were shown to be required for antibody formation. One cell type, derived from the thymus (T cell), was shown to be required, but neither

it nor its progeny were involved in the actual synthesis of antibodies. The other cell (B cell), derived directly from the bone marrow in mammals (or bursa in birds), was the precursor of antibody-producing cells and required the presence of T cells to become activated. Finally, a third nonlymphoid type of cell, the macrophage, was shown to be necessary and somehow involved in the effective presentation of antigen to the lymphoid cell compartment.

The importance of the thymus in immune responses first became evident from the independent studies of Good (6) and Miller (7). Both investigators demonstrated that thymectomy of adult mice had little effect on their abilities to reject foreign skin grafts or produce antibodies. However, adult mice which had been thymectomized at birth, exhibited marked deficiencies of these immune responses which could, however, be restored by the injection of thymocytes.

The definition of the B cell lineage in the production of antibody was first demonstrated by the effect of bursectomy on the immune capability of birds. Thus, Glick *et al.* (8) and later Cooper *et al.* (9,10) reported that bursectomy of chickens at hatching led to a marked decrease in antibody responses and, furthermore, resulted in a state of agammaglobulinemia. Skin graft rejection in these birds was normal, thus differentiating the effects of bursectomy from those of thymectomy.

Thus, in the early 1960's, a dichotomy of lymphocyte function was becoming apparent. Lymphocytes requiring the presence of the bursa (or its equivalent in mammals) appeared to control some aspect of antibody formation, whereas those requiring the thymus were involved in both antibody formation and graft rejection. The developmental aspects of B cell and T cell formation will be discussed in more detail in the

ontogeny section of this Introduction.

One of the popular interpretations held at the time concerning the effects of neonatal thymectomy on antibody formation envisaged the function of the thymus to relate to the seeding of the secondary lymphoid organs with functional precursors of antibody-producing cells. Thus, its removal prior to such seeding led to the observed state of immunodeficiency. The first clear-cut demonstration that the induction of antibody formation was rather more complicated was reported by Claman and co-workers (11). They demonstrated that neither thymus cells nor bone marrow cells were capable, by themselves, of restoring the ability of lethally X-irradiated mice to produce antibodies to SRC. However, when both cell populations were injected, the restoration of the antibody response was achieved. Similar observations were reported by Davies *et al.* (12) who, using a slightly different system demonstrated that both thymus and bone marrow cells were necessary for optimal antibody formation in irradiated recipients. In this way the synergistic effect of thymus and bone marrow cells led to the concept of cellular cooperation as a basic prerequisite for the induction of antibody responses. Claman postulated that the bone marrow contained "effector cells" capable of producing antibody, but only in the presence of "auxiliary cells" present in the thymus population.

Support for this interpretation was subsequently reported by Mitchell and Miller (13). They observed that when neonatally thymectomized CBA mice ($H\text{-}2^k$) were reconstituted with C57BL/6 ($H\text{-}2^b$) thymus cells and challenged with SRC, an anti-SRC plaque-forming cell (PFC) response was elicited. Treatment *in vitro* of the antibody-forming cells found in the spleen with complement and anti-$H\text{-}2^k$ antibodies directed against the

histocompatability antigens of the CBA host (source of B cells), led to a marked inhibition of the number of PFC visualized; however, antibodies directed to the cells of the thymus cell donor (anti-H-2^b) produced no such inhibitory effect. These results demonstrated that the antibody-producing cells were derived from the host and, therefore, were of bone marrow and not thymus origin. Additional studies reported by these investigators using chromosomal markers (14) supported the interpretation that the antibody-forming cells and their precursors were derived solely from the bone marrow population, whereas thymus cells reacted specifically with antigen but performed an auxiliary role in the production of antibody by B cells.

c) The Requirement of an Antigen-Processing System

The advent of tissue culturing techniques in the mid-1960's allowed the successful development of *in vitro* correlates of antigen recruitment and antibody production. Using such a system, Mosier and colleagues (15-17) demonstrated that mouse spleen cells could be activated by SRC to produce antibodies. Furthermore, such spleen cell populations could be separated into two subpopulations on the basis of their relative abilities to adhere to plastic or glass surfaces. The separation of spleen cells into adherent and nonadherent fractions led to the inability of either cell population to respond to SRC; recombination restored antibody-producing ability. Whereas the nonadherent population contained T and B lymphocytes, the cell type in the adherent fraction necessary for antibody production was the macrophage. The ability of such cells to phagocytose antigens and to display antigen both internally and on their membranes led to the concept that such a cellular requirement in the induction of immunity was related to antigen "processing" or antigen "presentation".

Thus, some investigators coined the term "the antigen-processing system" to describe the general aspects of this cellular requirement, whereas others used terms describing particular characteristics, i.e. macrophages (morphological classification), adherent cells (physical property), or accessory cells or A cells (functional property).

The concept that appropriate antigen presentation was a prerequisite for antibody induction *in vivo* was first suggested by the experiments of Dresser (18) in 1962. While studying the ability of mice to mount an immune response to bovine gammaglobulin (BGG), he reported that the "adjuvantivity" of BGG preparations could be removed by ultracentrifugation; a finding confirmed by Claman (19) in mice and by Battisto and Miller (20) in guinea pigs. Thus, following removal of the aggregated material, the BGG preparations were found to primarily induce unresponsiveness, as opposed to immunity. Subsequently, Frei *et al*. (21) reported that the removal of phagocytizable elements from bovine serum albumin (BSA) by *in vivo* biological filtration resulted in BSA preparations which were much less immunogenic than native BSA and could cause specific unresponsiveness in animals. Since (i) it was shown that aggregated or denatured antigens were rapidly phagocytosed by macrophages of the reticuloendothelial system (22,23), and (ii) it appeared that the aggregated or phagocytizable form of the antigen was responsible for immunity, it was proposed that antigen uptake and processing by macrophages were crucial steps in the induction of antibody formation (21). Thus, antigens which were processed by macrophages in some manner led to immunity, whereas antigens which remained in circulation resulted in unresponsiveness.

Direct experimental evidence to support this concept was reported by a number of investigators in the latter 1960's and early 1970's (24-29).

The basic experimental protocol consisted of obtaining mouse peritoneal macrophages, pulsing these with radioactive antigen, and subsequently assaying the immune response to the macrophage-bound antigen following transfer to normal syngeneic mice or irradiated recipients reconstituted with lymphocytes. By using radioactive-labelled antigen, the amount of antigen associated with the macrophages could be determined. The results indicated that macrophage-bound antigen was immunogenic, by definition capable of eliciting a strong immune response. The role of macrophages was particularly evident when relatively weak antigens were used. For example, albumins associated with macrophages were found to be 1,000 - 10,000-fold more immunogenic than when given in solution (27-29). Moreover, the strong immunogenicity of macrophage-associated antigen was found for a variety of antigens, whether particulate or soluble, high or low molecular weight proteins (30), suggesting a phenomenon of general significance.

Similar results were obtained using *in vitro* antibody responses to assess the immunogenicity of macrophage-bound antigen. Katz and Unanue (31) cultured DNP-KLH-pulsed macrophages or soluble DNP-KLH with primed mouse spleen cells and found the macrophage-associated antigen to be much more immunogenic than soluble antigen. Moreover, the strong antibody response to macrophage-bound antigen could be inhibited by the addition of free or soluble antigen, indicating two distinct forms of antigen — one immunogenic (macrophage-associated) and the other non-immunogenic (soluble).

Following the initial report by Mosier (15) of the requirement of an accessory cell for *in vitro* antibody responses, a number of investigators (32-35) confirmed this finding and demonstrated that the function

of such cells was radiation-resistant up to doses of at least 1,000 rads. In 1971, Gorczynski, Miller and Phillips further documented the *in vivo* requirement of such a radiation-resistant cell in the response of mice to SRC (36). They reported the development of two experimental protocols for the detection and quantitation of accessory cells *in vivo*. The first stemmed from the observation that although irradiated recipients could provide an intact accessory cell function shortly after irradiation, they were unable to do so 72 hours following irradiation. Thus, two hours after irradiation, recipients required only B cells and T cells to respond to SRC; whereas if reconstitution was attempted 72 hours following irradiation, a source of accessory cells (usually heavily irradiated spleen cells) was required. In this situation it was thought that the accessory cells left the spleen of the irradiated recipient, thus rendering them accessory cell deficient. The second protocol developed for the *in vivo* detection of accessory cell function was based on the finding that the injection of large numbers of horse erythrocytes (HRC) into mice led to a marked suppression of the antibody response to the antigenically unrelated SRC. When such HRC-treated mice were irradiated and used as adoptive recipients, it was found that, functionally, they were accessory cell deficient, and required the injection of radiation-resistant cells (accessory cells) as well as B and T lymphocytes in order to respond to antigen. In addition, the logarithm of the PFC response obtained with a fixed number of immunocompetent lymphocytes was shown to be directly related to the number of accessory cells injected, thus providing a basis for the determination of relative accessory cell frequencies in various cell suspensions. This technique was utilized in portions of this thesis

to compare the relative accessory cell frequencies in neonatal and adult mouse spleens. The results from such experiments are reported in Chapter III of the Results section.

d) The "Carrier" Effect

Following the demonstration in 1967 of cellular cooperation involving two distinct types of lymphocytes, numerous investigators addressed themselves to the nature of the cellular interactions which were apparently necessary for antibody production. Much of the initial work involved the study of antibody responses to hapten-carrier conjugates. In 1969, Mitchison (37) reported the first direct evidence for the cooperative participation of two cell types in the antibody response of mice to such conjugates. This was accomplished by analyzing the cellular requirements of the "carrier effect" in irradiated recipients. The carrier effect had been documented previously (38) and described the inability of animals to mount secondary anti-hapten responses unless the same carrier was used for both primary and secondary immunizations. Thus, animals primed with DNP-BGG mounted a secondary anti-DNP response following a second injection of DNP-BGG; whereas animals receiving DNP on an unrelated carrier such as ovalbumin did not develop secondary anti-DNP responses. Mitchison (37) reported that this carrier effect could be transferred with spleen cells to irradiated recipients. When irradiated mice were reconstituted with spleen cells from NIP-OA-primed mice a secondary anti-NIP antibody response was obtained on challenge with NIP-OA, but not with NIP-BSA. He further demonstrated that this carrier effect could be overcome by the injection of cells from mice primed to the unrelated carrier, in this case BSA. Thus, mice receiving both NIP-OA primed cells and BSA primed

cells could respond with a secondary anti-NIP response to NIP-BSA. These observations gave rise to the concept that, functionally, there were two populations of cells involved in responses to hapten-carrier conjugates; one which recognized the hapten and one which recognized the carrier. In addition, Mitchison demonstrated that both hapten and carrier determinants had to be on the same immunogenic molecule, suggesting a need for close proximity of both sets of determinants for effective collaboration to occur (39).

Shortly thereafter, Raff, using anti-Thy-1 alloantiserum plus complement as a method of selectively killing T cells, demonstrated that the carrier-specific cooperating cells or "helper" cells were thymus derived; by implication the hapten-specific antibody-forming cell precursors belonged to the B cell category (40).

e) The Discovery of Suppressor T Cells

In the early 1970's, while a great deal of effort was involved in the investigation of the mechanisms underlying T-B cell collaboration in humoral immunity, evidence was being obtained which suggested that T cells were concerned with suppression or regulation of the immune response as well as with providing helper function. One of the first demonstrations of this suppressor T cell function was provided by Gershon and Kondo in 1970 (41,42). Two groups of thymectomized, irradiated mice were reconstituted with either bone marrow and thymus cells or bone marrow alone. Both groups then received large doses of SRC repeatedly, in an attempt to induce tolerance. Following this course of injections all mice received normal thymus cells and were subsequently challenged with an immunogenic dose of SRC. It was found that recipients initially recon-

stituted only with B cells could subsequently mount a high anti-SRC response. Recipients which had both bone marrow and thymus cells present during the SRC treatment were unable to produce antibody, even following the injection of normal thymus (helper) cells. The involvement of a T cell in this lack of responsiveness was, therefore, indicated.

Since the initial discovery of suppressor T cells, a number of phenomena have been described which appear to involve the regulatory effects of such cells. These include: the responses of mice to the T-independent pneumococcus polysaccharide type III antigen (43); the response to hapten-carrier conjugates in both the IgG (44,45) and IgE (46,47) classes of antibody; the phenomena of allotype (48) and idiotype (49) suppression; acquired hypogammaglobulinemia in humans (50); and the impaired capacity of newborn mice to produce antibody (51,52). This latter finding will be examined in more detail in the Discussion of this thesis as it relates directly to the investigations reported in this thesis.

f) Ir Gene Control of Immune Responsiveness

(i) Ir genes.

When Burnet (5) advanced the clonal selection theory, precursors of antibody-producing cells were thought to react with antigen through appropriate immunoglobulin receptors on their membranes, resulting in their differentiation into antibody-producing cells. A simple genetic control of specific immune responsiveness in a classical Mendelian pattern was unexpected, since antibody responses to multivalent antigens and even simple haptens were highly heterogeneous with respect to the structural variability of the antigen combining sites. However, it had been generally observed that some individuals of random bred populations did not

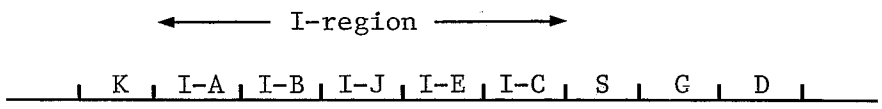
respond to specific antigenic challenges. This phenomenon of specific unresponsiveness was first studied in a systematic fashion by immunizing inbred strains of animals with immunogenic homopolymers or copolymers of L-amino acids. By using such relatively simple structures, the antigenic challenge presented to the animal was of sufficiently limited heterogeneity to allow the characterization of inbred strains or individual outbred animals as responders (R) or nonresponders (NR) (53).

Initial studies were on the responsiveness of random bred guinea pigs to dinitrophenylated-poly-L-lysine (DNP-PLL). Individual animals either responded with specific delayed type hypersensitivity (DTH) and a highly heterogeneous anti-DNP antibody response or did not respond (54). Breeding studies indicated that responsiveness to DNP-PLL was controlled by a single autosomal dominant gene which was designated an immune response gene or Ir gene (55,56). Responders were characterized by the ability to recognize PLL as a carrier and to produce antibodies to the DNP residue and other haptens coupled to PLL (57). On the other hand, nonresponders did not lack the ability to produce anti-DNP antibodies since these animals could so respond when challenged with DNP-PLL coupled to a normally immunogenic T-dependent carrier such as bovine serum albumin (58). Therefore, it appeared that the genetic control was related to the ability to recognize the PLL carrier as an immunogen.

McDevitt and Sela (59) studied the responsiveness of inbred strains of mice to synthetic polymers of amino acids, i.e. (T,G)-A--L, (Phe,G)-A--L and (H,G)-A--L. They demonstrated that responsiveness to such antigens was controlled by dominant single genes which were closely linked to the H-2 complex — the major histocompatibility complex (MHC) in the mouse (60). The Ir gene controlling responsiveness to (T,G)-A--L was mapped

into a region of the H-2 complex between the K and S regions and was designated the I region (61,62). Analogous I regions were described in the guinea pig (63) and the rhesus monkey (64).

Based on the studies of Ir gene control of responsiveness to many different antigens, the I region of the mouse has been divided into several distinct subregions, designated I-A, I-B, etc., and is schematically presented below:



Ir gene control for most of the responses studied have mapped in the I-A subregion, others in the I-B subregion, whereas control for some responses requiring complementing genes have mapped in both the I-A and I-C subregions.

Much of the initial work on the mapping of Ir genes involved the responses to antigens which were structurally relatively simple, i.e. PLL, GT and GAT. Recently, reports have described Ir gene control of the responsiveness of several strains of mice to the relatively more complex antigen, sperm whale myoglobin (65,66), an antigen for which five antigenic determinants have been well characterized. These determinants were sequenced, synthesized and used for *in vitro* challenging of cells from mice initially primed with intact myoglobin. The response pattern to the different antigenic determinants was found to depend on the particular H-2 haplotype expressed. For example, mice bearing the H-2^d haplotype responded to determinants 1,2,3 and 5, whereas haplotypes H-2^a and H-2^f correlated with responsiveness to determinants 1,2 and 3 but not 5. All were classified as high responders. Low responder mice with H-2^b,

$H\text{-}2^k$ and $H\text{-}2^r$ haplotypes responded only to determinant 4. Analysis of the data indicated that control of responsiveness to determinants 1,2,3 and 5 mapped in the I-A subregion, whereas the Ir gene controlling responsiveness to site 4 was in the I-C subregion. Thus, it could be concluded that different Ir genes controlled responsiveness to different regions of the myoglobin molecule and that the response to any given determinant was determined by the presence or absence of the particular Ir gene involved.

Although in many cases it appears that a single Ir gene is involved in responsiveness, there are some documented cases where two genes are apparently required. One example of such dual gene control is the response of mice to the synthetic terpolymer GLØ. It was demonstrated that the mating of two nonresponder strains could produce an F_1 hybrid with high responsiveness to this antigen. Furthermore, some recombinant strains derived from nonresponder mice were GLØ responders. Although the nonresponders clearly had one or the other GLØ Ir genes, both were required for responsiveness. Mapping studies located one gene in the I-A subregion and the other in the I-C subregion (67).

(ii) Is genes.

From the initial studies of Gershon and Kondo (42) and Cantor and and Boyse (68), it became evident that T cells regulated immune responses not only through the generation of helper function, but by the stimulation of antigen specific T cells which could modulate or suppress the immune response. Such suppressor T cells were shown to be distinct from helper T cells (69). These discoveries raised the question of whether specific suppressor T cell functions were controlled by I region genes

in a manner similar to the Ir gene control of helper function. This question was addressed by Benacerraf and co-workers (70), who studied the response of mice to a copolymer of L-glutamic acid and L-lysine (GT). It was found that GT was not immunogenic in several different inbred strains of mice bearing different H-2 haplotypes, although some individual random-bred Swiss mice were capable of giving a primary anti-GT IgG PFC response. All the inbred strains investigated, however, could respond to GT complexed to methylated bovine serum albumin (GT-MBSA). It was further shown that for some but not all of the mouse strains tested, preimmunization with GT resulted in a marked diminution of the ability of such mice to respond subsequently to GT-MBSA. This effect of GT pretreatment was shown to be antigen specific, since the responsiveness to unrelated antigens was unaffected, and related to the development of active suppressor T cells. Although the unresponsiveness to GT was shown to be due to the stimulation of suppressor cells in some inbred strains of mice ($H\text{-}2^k$, $H\text{-}2^d$ or $H\text{-}2^s$), this was found not to be the case in others ($H\text{-}2^a$ or $H\text{-}2^b$). Thus, based on the ability to respond to GT preimmunization with the activation of suppressor T cells, the inbred strains of mice were classified as "suppressors" or nonsuppressors". It was subsequently found that the capacity to develop GT-induced suppression of GT-MBSA responses was inherited as a dominant trait in (suppressor x nonsuppressor)F_1 hybrids, and that the gene or genes, referred to as Is genes, mapped to the I region of the MHC between the K and D regions. Moreover, GT-specific suppression was found to be controlled by two complementing Is genes, since F_1 hybrids of two GT-nonsuppressor haplotypes demonstrated GT-induced suppression of GT-MBSA responses (71). Thus, as with complementing Ir genes, Is genes also demonstrate comple-

mentation for some antigens. The exact relationship between the genes controlling helper or suppressor T cell responses has not yet been resolved.

(iii) The Ia antigens.

In order to analyze the mechanisms by which Ir genes exert their effects in immune recognition, attempts were made to define the phenotypic manifestations of such genes. To this end, alloantisera were prepared by the reciprocal immunization of mouse strains which were known to differ genetically only in the I region. This resulted in the serological definition of a series of antigens, designated Ia, of which many specificities were characterized (72). Ia antigens were subsequently detected on B cells (73), macrophages (74,75) and T cell helper factors (76,77), and the loci coding for these specificities have been mapped to the I-A, I-C and I-E subregions. The I-J subregion has been found to code for specificities on suppressor T cells (78) and their soluble factors (79-81).

It was found that the expression of certain Ia specificities by an individual could be correlated with the ability to mount a particular antibody response. In addition, antisera directed to these Ia specificities could block or inhibit the appropriate immune response. Such observations suggested a close association (or identity) between the Ir genes and the genes coding for the Ia specificities.

g) Ir Gene Control of Cellular Interactions

As the genetic restrictions of T cell responses to thymus-dependent antigens were being discovered, a series of investigations addressed the concept of I region gene control of cellular interactions.

(i) Macrophage-T cell interactions.

Rosenthal and Shevach (82), Shevach *et al.* (83) and Shevach (84) investigated the interrelationships between Ir gene control of responsiveness and macrophage-T cell interactions. They compared the ability of macrophages isolated from responder and nonresponder guinea pigs to effectively present antigen to sensitized T cells. Thus, using DNP-GL as antigen, for which strain 2 guinea pigs but not strain 13 are responders, (2 x 13)F_1 T cells could be stimulated if DNP-GL was presented on strain 2 but not strain 13 macrophages. Alternatively, using GT as antigen, macrophages from the responder strain 13 were shown to effectively stimulate (2 x 13)F_1 T cells. GT-macrophages from the nonresponder strain 2 could not. Subsequently, Shevach *et al.* (85) demonstrated that effective stimulation by antigen-pulsed responder macrophages could be inhibited by using the appropriate anti-Ia allosera, thus correlating Ir gene control and Ia specificities with macrophage-T cell interactions. Studies in the mouse also substantiate this correlation. Erb and Feldmann (86) demonstrated that helper T cells which had been primed with syngeneic macrophages and antigen could provide helper function to B cells in subsequent culture only if the antigen was presented with macrophages which were identical in the I-A subregion. Similarly, Pierce *et al.* (87) were able to show that syngeneic or allogeneic GAT-pulsed macrophages were equally able to elicit primary anti-GAT IgG PFC responses *in vitro*. However, spleen cells from mice primed with either syngeneic or allogeneic GAT-pulsed macrophages could respond in subsequent culture only if the antigen was presented on the same type of macrophages as that used for priming. Pierce and Kapp (quoted in Ref. 88) further demonstrated that the inability to respond to antigen presented on macrophages other than

those used for the initial *in vivo* priming was due to the generation of GAT-specific suppressor T cells. In addition, spleen cells from (responder x nonresponder)F_1 responder mice immunized *in vivo* with GAT were shown to be responsive, in subsequent culture, only to GAT on F_1 or responder macrophages, but not to GAT on nonresponder macrophages. These results indicated that following immunization with GAT *in vivo*, the F_1 macrophages were able to present GAT in an immunogenic form only in conjunction with the Ir gene products or Ia determinants present on their cell membrane which were derived from the responder and not from the nonresponder genotype.

In summary, it has been concluded from this data that the immunogenic presentation of antigen by macrophages is under Ir gene control. In conjunction with suitable antigenic determinants, Ir gene products or Ia antigens must be recognized by the responding T cell population in order to generate helper function; the lack of this dual presentation can result in suppressor T cell activation. Thus, the stimulation of helper and/or suppressor T cells largely depends on the presence or absence of appropriate macrophages. This concept of a balance between activation of helper or suppressor T cells has recently been supported by Pierres and Germain (89), who reported that "macrophage depleted" responder mice responded to GAT predominantly with the activation of suppressor T cells.

(ii) T-B interactions.

The role of Ir genes in T-B collaboration has also been investigated. Katz and co-workers (90,91) using antigens for which the response had been shown to be under Ir gene control demonstrated that (responder x nonresponder)F_1 helper T cells interacted efficiently with responder but

not nonresponder hapten-primed B cells and that this restriction of T-B collaboration was controlled by genes which mapped with the Ir genes controlling responsiveness to the antigens used, thus indicating a critical role for the Ir genes in this process.

h) Models for Ir Gene Function

There are currently two models to account for the functions of Ir genes. Since each model can be supported by a great deal of evidence, both are probably correct and represent two distinct mechanisms for I region control of specific immune responses.

The first model postulates that Ir genes control Ia molecules on macrophages and B cells. These Ia molecules are capable of interacting with T dependent antigens to form a complex which can be recognized by T cells. For effective triggering of specific T cells, both antigen and Ia must be recognized. Thus, an animal whose macrophages possess Ia determinants which cannot form an appropriate complex with a particular antigen will be a nonresponder to that antigen. This antigen will preferentially stimulate suppressor cells, providing no genetic defect (i.e. nonsuppressor genotype) exists in the ability to generate these cells.

This model further postulates that the same Ia specificities expressed on macrophages are also expressed on B cells. Thus, antigen-Ia complexes on the B cell could serve as a target for primed T cells which were activated by the identical antigen-Ia complex on a macrophage. Therefore, the genetic restrictions seen in secondary antibody responses could well be a reflection of the restrictions occurring during the initial T cell priming.

The second model postulates that Ir genes are primarily expressed

on T cells, and that helper and suppressor T cell factors bear determinants coded by the I-A and I-J subregions, respectively. An immune defect can be determined by the I region control of: (i) the ability to produce suppressor factors (79,92), (ii) the inability to produce specific helper factors (76,77) or (iii) the presence of B cells which are unable to recognize T cell derived factors (76,77).

i) Cellular Mechanisms for Antibody Formation

As information became available concerning the structural requirements for immunogenicity, the types of cells involved in antigen recognition and processing, and the genetic restrictions involved in the induction of immunity, many mechanisms were proposed to describe the cellular aspects of antibody production. In general, these can be subdivided into two broad categories: (i) those requiring cell-to-cell contact and (ii) those involving soluble mediators.

(i) Cell-to-cell contact. One of the first models for T-B cell collaboration stemmed from the work of Mitchison (37) and others concerning the antibody responses to hapten-carrier conjugates. The T cell was envisaged in an antigen-focussing role due to its ability to specifically combine with carrier determinants and thereby direct haptenic determinants towards the B cell. The observations that both haptenic and carrier determinants had to be on the same molecule and that primed T cells were radioresistant suggested that cell contact was necessary for the rather passive display of bound haptenic determinants on the surface of the T cell.

A more sophisticated version of this model has been advanced by Katz and colleagues (93,94), while studying the genetic restrictions of T-B

cell interactions. They demonstrated that successful T-B cell collaboration could occur between allogeneic T and B cells only if the cell donors were compatible in the I region of the MHC. Cells from mice with differences mapping outside the I region were found to act synergistically as well as cells from syngeneic donors. In order to account for this, they proposed the existence of cellular interaction genes which mapped in the I region of the MHC. These genes are expressed on the membranes of both cell types and identity of such gene products is required for effective collaboration, either by direct physical contact or by membrane interactions at a very close distance.

(ii) Models involving soluble factors. One of the first models which implicated the action of a soluble factor in the activation of B cells was based on what was to become known as the allogeneic effect. While investigating the response of guinea pigs to hapten-carrier conjugates, Katz and co-workers (95) observed that the well known carrier effect could be overcome by the injection of allogeneic immunocompetent cells. Thus, animals primed with DNP-carrier$_1$ would normally produce a secondary response to DNP if challenged with the homologous DNP-carrier$_1$ conjugate, but not with a heterologous conjugate DNP-carrier$_2$ (the carrier effect). Injection of allogeneic cells with the heterologous conjugate DNP-carrier$_2$ subsequently resulted in a high anti-DNP response (the allogeneic effect). Moreover, supernatants of allogeneic cell cultures were able to substitute for T cells in generating helper function. Such factors were capable of providing help for any antigen-primed B cells and were, therefore, not antigen specific in their action. They were not immunoglobulin in nature but possessed determinants coded by the I region of the MHC (96). Although this model is based on observations produced under rather artificial conditions, such as the injection of allogeneic cells, it is pro-

posed that the factors elaborated by T cells in response to carrier determinants on an immunogen will be the same as those elaborated in response to allogeneic determinants.

A second model involving soluble factors was proposed by Feldmann and co-workers following the study of *in vitro* antibody responses in which the cell populations under study were physically separated from each other by cell-impermeable membranes. By placing carrier-primed T cells in one compartment, hapten-primed B cells in the second compartment and hapten-carrier antigen in both, they demonstrated that anti-hapten antibody could be produced under conditions where no direct cell contact was possible (97). The action of a soluble helper factor derived from the T cells was proposed. Moreover, this factor exhibited antigen (carrier) specificity since it was shown that for an effective anti-hapten response to be produced, the T cells had to be primed with the same hapten-carrier conjugate as the B cells (98). Furthermore, when carrier-primed T cells plus antigen and macrophages were incubated on opposite sides of a cell impermeable membrane, the macrophages could subsequently trigger B cells to produce antibody (99). The soluble factor was absorbed by anti-immunoglobulin and since it was derived from T cells was termed IgT. In this model, antigen was thought to react with T cells which shed antigen-IgT complexes that became associated with macrophages. The macrophages, in turn, presented the antigen to the appropriate B cells.

More recent models involving T cell soluble factors have relied heavily on the ability to distinguish T cell subpopulations on the basis of membrane expressed alloantigens, of which the thymus leukemia (TL) and Ly antigen systems are very prominent. Cantor and Boyse (100) with the use of appropriately absorbed antisera revealed that about 90% of

mouse thymocytes carry three Ly antigens, whereas in the spleen, approximately 45% of the cells expressed some form of Ly antigen. The TL antigen which was normally found on cortisone-sensitive cortical thymocytes, was generally thought to be a marker for the immediate progenitors of the functionally mature peripheral T lymphocyte (101-103). The serological detection of these alloantigens on T cells allowed a refinement of the older but still widely used classification of T lymphocytes proposed by Raff and Cantor (104). Thus, the antigen independent differentiation process of T cell ontogeny was thought to proceed from the TL^+ Ly-1,2,3^+ thymocyte (T_0), through the TL^- Ly-1,2,3^+ stage (T_1) to the Ly-1^+ or Ly-2,3^+ peripheral T lymphocyte (T_2):

$$T_0 \longrightarrow T_1 \longrightarrow T_2$$

TL^+Ly-1,2,3^+ TL^-Ly-1,2,3^+ TL^-Ly-1^+ or TL^-Ly-2,3^+

Whereas T_1 cells were found to be short-lived, spleen-seeking and anti-thymocyte serum (ATS) resistant, the T_2 category described cells which were long-lived, recirculating and ATS sensitive. Operationally, only T_1 cells were shown to be present in mice two days after injection of ATS, and only T_2 cells eight weeks after thymectomy. T_1 and T_2 cells could also be separated on the basis of adherence properties to nylon wool; the former being adherent and the latter, nonadherent. In recent years, Tada (105-109) and Feldmann (110-115) and their colleagues have independently studied the relationships between these cell types, the soluble factors elicited by them, and their roles in the regulation of antibody induction and synthesis. The combined data of these investigators are represented in the scheme illustrated in Figure A. The central

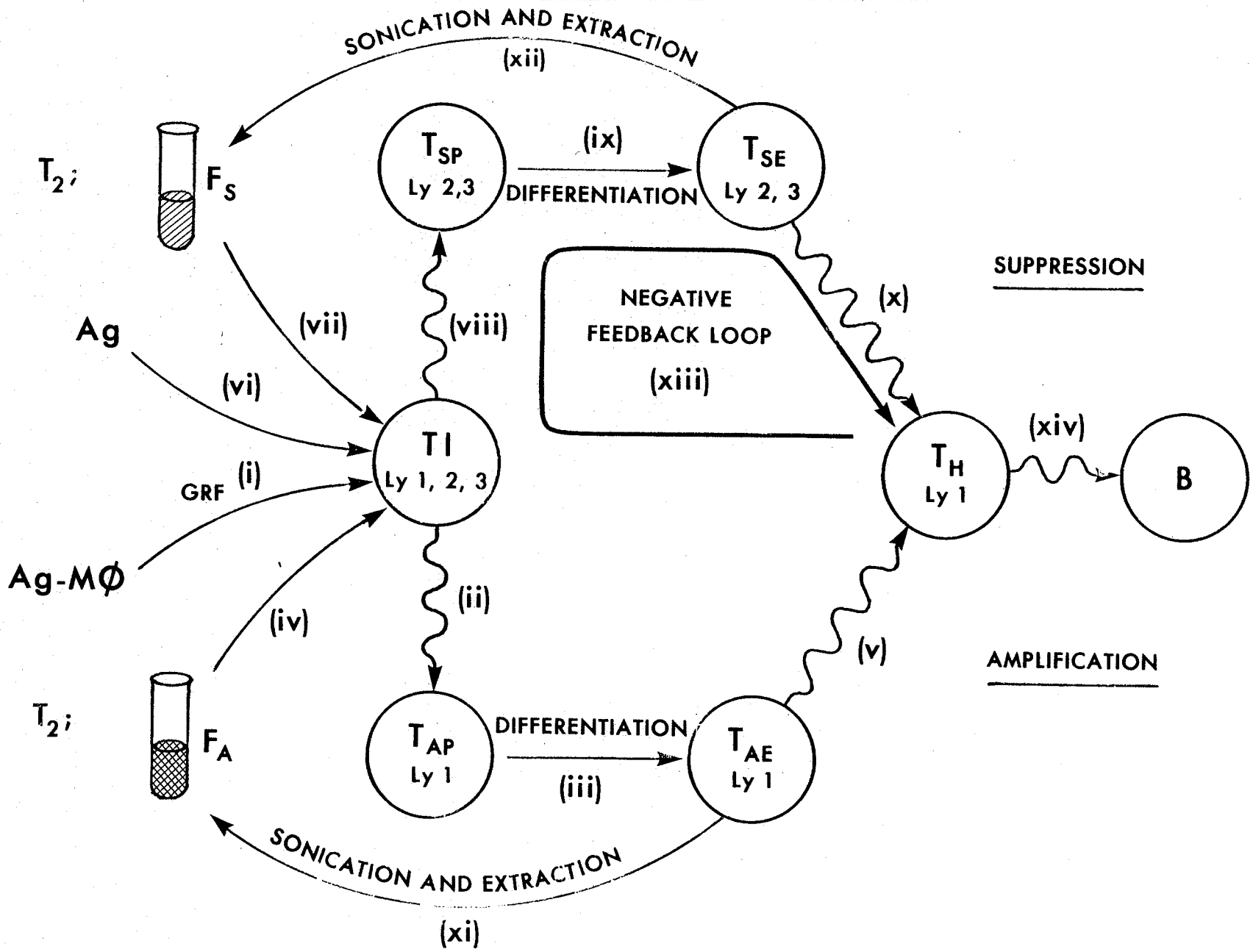


FIGURE A: AMPLIFICATION AND SUPPRESSION OF T HELP

cell in this scheme is the TL^- Ly-1,2,3^+ cell defined as the T_1 or adherent cell by Feldmann and Tada, respectively. When this cell encounters macrophage-processed antigen (see (i) in Figure A), it is thought to secrete a soluble factor (ii) which causes the differentiation (iii) of T amplifier precursor cells (T_{AP}) to T amplifier effector cells (T_{AE}), both of which are Ly-1^+. The interaction of macrophage-associated antigen with T_1 cells is genetically restricted inasmuch as both the macrophages and T_1 cells must have identical I-A (or I-C) genotypes. This interaction is mediated through a soluble factor designated "genetically restricted factor" (GRF). T_1 cells can also be activated by a soluble factor obtained by sonication of Ly-1^+ cells. This amplifying factor's (F_A) interaction with T_1 cells (iv) is antigen-specific and genetically restricted since both the cells producing this factor (Ly-1^+) and accepting it (Ly-1,2,3^+) must have been primed with the same antigen and possess identical I-A (or I-C) genotypes. Once formed, the T_{AE} cells augment the activity of helper T cells (T_H) by the action of a soluble factor which is neither antigen specific nor genetically restricted (v). The T_H cells then react with B cells in an antigen-specific manner by the elaboration of yet another soluble factor (xiv).

A suppressive counterpart to this amplification phenomenon has also been delineated. Thus, if unprocessed antigen (vi) or a suppressor factor (F_S) isolated from Ly-2,3^+ cells (vii) interacts with T_1 cells, a factor is produced (viii) which causes the differentiation (ix) of Ly-2,3^+ suppressor cell precursors (T_{SP}) into Ly-2,3^+ suppressor effector cells (T_{SE}). These cells, in turn, regulate the population of primed helper cells through the action of a soluble factor which acts in an antigen-nonspecific and genetically unrestricted fashion (x). Although this nonspecific

factor may be produced by the same cells (T_{SE}) which yield F_S, these two factors are clearly different. In contrast with the nonspecific factor (x), the interaction of F_S with T_1 cells (vii) has been shown to be antigen specific and genetically restricted since the cells producing it (Ly-2,3$^+$ and accepting it (Ly-1,2,3$^+$) must have been primed with the same antigen and sharedidentical I-J genotypes.

If the sources of F_A and F_S are the T_{AE} and T_{SE} cells as shown (xi and xii), this sytem would have the potential, following antigenic challenge, for continuous amplification of the immune response. Thus, T_{AE} and T_{SE} cells could produce factors for the generation of more T_{AE} and T_{SE} cells, respectively, thereby resulting in a system lacking homeostatic regulation. However, Gershon, Cantor and colleagues (116,117) have recently demonstrated that if primed T_H cells were added to a population of unfractionated normal T cells, the normal T cells developed, through an Ly-1,2,3$^+$ T cell, a very potent suppressor activity. This observation, therefore, allows one to picture a feedback mechanism whereby helper T cells can induce the formation of suppressor T cells, thereby resulting in homeostatic regulation (xiii).

(iii) Summary

In summary, several mechanisms have been proposed to describe the cellular interactions which are known to be necessary for the induction of an antibody response. Some stress the requirements of cell-cell contact while others describe the role of various soluble factors produced by the cells involved. The relative complexities of some of the current models can be considered a direct result of recent technological advances in the general area of *in vitro* cell culturing, immunochemistry

and immunogenetics. At present, there is no reason to believe that any one of the mechanisms proposed is operational to the exclusion of the others, or that new mechanisms will not be forthcoming. Undoubtedly, they all reflect different aspects of a multifaceted and complex physiological phenomenon.

The model in Figure A, describing the possible interaction of T cells and their factors, does take into consideration one aspect that must be considered vital to any proposed mechanism of antibody regulation; that is, the concept of homeostatic regulation. Both positive and negative feedback loops have been proposed, however, one reservation should be introduced at this time concerning the physiological role played by factors F_A and F_S. To date, their existence has been demonstrated only through the artificial means of cell extraction. It remains to be demonstrated that they can be naturally secreted by the amplifier and suppressor effector cells and, therefore, be considered relevant factors in the physiological milieu of cellular interactions.

II. ONTOGENY OF LYMPHOCYTES

a) Introduction

In the latter 1960's and early 1970's, as the mechanisms and cellular requirements of antibody formation were gradually being revealed, a large number of investigators turned to studying the general area of the ontogeny of lymphocytes. It was generally felt that this would provide a better understanding of the cellular mechanisms of immunity. In addition, an insight would be gained into the more general and fundamental phenomena of cellular differentiation, which control the pro-

gressive generation of mature, highly specialized cells from pluripotent stem cells.

It is now well documented that stem cells can be found in embryonic tissues, such as the yolk sac and liver (118-120) or the bone marrow of adults (121). Following migration to primary lymphoid organs, these cells are induced to differentiate into unipotential lymphoid cells. In avian species, the primary lymphoid organs have been defined as the bursa of Fabricius and the thymus, which give rise to B cells and T cells, respectively (8,122,123). In mammals, no distinct bursal equivalent has been found. Available evidence indicates that, in contrast to birds, the development of B cells in mammals cannot be anatomically associated with a single organ but rather appears to be multifocal in nature (124, 125). As with birds, the thymus in mammals is the site of generation of the T cell line of lymphocytes (118).

By and large, the study of the differentiative events leading to the formation of fully mature immunocompetent lymphocytes has relied heavily on the discovery and serological definition of plasma membrane antigens. Many surface antigens have been characterized and have allowed the serological distinction of B cells and T cells. For example, the TL (thymus leukemia), Ly-1, 2, 3,5, MSLA (mouse-specific lymphocyte antigen), Thy-1 and G_{IX} (Gross virus) antigens have been found to be expressed on T cells and not B cells, although the Thy-1 and G_{IX} markers do occur on some types of nonlymphoid cells. Cells of the B cell category are characterized by the presence of readily detectable amounts of surface Ig and several other markers including the alloantigens Ly-4,6,7, the heteroantigens, mouse-specific B lymphocyte antigen (MBLA) and mouse-specific plasma cell antigen (MSPCA) and the receptor for the

third component of complement or complement receptor (CR). In addition, some groups of antigens have been found on both B cells and T cells and include antigens controlled by the K and D regions of the MHC, Ia antigens and Fc receptors (FcR) (126).

The appearance (or disappearance) of these cell markers on lymphoid cells has allowed the delineation of the ontogenic development of both B and T cell lines and the definition of discrete stages in their respective development. In certain cases, the presence of particular markers has been directly correlated with specific immunological function, such as the different Ly phenotypes of helper and suppressor T cells described in the previous section; whereas the presence of other markers, such as TL, identify relatively immature cells. Some of the recent developments describing the ontogenic development of lymphocytes will now be described.

b) The Ontogeny of T Cells

(i) Conversion of prothymocytes to thymocytes

The precise mechanism whereby the thymus influences the differentiation of stem cells to thymocytes is not known, however, considerable evidence implicates the action of thymic hormone-like factors (127-130). One such factor is thymopoietin, a polypeptide hormone produced by the epithelial cells of the thymus. Komuro and Boyse (131,132) and Basch and Goldstein (128) demonstrated the ability of thymopoietin to induce, *in vitro*, the expression of markers characteristic of mature T cells, by acting on precursor cells or prothymocytes lacking such markers. Such inducible cells were found in the bone marrow and spleens of normal or athymic nude mice (133) and in the liver of 18-day-old embryos (132).

The presence of prothymocytes in athymic mice suggested that their differentiation from stem cells was not dependent on the presence of a thymus. The *in vitro* induction of prothymocytes by thymopoietin is accompanied with an increase in intracellular cyclic-AMP (134), and results in the differentiation of prothymocytes into phenotypically defined thymocytes. This conversion process occurs in the absence of cell proliferation, is very rapid, being complete in 2.5 hours (131), and requires transcription of DNA and translation of RNA but not replication of DNA (135). In addition, the generation of T cell markers by thymopoietin is accompanied by the acquisition of responsiveness to the T cell mitogens Con A and PHA but not to the B cell mitogen LPS (136). Taken together, these findings suggest that the prothymocyte found in fetal liver and adult bone marrow or spleen is precommitted to T cell development before migration to the thymus, and is discrete from the cells which are the immediate precursors of B lymphocytes.

(ii) Intrathymic differentiation

The early differentiative events which occur in the thymus were initially described by Owen and Raff (120,137), and subsequently by others. Studies in fetal mice demonstrated that large cells devoid of the TL, Thy-1, Ly and G_{IX} markers entered the thymic cortex at about day 11 of gestation. By day 18, most of the cells in the thymus were TL^+, Thy-1^+, Ly-$1,2,3^+$, H-2^+ and G_{IX}^+ (137-139). During subsequent differentiation, these cortical thymocytes were thought to form another population of cells which were TL^-, G_{IX}^-, Ia^+, expressed lower amounts of Thy-1, higher amounts of H-2 antigens, expressed one of three Ly phenotypes (Ly-$1,2,3^+$, Ly-1^+ or Ly-$2,3^+$), and resided predominantly in the medullary regions of the thymus. These transformations are summarized

in Figure B. In contrast to the cortical cells which accounted for 85% of all thymocytes, were large, frequently dividing and cortisone-sensitive, the medullary thymocytes tended to be medium-sized, nondividing and cortisone resistant. It is thought that these medullary thymocytes, which represented 10-15% of the thymocyte population, were the immediate progenitors of the functionally mature peripheral T lymphocytes (101, 140-142). At some point, they migrated from the thymus to the peripheral lymphoid organs (143-146).

A number of investigators have analyzed the immune potential of fetal and neonatal thymocytes by determining their responsiveness to T cell mitogens. Stobo and Paul (147) demonstrated that cortisone-resistant thymocytes from neonatal mice, which expressed relatively low quantities of Thy-1 antigen (medullary thymocytes), could respond to PHA. Mosier (148) reported that fetal mouse thymocytes could respond to PHA, Con A and PWM between days 16 and 18 of gestation. Thus, thymocytes were shown to achieve immunocompetence, at least to some degree, while within the thymus, prior to migration to the peripheral lymphoid organs.

(iii) Peripheral T cells

The seeding of the spleen by the thymus has been shown to begin in the mouse shortly after birth (121,149-151). Thus, thymectomy immediately following birth resulted in the subsequent inability to develop T cell dependent functions, such as antibody production to thymus dependent antigens, delayed hypersensitivity and homograft rejection (152). Chison and Golub (153) analyzed T cell function in newborn mice by determining the ability of such cells to interact with adult bone marrow to produce anti-SRC responses in irradiated recipients. They reported that at birth, T cell function in the thymus was less than 10% of adult

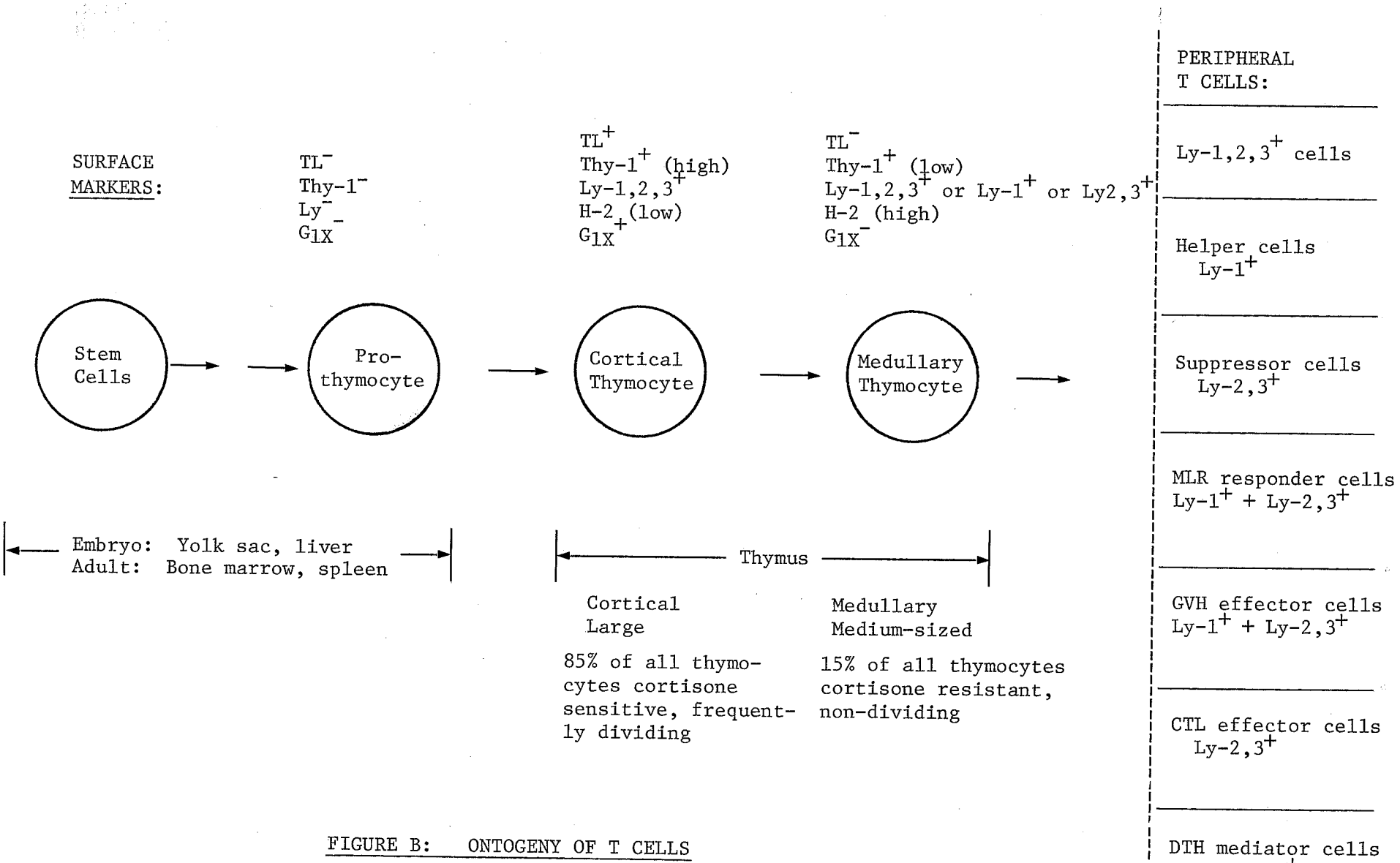


FIGURE B: ONTOGENY OF T CELLS

levels but increased to these levels within 48 hours. However, no helper T cell function was detectable in neonatal spleens until four days after birth, at which time such function became readily detectable.

An analysis of peripheral T cells has revealed a large degree of heterogeneity, evident both in terms of functional criteria and surface markers. Cantor and Boyse (100), using specific anti-Ly antisera, classified T lymphocytes into three broad categories: Ly-1,2,3$^+$, Ly-1$^+$ and Ly-2,3$^+$. They reported that virtually all Ly$^+$ spleen cells in mice one week after birth were of the Ly-1,2,3$^+$ category, whereas Ly-1$^+$ and Ly-2,3$^+$ cells were undetectable in neonatal life but increased gradually thereafter. These observations suggested that the Ly-1,2,3$^+$ cells were the immediate precursors of Ly-1$^+$ and Ly-2,3$^+$ cells in the normal antigen-independent differentiative processes described here. The Ly phenotypes of several categories of T cells have been determined and are summarized in Figure B.

c) The Ontogeny of B Cells

(i) General aspects of B cell ontogeny in birds and mammals

The ontogeny of B cells has been investigated extensively in both birds and mammals. In birds, a vital association has been demonstrated between the bursa of Fabricius and the development of B lymphocytes from stem cells (154). Thus, Cooper *et al.* (10) demonstrated that embryonic bursectomy resulted in agammaglobulinemia and a lack of identifiable B cells and plasma cells in the peripheral lymphoid tissues. During early embryonic development, hematopoietic stem cells have been shown to migrate from the yolk sac via the blood stream to the bursa, starting at the twelfth day of incubation; in later stages, the spleen and bone marrow apparently serve as a source of stem cells (155,155a). Within

two days (day 14), IgM-bearing cells can be detected in the bursa, whereas IgG- and IgA-bearing cells appear later in the course of development (day 21, hatching) (156). The finding that the administration of anti-μ during early embryonic life prevented the development of IgG- and IgA- as well as IgM-producing cells led to the concept of a sequential development of B cells, with the early-appearing IgM-bearing cells subsequently giving rise to cells expressing IgG and IgA (157). This sequence of heavy chain expression appeared not to be affected by antigenic challenge (156), suggesting that these differentiational changes in the bursal environment were antigen independent. Subsequent to the maturational influences of the bursa, the Ig^+ B cells migrate to the peripheral lymphoid organs (158). Since mature antibody-producing cells are not found in the bursa (159,160), it is likely that further maturational events occur in the periphery which result in the generation of fully reactive B cells.

In contrast to the situation in birds, the maturational development of B cells in mammals has not been associated with an anatomically distinct organ equivalent to the avian bursa. Since the bursa arises in embryonic development from the bird gut, investigators were led to examine lymphoid tissues associated with the gut as a possible mammalian equivalent. However, Silverstein has demonstrated that removal of the upper and lower intestinal tracts from fetal lambs at a time when no mature B cells were present, did not prevent the subsequent development of such cells (161).

In recent years, evidence has been obtained to indicate that the formation of phenotypically descernable B cells can occur from precursor cells in several different locations in the neonatal mouse. Nossal and Pike (124) using autoradiographic techniques, demonstrated the multi-

focal origin of B cells in CBA mouse embyros at all the major sites of erythromyelopoiesis, including the liver, spleen and bone marrow, commencing three days before birth. Moreover, liver cells taken from mice at 13 days of gestation, i.e. 3-4 days before the detection of surface Ig, were shown to be able to repopulate the B cell compartment of adult irradiated recipients. The mesenteric lymph nodes of fetal mice were essentially alymphoid, however, increasing numbers of first T and then B cells were found in these nodes shortly after birth. These findings demonstrated that the lymph nodes acquired B lymphocytes significantly later than the sites of erythomyelopoiesis, suggesting that B cells were not generated locally but were seeded into the nodes.

The multifocal nature of B cell generation was also demonstrated by Owen, Raff and Cooper (125,162). In their experiments, embryonic tissues were cultured *in vitro* and analyzed for cells expressing B cell markers including surface Ig, FcR and MBLA. They reported that organ explant cultures of fetal liver and spleen, not containing any phenotypically distinguishable B cells, developed such cells after 4-7 days of *in vitro* incubation. These results demonstrated that B cell precursors were not restricted to one anatomical site, thereby supporting the concept of a multifocal development of mammalian B cells.

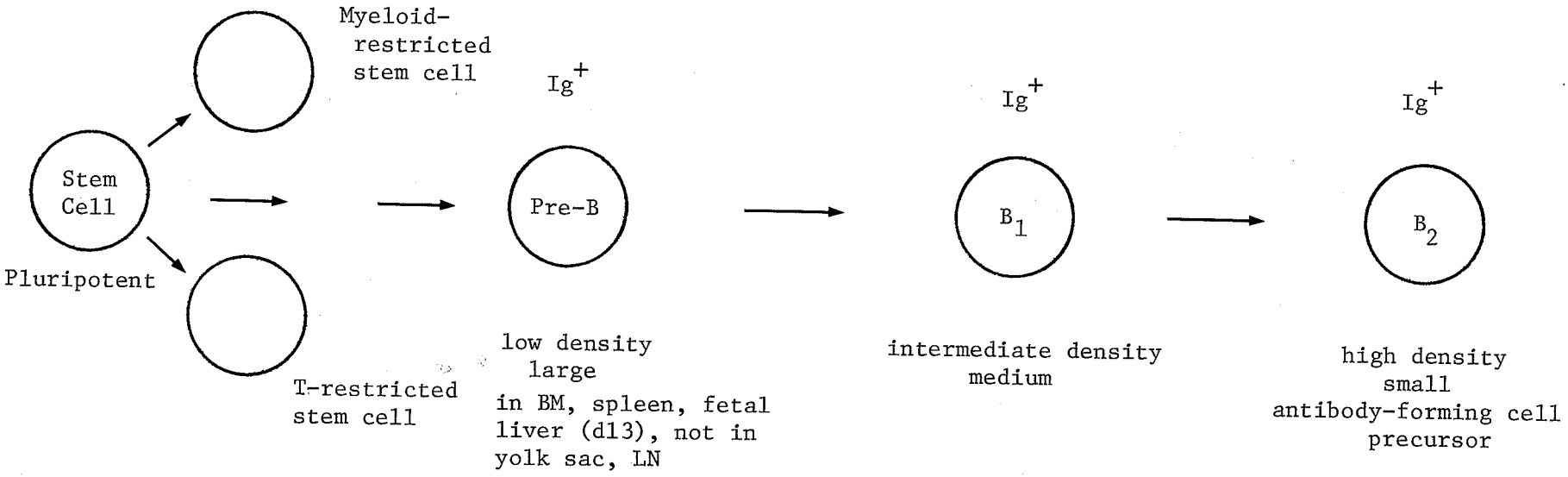
The ontogenic development of B cells is an area which has been investigated by a number of workers in recent years utilizing a variety of approaches. The general area of stem cell definition and differentiation has been studied using chromosome markers, whereas later stages of B cell differentiation have been defined by changes in physical properties such as size and density, the acquisition of cell surface markers and, lastly, immunological function. Several discrete stages have been id-

entified independently and by different investigators and an attempt to summarize these is presented in Figure C. Some stages have been defined in fetal and neonatal mice, whereas others have been observed in adults. The phenomenon of B cell differentiation therefore reflects not only the early stages of ontogenetic development, but also the on-going self-renewal processes which occur throughout life.

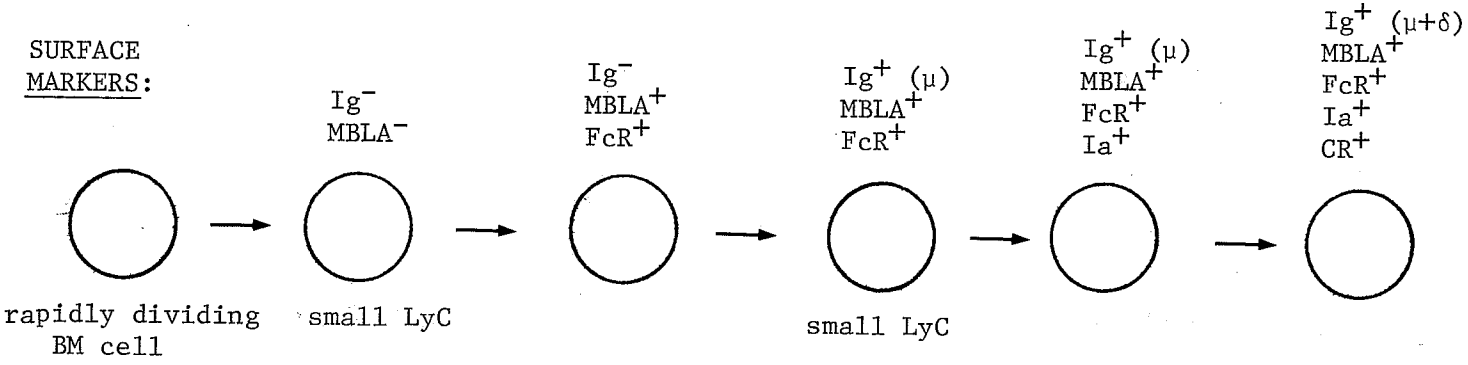
(ii) Definition of stem cells

Studies on the early stages of B cell development from stem cells in the mouse were originally concerned with a possible connection (or identity) of stem cells for the myeloid and lymphoid cell systems. Thus, stem cells when injected into irradiated recipients, gave rise to discrete nodules or cell colonies in the spleen. Analysis of such cell colonies demonstrated the presence of all myeloid cell types, however, the cells found in any one colony were predominantly of only one cell type (163). The original cell giving rise to these spleen colonies was termed a colony forming unit-spleen (CFU-S) and was considered to be functionally equivalent or identical with the pluripotent stem cell. It is noteworthy that no lymphoid cells were reported in such spleen colonies. However, indirect evidence has been reported to suggest that CFU-S functioned as stem cells for the lymphoid system (164,165). It was observed that cells from spleen colonies, bearing a chromosomal marker, could repopulate the lymphoid cell compartments of irradiated recipients. At least some of these lymphoid cells were shown to possess the chromosomal marker, indicating they were of donor and not host origin.

Recently, Abramson *et al.* (166) described a study intended to define pluripotent stem cells and stem cells which, following differentiation were committed to a particular cell line. Bone marrow cells were irra-



Lafleur, Melchers, Miller, Phillips (167-171)



Ryser and Vassalli (181)

Sidman and Unanue (183)

FIGURE C: ONTOGENY OF B CELLS

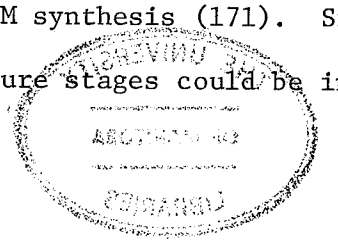
diated to induce chromosome markers and limiting numbers of such cells were injected into lightly irradiated recipients possessing the W/W^V genotype; mice of this genotype have a macrocytic anemia resulting from defective CFU-S. After allowing sufficient time for the small number of stem cells to repopulate the recipients, the animals were sacrificed and their cells analyzed for chromosomal markers. The presence of chromosomal markers in the myeloid cell line was established by transfer of bone marrow cells from these mice to a second group of irradiated recipients and searching for markers in the resultant spleen colonies. Markers in the B or T cell lines were assayed by culturing spleen lymphocytes with either LPS or PHA, respectively, and analyzing the chromosomes of the resultant blast cells. It was reported that some particular chromosome markers were found associated with all three cell types, i.e. myeloid cells, B and T cells. This was interpreted as direct evidence for the existence of a pluripotent stem cell which could give rise to all three cell types. Other characteristic markers were detected only in CFU-S and not LPS- or PHA-stimulated blasts. Still other markers were found in PHA-induced blasts and not in LPS cultures or CFU-S. These latter two observations suggested the existence of stem cells which were no longer pluripotent but could only give rise to myeloid or T cell lines, respectively. No markers were found which were restricted to the B lymphocyte cell line. The regenerating properties of the three types of stem cells were demonstrated by transferring bone marrow cells from the originally reconstituted irradiated mice to a second set of irradiated recipients. The distribution patterns for particular chromosome markers defined in the first set of irradiated recipients were preserved in the secondary recipients, thus confirming that the markers were indeed in-

duced in cells with stem cell properties.

In summary, the authors proposed the existence of a pluripotent stem cell pool from which the precursors of B cells, T cells and myeloid cells are derived. Furthermore, a stage of differentiation was defined at which pluripotent stem cells became committed to either the T cell or myeloid cell lineage, while still retaining the self-renewal properties of stem cells. Stem cells which were committed to B cell development were not detected but their existence was not ruled out. These findings are schematically summarized in Figure C.

(iii) Precursors of B cells

Lafleur *et al.* (167,168) reported studies on the definition of discrete stages of B cell development. They observed that adult bone marrow cells could be separated into three fractions based on sedimentation properties. Cells which were small and sedimented slowly were found to express readily detectable amounts of surface Ig, and could be stimulated by antigen in the presence of T cells in irradiated recipients to yield antibody-forming cells. These were considered "mature" B cells and were designated B_2. The large, fast sedimenting, low density cells isolated from bone marrow were not immunocompetent but could be converted to B_2 cells by adoptive transfer to irradiated recipients, or by *in vitro* culture (169). Such cells were termed pre-B cells and could be found in adult bone marrow, spleen, but not in peripheral lymph nodes. They were also detectable in fetal liver as early as 13 days of gestation, but were not found in the yolk sac (170). Pre-B cells were found primarily in populations depleted of surface Ig but were shown to be capable of 8s IgM synthesis (171). Since the differentiation of pre-B cells to more mature stages could be inhibited by anti-IgM, it was concluded that



at this stage of B cell differentiation, a small amount of surface Ig was expressed. A third stage of B cell differentiation was proposed to describe the transition of pre-B cells to B_2 cells. Designated B_1, this stage was characterized by cells with physical properties which were intermediate in their sedimentation velocities to those of pre-B and B_2 cells. The proposed differentiation scheme is summarized in Figure C.

(iv) Acquisition of B cell surface markers

Many other investigators have investigated the ontogeny of B cells by defining the sequential appearance of morphologically and phenotypically distinguishable lymphoid cells in the bone marrow of laboratory rodents, notably guinea pigs, rats and mice. Radioactive pulse experiments with ^{3}H-thymidine have demonstrated that 20% of the nucleated cells of adult bone marrow are lymphocytes, the majority of which are small, non-dividing cells. These cells are newly-formed, produced in the bone marrow and are rapidly replaced within approximately three days. These newly-formed small lymphocytes can be detected in the blood and tend to localize in the spleen and lymph nodes after migration from the bone marow. There is evidence to suggest that at least some of these cells may die in the bone marrow. It is thought that the precursors of these small lymphocytes are a group of large, rapidly-dividing "transitional" cells, which upon culture have been shown to give rise to small lymphocytes. Autoradiographic studies have shown that only 5-10% of the small lymphocytes in the bone marrow are long-lived, suggesting that they have entered the bone marrow from the pool of long-lived circulating lymphocytes (172-180).

Ryser and Vassalli (181) demonstrated that 50% of the small lymphocytes present in adult mouse bone marrow possessed neither B or T cell surface antigens. Pulse-labelling studies conducted *in vivo* with

^{3}H-thymidine indicated that these "null" cells were the first cells to become labelled and were derived from the large, rapidly-dividing "transitional" cells of the bone marrow. Transfer of null cells to irradiated recipients or *in vitro* incubation resulted in a rapid transformation (within 20 hours) of such cells into MBLA^{+}, Ig^{+} cells, and at no time were T cells detected in such progeny. Furthermore, such transformation did not appear to require cell division as judged by the lack of cell labelling in the continuous presence of ^{3}H-thymidine. (This latter finding suggests that null cells are already precommitted to the B cell lineage, and are similar in this regard to prothymocytes, which do not express T cell antigens but can be induced to do so without cell division.) These authors also reported finding higher numbers of MBLA^{+} cells than Ig^{+} cells, suggesting that MBLA antigen was acquired before surface Ig. In addition, cells bearing Ig heavy chains (predominantly μ) were consistently found in lower frequencies than kappa chain positive cells; a finding which led to the suggestion that perhaps light chains were the first Ig components to be expressed on the surface of B cells. Such an interpretation is difficult to rationalize inasmuch as it suggests final Ig assembly takes place in or on the cell membrane. Raff *et al*. (181a) have recently shown that intracellular, cytoplasmic IgM can be detected in mouse B lymphocyte precursors as early as day 12 of gestation, several days before surface Ig is first detected. Therefore, the preferential finding of kappa chains on early B cells may be due to some undefined artifact. Finally, Ryser and Vassalli reported that all Ig^{+} cells in the bone marrow were also FcR^{+} but 10-20% of the Ig^{-} cells were found to possess FcR, suggesting the existence of an FcR^{+} Ig^{-} cell. CR was not found on any bone marrow lymphocytes including Ig^{+} cells (181).

Gelfand *et al.* (182) demonstrated that the *in vitro* culturing of neonatal spleen or liver cells resulted in the generation of Ig^+ cells. Unlike the findings reported by Ryser and Vassalli discussed above, Gelfand reported that DNA and protein synthesis (and presumably cell division) was required for the conversion of Ig^- precursors to Ig^+ cells.

Hämmerling *et al.* (182a) recently reported studies on the sequential appearance of Ig, Ia and CR on mouse bone marrow cells. By exposing bone marrow cells to the cytolytic effects of anti-Ig, anti-Ia or to a rosetting procedure, Ig^+, Ia^+ or CR^+ cells could be selectively eliminated. Following a short *in vitro* culture with LPS, the reappearance of these markers was investigated. Their results suggested that the precursors of CR^+ cells were Ig^+, Ia^+ and that the precursors of Ia^+ cells were Ig^+ CR^-. They proposed the ontogenic development of B lymphocytes could be categorized into at least four phenotypically distinct stages with the maturational sequence being: $Ig^-Ia^-CR^- \rightarrow Ig^+Ia^-CR^- \rightarrow Ig^+Ia^+CR^- \rightarrow Ig^+Ia^+CR^+$. Kearney *et al.* (182b) have also suggested that the precursor of Ia^+ cells are Ig^+, and furthermore demonstrated that Ig D^+ cells appear later during ontogeny than Ia^+ cells.

Sidman and Unanue (183) used autoradiography and immunofluorescence to characterize the cells in spleens of neonatal C57BL/6 mice. They reported that during the first ten days of life, 80% of the spleen lymphocytes carried neither Thy-1 or Ig, i.e. were null cells. However, with increasing age, both the percentage and total numbers of such null cells dropped, with the numbers of $Thy\text{-}1^+$ and Ig^+ cells showing a concomitant increase. In addition, they demonstrated that Ig^+ cells present in the spleen during the first week of life were CR^-, and began to acquire this receptor at about ten days of age. This period of time also

coincided with the acquisition of membrane-bound IgD by mouse B cells (184).

The proposed sequence of acquisition of the above-mentioned surface markers is summarized in Figure C. This undoubtedly is an oversimplification since the studies of Cooper and collaborators (185) and others (186) suggest that, in addition to the expression of IgM and IgD, there exist subsets of B cells individually expressing IgA, IgE or all the subclasses of IgG. The acquisition of surface Ig is thought to be antigen-independent and follows the sequence $IgM^+ \rightarrow IgM^+$ IgG, A or $E^+ \rightarrow IgM^+$, IgG, A or E^+, IgD^+. The acquisition of IgD is regarded as a characteristic of mature B cells. Accordingly, IgD^+ cells have not been found in adult mouse bone marrow. IgD, however, is the major Ig expressed on peripheral B cells, accounting for 60-70% and 85-95% of the Ig on cells in the spleen and Peyer's patches, respectively (184,187).

d) Summary

A great deal of information has been gained in the last ten years concerning the ontogenic development of both major classes of immunocompetent lymphocytes. A pluripotent stem cell has been defined and studies have revealed the early dissociation of B and T cell development by the definition of a T cell-restricted self-renewing stem cell. The study of subsequent differentiation, in the microenvironments of the thymus, bone marrow and fetal liver and spleen has been facilitated by the detection of cell surface markers which have been used to define discrete stages of cell maturation. In some instances, the acquisition of membrane components (e.g. IgD) has been correlated with responsiveness; this aspect will be considered in the Discussion, as it relates

to the development of antibody-forming potential in neonatal mice.

III. SCOPE OF THE THESIS

At the onset of this investigation in 1970, it was known that newborn and neonatal mice lacked the capability of developing humoral antibody responses, but began acquiring immunocompetence several days after birth (188-190). One factor which contributed to this inability was the apparent lack of thymus-derived cells in the peripheral lymphoid organs of the newborn mouse. The seeding of peripheral lymphoid organs by the thymus was shown to be initiated shortly after birth, since neonatal thymectomy was effective in producing subsequent immunoincompetence only if performed within 24 hours of birth, but not later (121,149-151).

Another factor which was implicated in the lack of responsiveness of newborn mice was the possible inability of such mice to capture and "process" antigen effectively. The studies of Williams and Nossal in 1966 (191,192), on the ontogeny of the antigen-capturing structures in rats, visualized by autoradiographic techniques, demonstrated that newborn rats lacked the necessary lymphoid structures to capture and retain antigen. Furthermore, the acquisition of the ability to retain antigen during neonatal development coincided with the onset of immune responsiveness.

The requirement of an antigen-processing system in mice for humoral antibody production had been documented in 1968 and 1969 (23,24). At about the same time, Argyris (190) reported that the antibody response of 3-4-day-old C3H mice to SRC could be increased by the injection of macrophages from adult mice prior to challenge with SRC. Subsequently,

this effect was reproduced and reported by other investigators in 1971 (193). The interpretation offered by both groups was that the newborn mouse lacked sufficient numbers of "mature" macrophages to effectively develop an immune response and this deficiency could be overcome by providing macrophages from adult mice. However, a critical examination of the data indicated that the effect reported by these investigators was small (an increase of about five PFC/spleen to 30 PFC/spleen) and doubt was raised as to the physiological relevance of these findings.

Therefore, at the onset of the research described in this thesis, the reason(s) for the inability of newborn mice to respond to thymus-dependent antigens such as SRC was not at all clear. Two questions were posed and the initial experiments were designed to answer these:
(1) Based on the functional ability to produce antibody, were there any detectable antigen-sensitive cells in the spleens of newborn mice and, if so, how did their numbers vary during the early stages of life?
(2) What was the relative state of maturation of the antigen-processing system in newborn and neonatal mice, and was it a factor in the unresponsiveness of young mice to SRC?

The initial approach taken to answer these questions was to analyze, by adoptive transfer to adult irradiated recipients, the antibody-producing potential of spleen cells from neonatal mice. It was felt that this would allow an assessment of the immunocompetence of the lymphocyte pool of neonates, independent of their antigen-processing systems, since the irradiated recipient would be providing this latter requirement.

These studies form the basis of the first two chapters of the Results section, and were the basis for two papers published in the Journal of

Immunology in 1972 and 1973 (194,195). Since this work supported the interpretation that neonatal mice did not possess fully functional antigen-processing systems, additional approaches were taken in an attempt to assess the extent of maturation of this system in young mice. These included: (i) the determination of relative accessory cell (A cell) frequencies in neonatal and adult mouse spleens (Chapter III-1), using the assay system developed and reported by Gorcyznski, Miller and Phillips (36) in 1971; the results of this investigation were published in 1976 in the Journal of Immunology (196); (ii) an assessment of the ability of the antigen-processing systems of neonatal mice to retain passively administered antibody for long periods of time (Chapter III-2); and (iii) a comparison of the distributions and uptake of radioactive-labelled SRC in neonatal and adult mice (Chapter III-3). Two additional factors which were investigated are described in Chapters III-4 and III-5. These relate to a possible T cell deficiency in neonatal mice and a demonstration of crossreactivity between the environmental antigen LPS and the test antigen used in these studies, SRC.

Each chapter in the Results section contains an introduction and a discussion, which reflect the interpretations of the results at the time the work was completed. In the General Discussion at the conclusion of this thesis, a summary is made of the literature published during and subsequent to the completion of this work (1974-78) which relates directly to the maturation of antibody-forming potential in neonatal mice. The relationships between these more recent findings and the results reported in this thesis are also discussed.

MATERIALS AND METHODS

Animals

All C57BL/6 HC mice were obtained from North American Laboratory Supplies, Gunton, Manitoba. Twelve-day-old mice were obtained from brother-sister mating of pedigree stock animals. The time of birth for all mice was established to within 12 hours.

Red Cells

Sheep and horse blood, collected in citrated dextrose solution, was purchased from North American Laboratory Supplies, Gunton, Manitoba. Cells were washed three times in buffered saline solution before use.

Agarose

The Indubiose used in the localized hemolysis in gel technique was obtained from L'Industrie Biologique, Gennevilliers (Seine), France.

Guinea Pig Serum

Fresh guinea pig serum, used as a source of complement, was obtained from North American Laboratory Supplies, Gunton, Manitoba.

Fetal Calf Serum

Gammaglobulin-free fetal calf serum was obtained from Grand Island Biological Company, Grand Island, New York.

Tissue Culture Media

TC199 tissue culture medium and Hank's balanced salt solution, supplied in dry powdered form from Grand Island Biological Company, was dissolved in deionized double distilled water, adjusted with sodium bicarbonate and sterilized by pressure filtration through a 0.22 μ Millipore membrane.

Tissue Cell Suspensions

Mice were sacrificed by cervical dislocation and their spleens or thymuses removed and placed in iced TC199 supplemented to 20% with FCS. Using a loose fitting teflon-glass homogenizer, the tissues were gently suspended at 0°C. The suspensions were allowed to settle for 15 minutes, after which time the supernatant cell suspension was removed avoiding the sedimented debris. For adoptive transfer experiments, or thymus cell injections, the cells were washed once at 4°C, sedimented at 600 x g and resuspended in TC199 + 20% FCS. Leucocytes were enumerated after staining with Turk Solution (Hartman-Leddon Co., Philadelphia, Pa.). For the PFC assay, spleens were suspended either individually or collectively in TC199 + 20% FCS (2 ml/spleen). After allowing the debris to settle, the cell suspension was used as such or diluted 1/10 and 1/100 with TC199 + 20% FCS for plating.

Irradiation

Normal or accessory cell-depleted mice were individually confined in a compartmentalized lucite box, were exposed to 770 rads of whole body irradiation and then were reconstituted with cells at the time intervals specified in the Results section. Suspensions of cells were maintained at 0°C in a 100 ml siliconized beaker while being irradiated from above with a dose of 1200 rads. The source in each case was ^{60}Co, the dose rate approximately 50 rads per minute and the source-target distance 100 cm.

Immunization Procedures

For direct immunization of neonatal mice, all injections were made i.p. unless otherwise specified. Adult mice were injected using 25 or

27 gauge needles, 8 to 12-day-old mice with 30 gauge needles and newborn mice with extended glass capillaries adapted to 1 cc syringes.

For adoptive transfer experiments appropriate volumes of spleen cells and SRC were mixed at room temperature and injected into a lateral tail vein of the recipient mouse.

For experiments in which irradiated mice were injected with variable numbers of accessory cells (Chapter III), the actual volume of cell suspension injected was made constant, regardless of the total number of cells finally injected. In these experiments, irradiated mice were injected i.v. with heparin (15 units) approximately five minutes before the injection of cells. For the preparation of A-cell depleted mice, normal adult mice were injected i.p. with 2×10^{10} HRC.

Localized Hemolysis in Gel Assay

PFC were visualized using a modified procedure of the technique originally described by Jerne *et al.* (197). Into a 13 x 100 mm glass test tube at 45°C the following additions were made in the order given:

1) 0.4 ml agarose (0.83% in water) at 60°C
2) 0.4 ml Hank's solution (2x)
3) 0.1 ml SRC (5×10^9/ml PBS)
4) 0.1 ml PBS or 1/80 dilution in PBS of rabbit anti-mouse IgG-2a
5) 0.2 ml spleen cells in TC199 + 20% FCS.

The test tube was shaken gently and the contents poured over a base layer of 0.4% agarose (in PBS) in 60 x 15 mm plastic Petri dishes. After allowing to stand at room temperature for 5-10 minutes, the dishes were incubated at 37°C for one hour. Guinea pig serum, diluted 12-fold in PBS, was added (2 ml) and the dishes incubated at 37°C for a further

30 minutes. Plaques were subsequently enumerated with the aid of a stereomicroscope at a total magnification of 4x.

Rabbit antibody preparations used for developing the plaques produced by IgG antibody-secreting cells were obtained by immunizing rabbits with mouse γ-globulins (Winley-Morris, Montreal). From the antisera obtained, the γ-globulins were isolated by precipitation with Na_2SO_4 (198). The γ-globulin preparations were absorbed with insolubilized mouse kappa chains isolated from the urine of BALB/c mice bearing a MOPC 149 tumor (199). On immunoelectrophoresis against mouse γ-globulins, a single arc of precipitation corresponding to IgG-2a was observed. Antibody to kappa or lambda chains was not detected.

To establish the developing properties of this rabbit γ-globulin preparation, an incorporation assay was used (200). At the concentration of developing rabbit antibody which provided for maximal development of IgG PFC, the number of IgM plaques was reduced by 13%. Consequently, IgM and IgG PFC were calculated as follows:

IgM PFC = number of plaques obtained in the absence of rabbit anti-mouse IgG-2a

"Total" PFC = number of plaques obtained in the presence of rabbit anti-mouse IgG-2a

IgG PFC = (Total PFC) - (0.87) (IgM PFC)

Reagents for Labelling and Characterization of Red Cells

$^{51}CrCl_3$ — obtained from Atomic Energy of Canada Limited, neutralized and diluted with PBS before use

$Na^{125}I$ — carrier free, Amersham Corporation, Oakville, Canada

Lactoperoxidase — Sigma Chemical Co., St. Louis, U.S.A.

H_2O_2 — 3%, Fisher Scientific Company

HEPES — Calbiochem, San Diego, U.S.A.

Sodium Phosphate, 310 mOsm, pH 7.4 (Isotonic) —

prepared by combining 500 ml 0.103 M Na_2HPO_4 and 77 ml 0.155 M NaH_2PO_4

Sodium Phosphate, 20 mOsm, pH 7.4 —

prepared by diluting 64.52 ml of 310 mOsm buffer to 1 liter with water

Preparation of Radioactively Labelled SRC

^{51}Cr-labelled mouse red cells were prepared as follows. C57BL/6 mouse blood, collected in citrated dextrose solution, was centrifuged and the red cells washed 3x in 0.01M HEPES buffered Hank's solution, pH 7.2 (HEPES-HANKS). The red cells (4.5 ml at 2 x 10^9/ml) were incubated with $^{51}CrCl_3$ (0.5 ml; 1 mCi/ml) at 37°C for 30 minutes. After centrifuging at 600 x g the ^{51}Cr-mouse red cells were washed 6x with 10 ml portions of cold HEPES-HANKS and counted. Typically a specific radioactivity of 2300 cpm/10^6 SRC was obtained. Radioactivity was determined using a Nuclear Chicago gamma counter. A 3% counting efficiency was obtained for ^{51}Cr. Approximately 90% of the ^{51}Cr was found to be associated with the cytoplasmic components of the red cells.

^{125}I-SRC were prepared by utilizing the lactoperoxidase surface-labelling technique described by Phillips and Morrison (201), Vitetta and Uhr (202) and Marchalonis *et al.*(203). The following modified version of this technique described here was decided upon since it was found to optimize a) the incorporation of label and b) the localization of label on the surface of the cell. Into a 15 x 100 mm plastic disposable test tube the following additions were made in the order

given:

1) 200 µl SRC (5×10^9/ml PBS-G)

2) 750 µl PBS-G

3) 45 µl lactoperoxidase (2 mg/ml PBS-G)

4) 3 µl $Na^{125}I$ (200 µCi carrier free)

At 0 time the reaction was initiated by the addition of 3 µl of 1 mM H_2O_2. Additions were subsequently made at the times indicated:

+ 30 seconds -- 3 µl H_2O_2

+ 90 seconds -- 45 µl lactoperoxidase, 3 µl $Na^{125}I$

+ 120 seconds -- 3 µl H_2O_2

+ 150 seconds -- 3 µl H_2O_2

At 210 seconds, the reaction was stopped by the addition of 11 ml cold HEPES-HANKS. The ^{125}I-SRC were sedimented (600 x g for six minutes), washed 6x with 12 ml portions of cold HEPES-HANKS, and finally resuspended in HEPES-HANKS. The cell concentration was determined and the radioactivity of a suitable aliquot counted using a Beckman Model 300 Gamma Counter. Counting efficiencies for ^{125}I were 55-60%. Using this procedure specific radioactivities were typically 3000-4000 cpm/10^6 SRC. In order to prepare sufficient numbers of ^{125}I-SRC for injection into mice, the above reaction protocol was repeated sequentially six times and the SRC pooled after the first sedimentation. This approach was found necessary since, for reasons which were not entirely clear, attempts to simply scale-up the procedure were not uniformly successful.

Distribution of Label Associated with ^{125}I-SRC

In order to determine the extent of surface labelling as opposed to cytoplasmic labelling of the ^{125}I-SRC, the cells were hypotonically lysed according to the procedure described by Dodge *et al.*(204). Typ-

ically, 2.5 x 10^8 ^{125}I-SRC were combined with 1 x 10^{10} SRC (cold carrier) in a total volume of 2.0 ml 310 mOsm sodium phosphate, pH 7.4. The cells were poured into 28 ml 20 mOsm sodium phosphate, pH 7.4, and swirled in ice for five minutes. The stroma and hemoglobin were separated by centrifugation at 31000 x g for 25 minutes in the cold. After two washings with 20 mOsm phosphate, 84-91% of the ^{125}I was recovered in the stroma fraction, indicating that most of the ^{125}I label was associated with the membrane of the SRC.

Distribution of ^{125}I-SRC in Neonatal and Adult Mice

Both 12-day-old and 12-week-old mice received a dose of 2 x 10^8 ^{125}I-SRC. Adult mice were injected with 0.10 ml into a lateral tail vein. Twelve-day-old mice received 0.05 ml of the cell suspension injected into the retro-orbital sinus. After a specified time interval, a blood sample was taken from the tail, the mice sacrificed by cervical dislocation and the spleen, lungs and liver removed and placed in cold HEPES-HANKS. The blood, collected in a calibrated heparinized glass capillary, was transferred into a 20 ml counting vial with approximately 0.5 ml citrated saline. The lungs, liver and carcass also were placed in counting vials. The spleen was suspended and subdivided into three fractions as described below. All operations were performed at 0°C. Each spleen was gently suspended in 2 ml cold TC199 + 20% FCS using a loose fitting teflon-glass homogenizer. The resulting suspension was allowed to settle for ten minutes. The spleen cells which did not settle during this time were drawn off and designated the spleen cellular fraction. The settled splenic debris, designated the R (reticular) fraction, was washed 2x with 2 ml cold TC199 + 20% FCS using five-minute

settling times (1 x g). The washes were combined with the spleen cell fraction; the volume adjusted to 10 ml and the suspension centrifuged at 600 x g for five minutes. The pelleted spleen cells were designated the C (cellular) fraction. The clear supernatant was termed the cellular supernatant or *C supernatant*. The entire R and C fractions were counted for radioactivity, whereas an appropriate aliquot of the *C supernatant* was counted. The amount of radioactivity found in the various samples was determined and expressed as a percentage of the total activity injected.

Lipopolysaccharide Antigen from *E. Coli*

The lipopolysaccharide cell wall antigen (LPS) was extracted from *E. coli* (ATCC 11303) by the hot phenol-water method described by Staub (205). The purified product was detoxified in 0.25 N NaOH as described by Britton (206), and dissolved in PBS before use. Mice were injected i.v. with up to 2 mg/animal without any mortalities. For inhibition of PFC using LPS, the antigen was incorporated at various concentrations into the indicator layer containing spleen cells and SRC.

RESULTS

CHAPTER I

THE GENERATION OF ANTIGEN SENSITIVE CELLS IN THE NEWBORN MOUSE

INTRODUCTION

Relative to the response of adults, newborn mice mount only a feeble antibody response to sheep red cell antigens (188). The low responsiveness evident in neonates was reported by Playfair (189) to exist in several strains. Some strains of mice, notably NZB, produced IgM anti-SRC PFC when injected with antigen at birth, whereas other strains, for example C57BL/6, could not produce PFC when similarly injected and produced PFC only when antigen was administered several days after birth. This study was basically descriptive and the basis for the differences in responsiveness exhibited by the various strains was not determined. The data of Playfair indicated that non-responding strains of newborn mice lacked one or more of the necessary cellular components required for the generation of an immune response.

In order to elicit an anti-SRC response, three cellular compartments are required; a) the T-cell (thymus derived) pool, b) the B-cell (bone marrow-derived) pool and c) the antigen processing system. This latter requirement has been characterized *in vitro* severally as a macrophage, a radiation-resistant cell (32,33,34,35), adherent cell (15,16), and has been referred to also as an accessory cell (34) or simply A-cell. In addition, the *in vivo* requirement for such a cell has been extensively investigated by Gorczynski *et al.* (36). In this thesis this cellular component will by and large be referred to as the antigen processing system. A deficiency in any one or more of these compartments would result in a lack of responsiveness such as that exhibited by neonatal mice. The bases for two such deficiencies in neonatal mice have been documented in the literature. 1) It has been demonstrated, for example, that newborn mice have few thymic lymphocytes in their spleens

and that initial seeding of these cells into the spleen occurs during the first few days of life (121, 149-151). Thus, a lack of responsiveness in mice during the immediate post-natal period could be anticipated on the basis of a T-cell deficiency. 2) The anti-SRC response of neonatal C3H mice was investigated by Argyris (190). It was reported that the very feeble response of three-day-old mice could be enhanced by the injection of adult macrophages, thus implying a deficiency of the antigen processing system in these mice.

At the time the work in this thesis began (1970), there had been no comprehensive quantitative investigation related to the development of immune responsiveness by neonatal mice. One of the principal interests of the laboratory in which this work was performed was the effects of anti-immunoglobulin antibodies on the development of immunocompetence. One requirement for such a study was a clear definition of the maturational processes which occurred during neonatal life; the availability of such a reference would thus allow for the efficacy of the above experimental procedures to be accurately assessed. The scope of the study reported in this thesis includes studies on the rate of maturation of the different cellular compartments involved in an antibody response. The study of anti-immunoglobulin effects lies outside this scope and will not be considered further.

The initial experimental protocols were addressed to two aspects of the antibody response in developing newborn mice:

a) the rate of development of the capacity to produce IgM and IgG antibody forming cells and

b) the development of the antigen-processing system as it related to the production of antibody.

In order to determine the rate of development of the capacity to produce antibody, two test systems were utilized: 1) The response of intact mice and 2) the response of immunocompetent cells which were injected into irradiated syngeneic recipients. In system 1, the *in situ* response of neonatal mice of increasing age were determined. This system served to define the capability of the intact mouse to respond as a function of age. In system 2, immunocompetent cells from neonatal mice of increasing age were challenged with antigen in adult irradiated recipients.

The C56BL/6 strain of mice was chosen for this investigation since the report of Playfair (189) had indicated that this strain developed relatively late the ability to mount an anti-SRC response.

The SRC was chosen for this study as a representative thymus dependent antigen. In addition, the choice of this antigen allowed the enumeration of antibody-producing cells using the sensitive PFC assay in which both direct (IgM secreting cells) and indirect PFC (IgG secreting cells) are scored.

RESULTS

1. Response of Intact Neonatal Mice

The anti-SRC response of intact C57BL/6 mice was studied as a function of age in the following way. Groups of mice of various ages up to two weeks were formed with 15-20 mice to a group. The individual mice of each group were either littermates or born within 24 hours of one another. All mice were injected with an immunizing dose of 7.5×10^8 SRC i.p. This dose of antigen was selected since other results (see Chapter II) suggested that the optimal antigen dose for mice of this age was approximately 1×10^9 SRC. The resulting antibody responses were determined on days 5,6,7 and 8 following antigen injection. On each of these days 3-5 mice were withdrawn from each age group and the IgM and IgG-PFC appearing in the spleens were determined. Typical PFC responses of one such age group are illustrated Figure 1.

The salient features of the PFC responses are:

a) The responses increase gradually with time, reach a maximum level and then decline.

b) The maximum IgM PFC response occurs 6-7 days after injection, whereas the IgG PFC response peaked one day later, i.e. at 7-8 days.

c) The PFC responses of neonates developed more slowly than those given by adult mice inasmuch as the adult mice obtain a maximum response 1-2 days before the neonates. This was apparent for both the IgM and IgG PFC responses. This point is illustrated in Figure 2 for the IgM responses.

The maximum IgM and IgG PFC responses of each age group have been plotted as a function of age in Figure 3. It was clear that the abili-

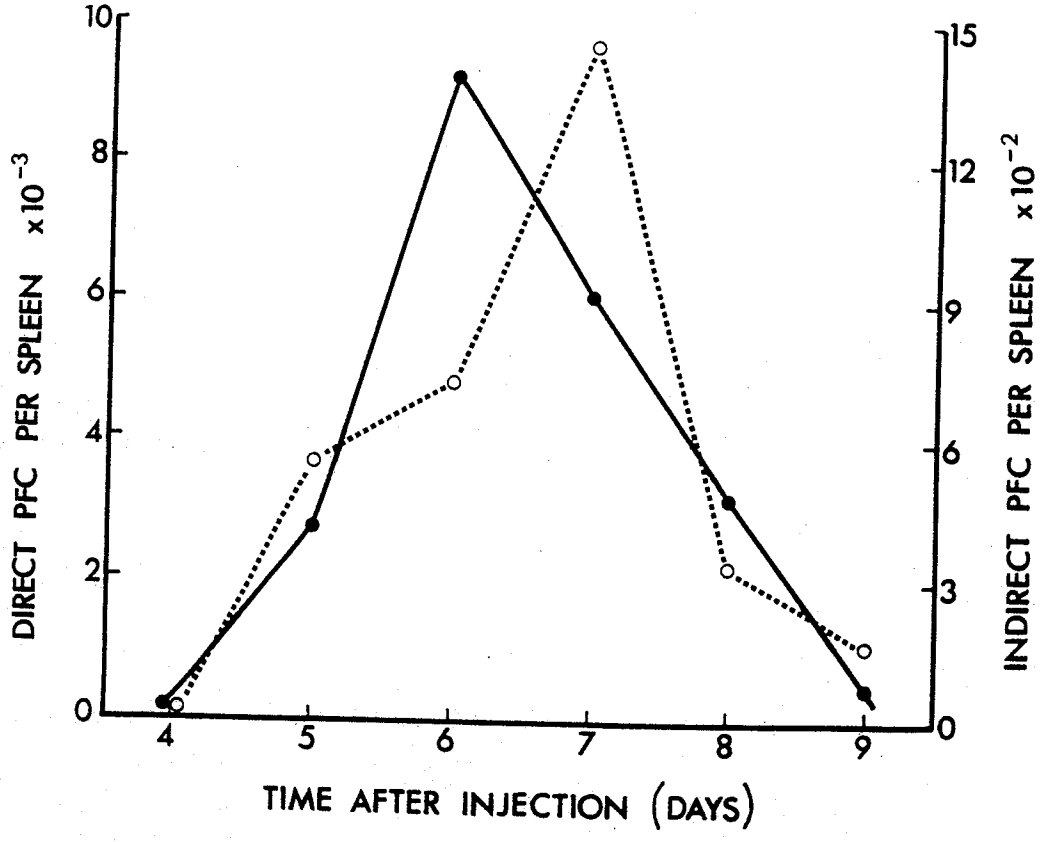


Figure 1:

The kinetics of the anti-SRC response in 12-day-old C57BL/6 mice. Animals were injected i.p. with 7.5×10^8 SRC and their spleens subsequently assayed for direct (●) and indirect (O) PFC. Each point represents the mean determination of 3-5 mice.

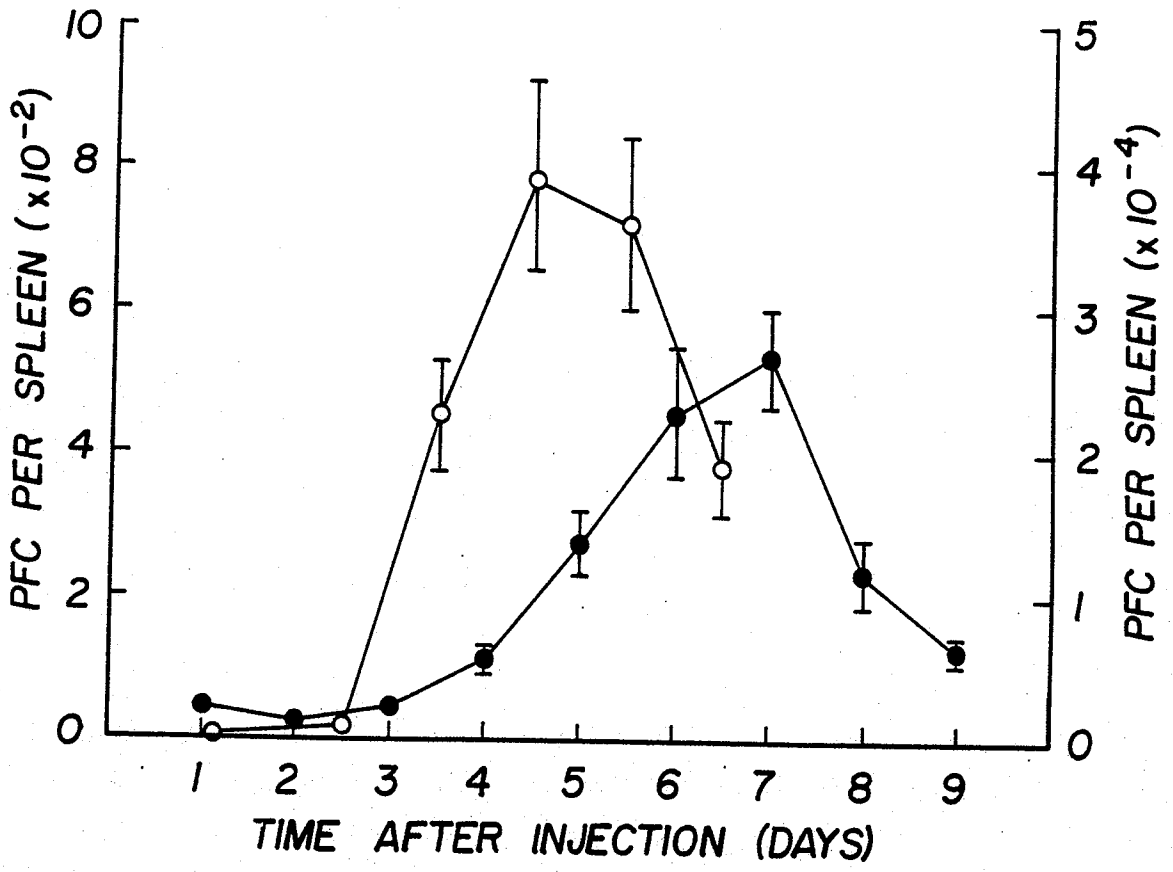


Figure 2:

The kinetics of the anti-SRC response in C57BL/6 mice of two different ages. Animals were injected i.p. with 1 x 10^8 SRC and their spleens assayed for PFC. Direct PFC only are illustrated: (●), 8 days old (left scale), 6 animals per point; (○), 28 days old (right scale), 4 animals per point. One standard error of the mean is defined for each point by a vertical bar.

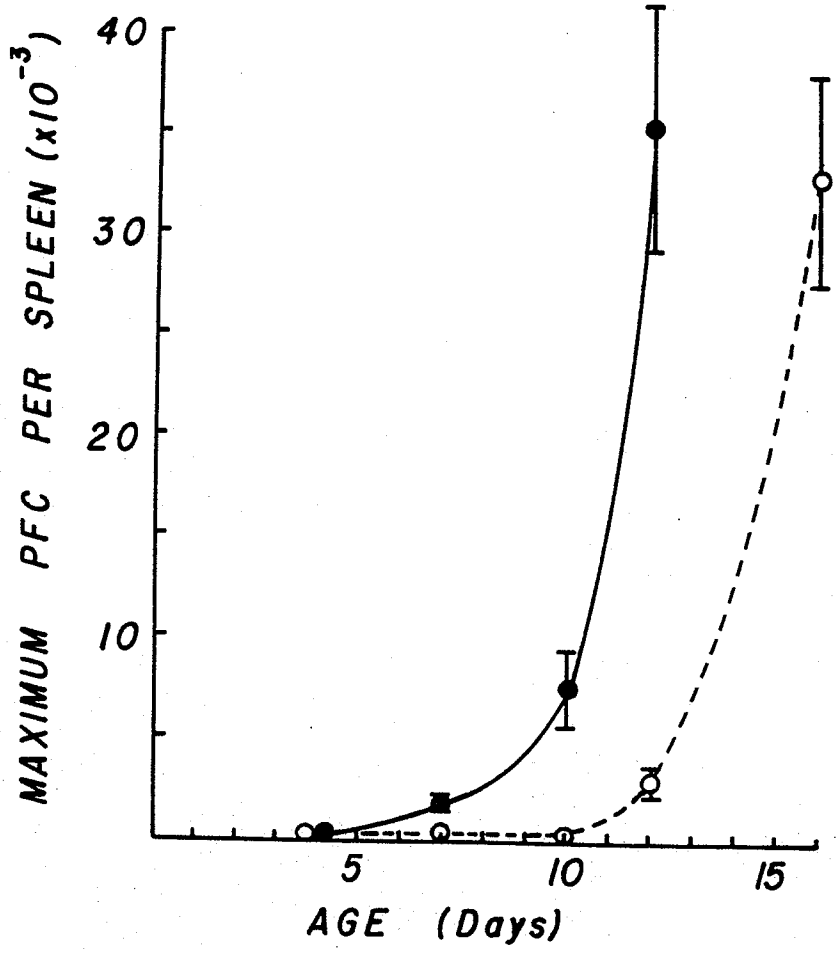


Figure 3:

The anti-SRC response of intact C57BL/6 mice as a function of age. Normal mice of a given age were injected i.p. with 7.5×10^8 SRC and their spleens assayed for the subsequent direct (●) and indirect (○) PFC responses; the maximum value attained by these responses is expressed as a function of the age of the animal (5 animals per point). One standard error of the mean is defined for each point by a vertical bar.

ties to produce IgM and IgG antibodies were acquired at different times after birth. The capacity to mount an IgM PFC response developed first. For example, when mice were injected at age four days, a weak but definite IgM PFC response was evident. On the other hand, the IgG PFC response was consistently apparent only in mice older than ten days. The age of ten days appeared to be the time when the ability to produce IgM and IgG PFC responses increased markedly. Twelve-day-old mice, for example, could produce both IgM and IgG PFC and appeared to be the youngest C57BL/6 which could do so reproducibly. The maximum IgM response was consistently greater than the IgG response.

2. Adoptive Transfer of Neonatal Immunocompetent Cells to Adult Irradiated Recipients

In the following experimental protocol spleen cells from neonatal mice were adoptively transferred to adult irradiated recipients, challanged with SRC and the subsequent PFC enumerated. Thus adult mice which had received 770 rads whole body irradiation 20 hours previously were injected i.v. with 5×10^7 spleen cells prepared from young mice of the selected age along with 2.5×10^8 SRC. The IgM and IgG PFC responses were subsequently determined. A typical PFC response of these recipients is illustrated in Figure 4. Under these conditions it was observed that the IgM PFC response attained a maximum at approximately 8 - 9 days and the IgG PFC response at 9 - 10 days after the adoptive immunization. No PFC responses were detected at these times in irradiated recipients receiving only spleen cells or SRC alone.

Figure 5 illustrates the maximum IgM and IgG PFC responses so obtained as a function of age. The salient features of these results are:

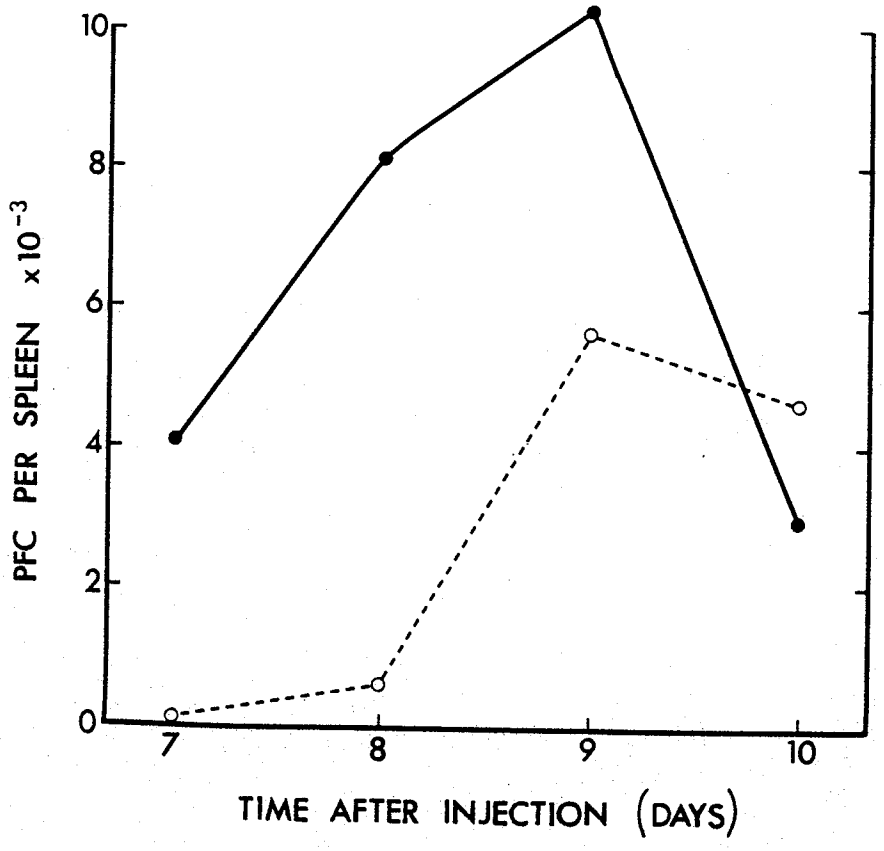


Figure 4:

The immune responsiveness of neonatal spleen cells in adult irradiated recipients. 5×10^7 spleen cells from 12-day-old normal donors were injected i.v. together with 2.5×10^8 SRC into irradiated recipients, and their spleens subsequently assayed for direct (●) and indirect (○) PFC 7-10 days after injection. Each point represents the mean determination of 3 mice.

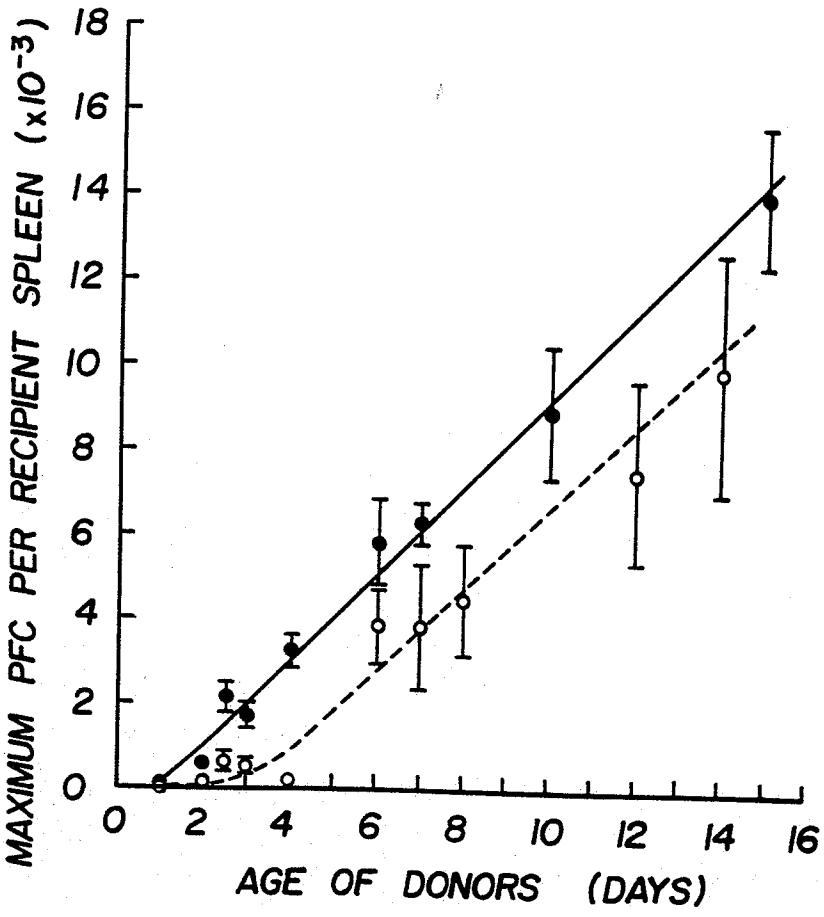


Figure 5:

The immune responsiveness of normal spleen cells in irradiated recipients. 5×10^7 spleen cells from normal donors of different ages were injected i.v. together with 2.5×10^8 SRC into irradiated recipients. The maximum value of the resulting direct (●) and indirect (○) PFC responses is expressed as a function of the age of the spleen cell donor (3-5 animals per point). One standard error of the mean is defined for each point by a vertical bar.

a) Antigen sensitive precursors for the IgM PFC response to SRC are already present in the spleens of animals which are only 24 hours old.

b) The number of such precursors in the fixed inoculum of 5×10^7 spleen cells increases rapidly after birth.

c) In contrast with the IgM PFC responses, recipients receiving spleen cells from one or two-day-old donors exhibited no IgG PFC responses. In fact, spleen cells from donors as old as four days could produce only a very feeble IgG PFC response. After this time, however, the rate of increase in IgG responsiveness was similar to that for the IgM response.

Additional information derived from these experiments is presented in Figure 6 in which the number of recovered cells per spleen is plotted as a function of donor age. The spleen cell count was found to increase rapidly from birth until age 4.5 days at which time the rate of increase declined sharply. Subsequently a much slower rate of increase was observed which was similar to the rate of increase in total body weight until at least ten weeks of age. The percentage of viable spleen cells remained constant with the age of the donor, being 90% or greater in all cases.

The results presented in Figures 5 and 6 suggested an additional experiment. It was clear that from birth to four days of age there existed a sizable cellular influx into the spleen, the termination of which appeared to correlate with the abrupt increase in IgG PFC responsiveness. Since it had been demonstrated that thymus derived lymphocytes began to migrate to the spleen shortly after birth (121, 149-151) and furthermore that this cell type was apparently more critically involved

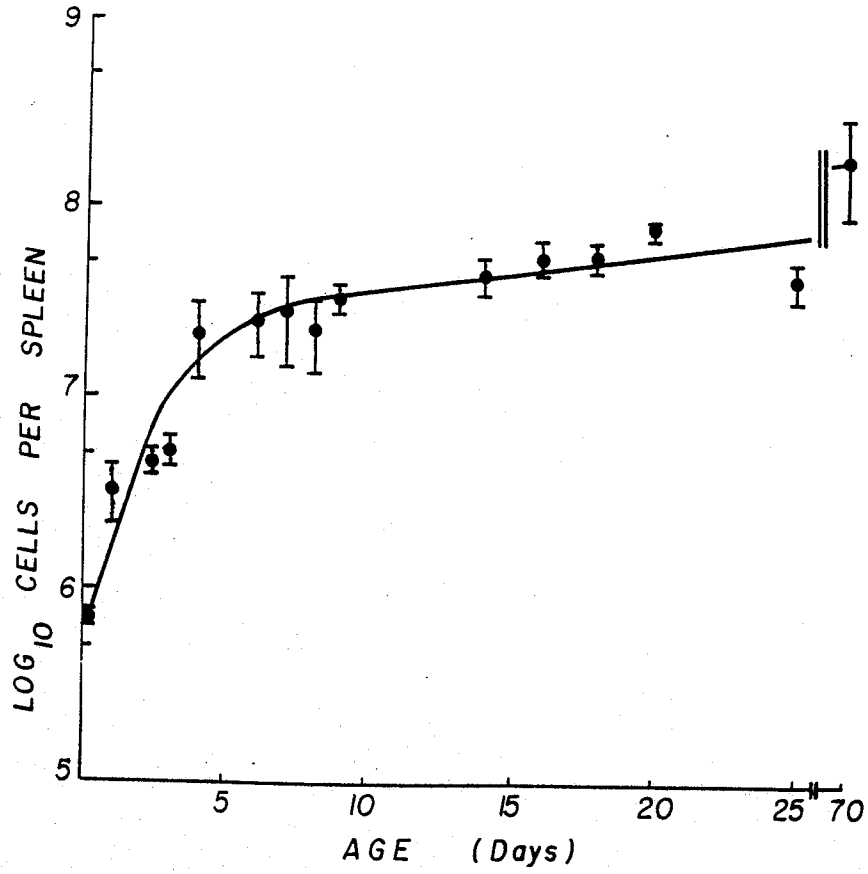


Figure 6:

The recovery of cells from the spleens of mice of increasing age. Each point represents the mean (±1 S.E.) of 10 or more spleens.

in the IgG than the IgM response (207), an examination was undertaken of the relative T-cell deficiency occurring in the spleen cell donors during the period shortly after birth. For this purpose two-day-old donors were selected. This choice represented a compromise of two factors: a) the fact that T-cell seeding of the spleen begins within a day after birth and b) the logistics of recovering sufficient numbers of spleen cells from mice one to two days old to repopulate adult irradiated recipients at a level of 5×10^7 spleen cells per recipient. In the results reported here, approximately one hundred two-day-old mice born within a 36-hour period were required for six irradiated recipients. Two groups of adult mice consisting of three to a group were irradiated (770 rads) and 20 hours later were injected i.v. with 5×10^7 spleen cells from two-day-old donors and 2.5×10^8 SRC. In addition the recipients of Group 2 (see Table I) received 1×10^8 syngeneic thymus cells prepared from ten-week-old normal mice. The spleens of recipient mice were assayed for IgM and IgG PFC 9.5 days after cell transfer. As had been demonstrated earlier in Figure 5, mice which had received only spleen cells from two-day-old donors and antigen (Group 1) generated a substantial IgM PFC response but no detectable IgG PFC response. In contrast, two-day-old donor spleen cells supplemented with normal adult thymocytes could generate an IgG PFC response (Group 2). This finding clearly indicated that:

1. antigen sensitive precursor cells for both the IgM and IgG PFC responses were present in the spleens of newborn mice and

2. the spleens of two-day-old mice apparently lacked the necessary T-cell function required to generate an IgG PFC response, but this deficiency was apparently overcome by age 5-6 days inasmuch as the IgG PFC

TABLE I

RESPONSE OF SPLEEN CELLS FROM 2-DAY-OLD DONORS AFTER TRANSFER TO IRRADIATED RECIPIENTS[a]

Group	PFC/Spleen ± 1 S.E.	
	IgM	IgG
1	3000 ± 1030	0
2	8600 ± 2900	3420 ± 1900

[a] Both groups (three animals each) received 5 x 10^7 spleen cells and 2.5 x 10^8 SRC; recipients in group 2 received in addition 1 x 10^8 thymus cells from normal 10-week-old mice. Recipient spleens were assayed 9.5 days after cell transfer.

response increased at the same rate as the IgM PFC response (Figure 5).

In order to more fully understand the contribution of the irradiated recipient in the results of Figure 5, the data were transformed. The relationship between age and a) the total number of cells per spleen (Figure 6) and b) the maximum PFC response given by 5×10^7 donor spleen cells in irradiated recipients (Figure 5) allowed the calculation of the maximum number of PFC produced per donor spleen which became apparent in the irradiated recipients. This parameter, termed the maximum apparent PFC per spleen, is plotted as a function of age in Figure 7. In this calculation it has been assumed that in the injected spleen cell suspensions the ability of immunocompetent units to repopulate the spleens of the irradiated recipients does not vary significantly with the age of the donor, at least not during the ten days immediately after cell transfer. It can be seen in Figure 7 that the maximum apparent IgM PFC response rises exponentially with age and, further, that the IgM and IgG responses have similar slopes within the age limits studied and for the antigen concentration used.

Thus, the maximum antibody response produced by an animal of any given age has been expressed in two ways: a) as PFC per spleen from an intact animal (Figure 3) and b) as apparent PFC per donor spleen (Figure 7) as measured by the number of PFC in the spleens of irradiated recipients. The ratio of these two values is expressed as a function of age in Figure 8. It can be seen that a steep increase in this ratio occurred, beginning at approximately ten days of age. This observation strongly suggested that beginning at ten days of age, a maturational process was initiated in the intact animal. This process involved a system which was a prerequisite for a vigorous antibody response

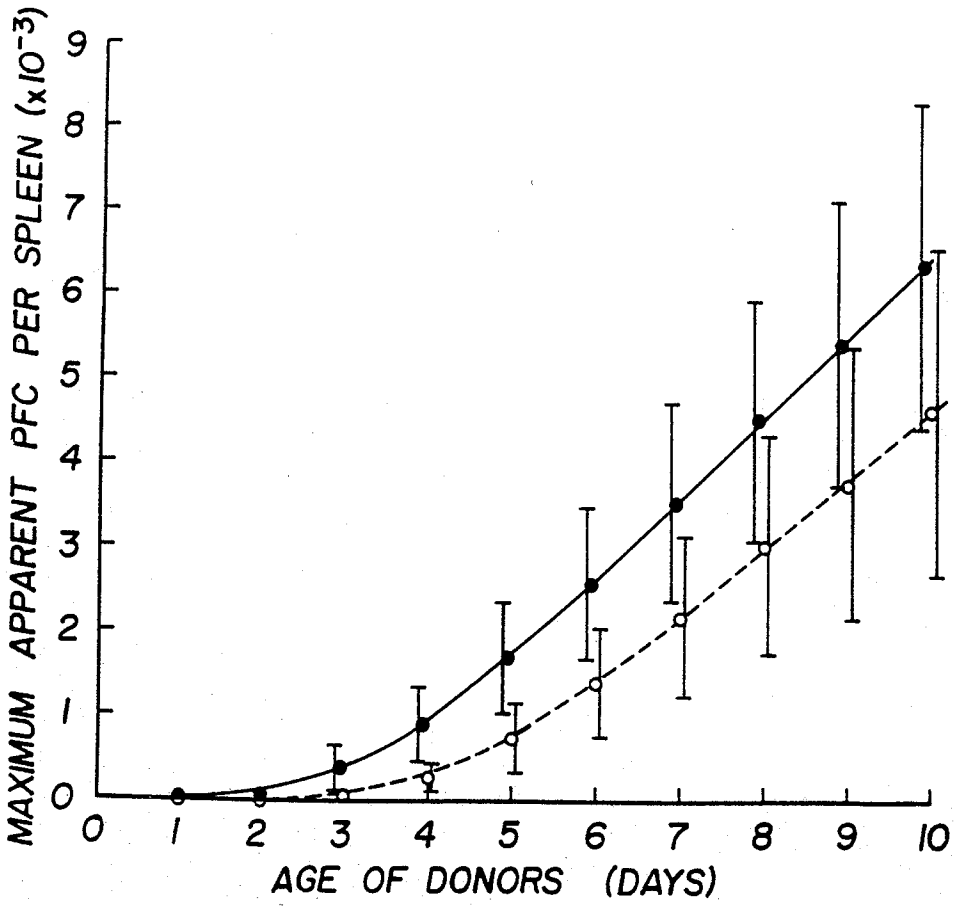


Figure 7:

The maximum apparent PFC per donor spleen as a function of the age of the donor. 5×10^7 normal spleen cells from donors of various ages were injected into each irradiated animal together with 2.5×10^8 SRC; the apparent PFC per donor spleen were calculated from data illustrated in Figures 5 and 6 and expressed as a function of the age of the donor, (●) direct PFC: (○) indirect PFC. Each vertical bar indicates the range of the apparent PFC/spleen when the values derived from Figures 5 and 6 are each varied by one standard error.

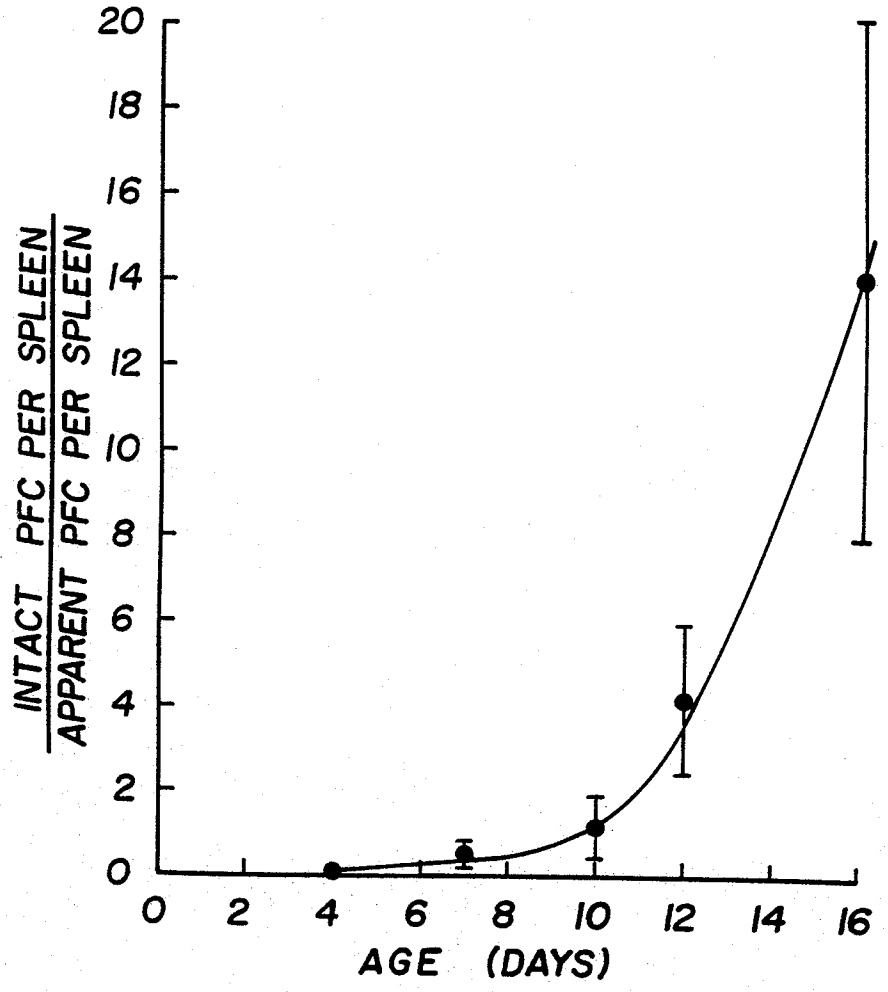


Figure 8:

The anti-SRC response of intact C57BL/6 mice relative to that of spleen cells stimulated in irradiated recipients. The ratio, maximum direct PFC per spleen (intact mice): apparent direct PFC per donor spleen, is expressed as a function of the age of the animal (intact mice and donor mice being of the same age). Each vertical bar defines the range encountered when the numerator and denominator are varied by the errors indicated in Figures 3 and 7.

and which was apparently still functional in the irradiated adult recipient. The nature of this system will now be discussed.

DISCUSSION

One of the principal aims of the experiments described in this Chapter has been to quantitate and define the basis for the immune responsiveness of intact neonatal mice during the first three weeks of life. The results in Figure 3 demonstrated that during this time period, the neonatal mice were transformed from a state of almost total unresponsiveness to a state of vigorous responsiveness. For example, mice injected at four days of age exhibited barely detectable numbers of IgM PFC (125 PFC/spleen) and virtually no IgG PFC, whereas at age twelve days, mice could respond with much higher levels of both IgM PFC (35,000/spleen) and IgG PFC (6,000/spleen). It was evident that during this time period a maturational process was occurring which was crucial to the development of an anti-SRC response.

The next question to be addressed was the identification of the system(s) responsible for the dramatic increase in responsiveness of neonatal mice. Was the sharp increase in PFC responsiveness which occurred at 1-2 weeks of age due to a sudden appearance of antigen-specific B- and/or T-cells in the spleens of these mice or a sudden maturation of the antigen-processing system; or was it a combination of these factors which was involved? In order to further define the immune capability of immunocompetent cells in neonatal mice, spleen cells from these mice were stimulated with antigen in syngeneic irradiated recipients. On the basis of the results presented in Figure 5 and Table I, it was concluded that both B- and T-antigen-sensitive cells were present in the spleens of C57BL/6 mice as early as 24 hours after birth.

These experiments were not addressed to the question of the origin

of the antigen-specific precursor B-cells. However, since it has been recently shown that the development of antigen sensitive B cells is a multifocal process in mice (124), it is probable that the antigen-specific B cells detected in these studies have arisen due to a combination of a) differentiation in the spleen of stem cells and b) migration of antigen specific B cells to the spleen from other hemopoietic sites. The magnitude of the IgM PFC response produced in the irradiated recipients increased linearly with the age of the donor (Figure 5) suggesting that the rate of appearance of the antigen-specific B cells occurred at a constant rate with donors aged from 1-14 days. This appeared also to be the case with the IgG PFC response using donors four days and older. Thus, there appeared to be no abrupt change in the rate at which B cell precursors appeared in the spleens of mice during the time intact mice were displaying a very rapid change in the rate of increase of their immune responsiveness, i.e. at 1-2 weeks of age. It was, therefore, concluded that the marked change in the rate of increase apparent in intact mice aged 1-2 weeks was not due to any sudden change in the rate of appearance of antigen-specific B cell precursors. It has been demonstrated (124,125) that fetal liver, spleen and bone marrow are sites of B lymphocyte differentiation in the developing embryo. The results described here demonstrated that antigen-specific B cells have already differentiated to this state from more primitive progenitor cells and are present in the spleen in relatively small numbers 24 hours after birth.

In the study presented in Figure 5 it was found that the kinetics of the PFC responses given by the spleen cells of neonatal mice in irradiated recipients did not vary with the age of the donors. This

finding suggested that the cells present in the donor spleens at the time of transfer were already antigen specific and that within the period of study development in the irradiated recipient to this state from more primitive progenitors was not a significant factor in these results.

In contrast to the multifocal development of B cell precursors, T cells are generated in the thymus and are seeded in the spleen in an abrupt manner immediately following birth. Seeding of the spleen by thymic lymphocytes has been reported to occur in the mouse predominantly in the first 24-48 hours, with the rate reducing markedly by four days after birth (121,149-151). Subsequent to this post-natal period, seeding occurs at a much lower rate. The effect of this sudden influx of T cells into the spleen was apparent in the IgG PFC responses presented in Figure 5. In contrast with the linear increase of the IgM PFC responses with age observed in irradiated recipients, the IgG PFC responses exhibited a biphasic pattern with increasing age. At approximately 3.5 days of age a marked increase in IgG PFC responsiveness occurred which coincided with the time of maximum rate of increase of lymphoid cells in the spleen (Figure 6). On the assumption that the observed increase in cellularity of the spleen is a reflection of T cell seeding, it can be concluded that the IgG response is more dependent on T cells than the IgM response. This is in agreement with the conclusions reached by Taylor and Wortis (207) who demonstrated that the IgG response was much more susceptible to the effects of thymectomy than the IgM response. In order to determine whether a T cell deficiency could indeed be demonstrated in the spleens of mice from birth to four days of age, spleen cells from 2-3-day-old donors were chal-

lenged with SRC in syngeneic irradiated recipients with and without the addition of adult thymocytes. It was shown in Table I that spleen cells from two-day-old donors failed to mount an IgG PFC response in the absence of added thymus cells; however, an IgG response was facilitated after 1×10^8 thymus cells were added to the inoculum. Thus, it was concluded that in terms of the anti-SRC response, the spleens of 2-3-day-old mice had less than optimal numbers of antigen-specific T cells. This relative deficiency appeared to have been overcome by age 4-5 days since both the direct and indirect PFC responses increased linearly in a parallel manner with increasing age. Thus, it was considered unlikely that the major factor involved in the maturation of immune responsiveness of intact mice which occurred between 7-14 days of age was due to a sudden influx of specific T cells into the spleens of these mice.

In order to evaluate the contribution of the antigen processing system in the results reported here, the responses of intact mice and reconstituted irradiated recipients were compared (Figure 8). This comparison was made by calculating the ratio of PFC/spleen in intact mice to apparent PFC/spleen as a function of age. Since it had been previously demonstrated that irradiated recipients can provide a functional antigen processing system (11,24), the response of neonatal cells in such recipients (apparent PFC) was determined with a constant antigen processing system background. If this system was constant in the intact mice, it would be predicted that the ratios calculated in Figure 8 would be constant as well. In fact, while remaining almost constant for the first week of life, the ratios increased dramatically starting at about ten days of age. It was, therefore, reasoned that the antigen processing system was not a constant factor in the response of intact mice, and it was this system that provided the basis for the increased

responsiveness observed in Figures 3 and 8.

It has been reported that macrophages are essential participants in the response to antigens such as the SRC (34) and, furthermore, under certain conditions macrophages have been reported to confer responsiveness on otherwise unresponsive mice (190). However, simply the presence or absence of macrophages may not be the deciding factor in determining the responsiveness of young animals since it has been shown also that phagocytic cells taken from fetal mice can have similar properties to those of peritoneal macrophages from adult mice (208). One possibility is that macrophages are not functioning as accessory cells. A second is that a basic change in the architecture of the lymphoid areas within the spleen may be occurring in addition to the increasing migration of lymphoid cells from the central lymphoid organs. In relation to this point, Williams and Nossal (191) previously demonstrated in young rats that follicle formation increases only gradually over the first few weeks of life as does the ability to retain antigen in lymphoid tissue (192). Antigen localization and distribution in young C57BL/6 mice will be examined in Chapter III.

It was concluded from the results presented above that the antigen processing system was crucially involved in the increasing immune potential displayed by neonatal mice. Subsequent studies which were undertaken to further define some of the functional characteristics of this system and how they changed with age will be described in the following Chapter.

CHAPTER II

AGE DEPENDENT CHANGES IN SENSITIVITY TO ANTIGEN IN THE MOUSE

INTRODUCTION

In the previous Chapter, the development of the immune potential of neonatal C57BL/6 mice aged from birth to two weeks of age was described in terms of the magnitude of an antibody response which could be elicited by a single dose of antigen. It was demonstrated that antibody responsiveness increased markedly after birth. Bosma *et al.* (188) and Price and Makinodan (209) have shown that with increasing age antibody responsiveness in mice reaches a maximum during adult life and subsequently declines. In addition, these authors reported that the decline in responsiveness during senescence, as manifested by a decreased ability to produce antibody, was accompanied by a reduction in sensitivity to antigen. This reduction in sensitivity was demonstrable as an increase in the amount of antigen required to stimulate a maximal antibody response.

In order to establish whether the relatively low responsiveness of neonatal mice was similarly associated with a low sensitivity to antigen, adult and neonatal mice were compared in terms of antigen sensitivity. This parameter was defined by constructing dose-response curves for the IgM and IgG-2a PFC responses. These functions related the dose of antigen to the maximum PFC response which that particular dose was able to elicit, regardless of any variations in the kinetics of the response. The dose of antigen which provided the maximum PFC response was considered to be a measure of antigen sensitivity, i.e. a low optimum dose indicated high sensitivity and vice-versa.

The maturation of antibody responsiveness in neonatal mice was studied by comparing the antigen sensitivities of seven-day-old and twelve-day-old mice to twelve-week-old mice. The following considera-

tions provided the rationale for choosing these particular age groups. Twelve-week-old mice were considered to be fully developed adults since a) their body weights were maximal or nearly so at this age and b) they exhibited maximum levels of antibody production (188). These mice, therefore, provided a reference for the evaluation of neonatal responsiveness. As had been shown in Chapter I (Figure 3), the earliest age at which intact C57BL/6 mice could reproducibly generate both IgM and IgG PFC responses was at twelve days of age. The determination of antigen sensitivity in these mice allowed comparisons to be made for both classes of antibody. Mice younger than twelve days of age either did not respond at all to SRC or responded with IgM PFC only. Seven-day-olds were found to be the youngest mice capable of producing a reproducible PFC response *in situ*. This response was found to be exclusively IgM.

The antigen sensitivities of neonatal and adult mice were established and compared in two different ways:

1. Immunization of Intact Mice

In this approach, antigen dose-response curves were determined by injecting varying doses of antigen into intact mice and quantitating the ensuing antibody responses. It was predicted that the sensitivities exhibited by the different age groups would reflect the state of maturation and the antigen processing system.

2. Adoptive Immunization of Adult Irradiated Recipients

A second approach involved the adoptive transfer of spleen cells from 12-day-old or 12-week-old mice to syngeneic adult irradiated recipients. The sensitivity of these cells to antigen in the irradiated recipients was then determined. Since the irradiated recipients provided a functional antigen processing system, the stimulation of immunocompetent cells on a common antigen processing background was achieved.

Thus, differences observed between the two age groups would be expected to be solely derived from the immunocompetent cell compartment, and not due to differences in antigen processing.

The remainder of this Chapter deals with the derivation of antigen dose-response curves in the two aforementioned systems.

RESULTS

1. Immunization of Intact Mice

In order to establish a dose-response curve for neonatal mice, several groups of twelve-day-old mice were injected i.p. with SRC covering the dose range of 1×10^8 to 1×10^{10} SRC/mouse. Within any one group all mice were injected with the same dose of SRC.

Between four and nine days following injection of antigen, mice in each group were killed on a daily basis, their spleens removed, and the number of IgM and IgG PFC present in the corresponding cell suspensions determined. The maximum PFC values attained by each group during this time were used to compute the dose-response curves. For twelve-day-old mice the maximum IgM responses usually occurred on day 6 or day 7 following antigen injection, whereas the maximum IgG responses occurred on day 7 or day 8. A similar experimental protocol was followed using twelve-week-old mice. In this case, however, the maximum IgM and IgG PFC responses usually occurred on days 5 and 6, respectively.

A common feature of the antigen dose-PFC response relationships thus obtained was that with increasing doses of antigen, increasing maximal responses were obtained. Further increases in antigen concentration, however, led to reduced maximal responses. Thus, the dose-response relationships appeared to be parabolic in nature.

In order to obtain a more comprehensive definition of this relationship, the data were subjected to a polynomial regression analysis using a CDC-1700 computer. It was found that over the dose range studied the logged data conformed very closely to an equation of the general form $y = a + bx + cx^2$, where $y = \log_{10}$ maximum PFC response

and $x = \log_{10}$ (SRC injected). The partial regression coefficients a, b and c were computed for each dose-response curve by the method of least squares. The treatment of the data in this way allowed the calculation of the dose of SRC (x_{OPT}) which elicited the maximum PFC response Y_{MAX}. Thus equating the first derivative of the parabolic function to zero and solving for x, the relationship $x_{OPT} = \frac{-b}{2c}$ was obtained; the antilog of which yielded the optimum dose.

The computed dose-response curves derived from the IgM and IgG PFC responses of twelve-day-old mice are presented in Figure 9. In both cases it was found that the data conformed very closely ($p < .001$) to parabolic functions. In addition, it was found that the optimum doses for the IgM and IgG PFC responses were different by a factor of 2.8; the IgG PFC response having a higher optimum (3.4×10^9) than the IgM PFC response (1.2×10^9).

The computed dose-response curves obtained using twelve-week-old mice are illustrated in Figure 10. As found in the previous experiment, parabolic functions provided a close fit for both the IgM PFC ($p < 0.001$) and the IgG PFC ($p < 0.01$) responses. The optimum antigen doses were calculated as 2.9×10^8 for the IgM PFC response and 1.0×10^9 for the IgG PFC response - a 3.4-fold difference.

A summary of the optimum antigen doses derived from Figures 9 and 10 is presented in Table II. A comparison of the IgM PFC responses indicated that in order to mount a maximum response the twelve-day-old mice required a 4.1-fold higher antigen dose (1.2×10^9) than the twelve-week-old mice (2.9×10^8). For the IgG PFC responses, the young mice responded maximally at a dose of 3.4×10^9 SRC/mouse. This figure can be compared to 1.0×10^9 obtained for the older mice - a change in

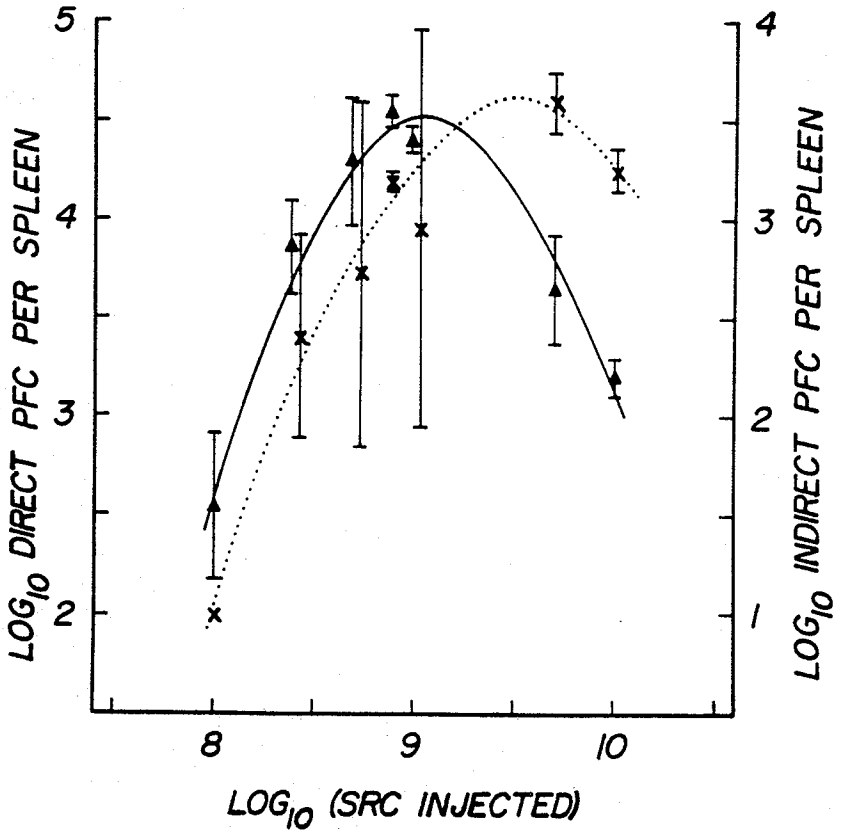


Figure 9:

Maximum anti-SRC PFC responses by 12-day-old C57BL/6 mice. Seven groups of 12-day-old C57BL/6 mice were injected i.p. with different doses of SRC and the maximum number of direct and indirect PFC appearing subsequently in the spleens were determined. The direct PFC dose-response curve (——) was computed by the method of least squares and was found to fit ($F_{2,29}$ = 18.17, $p < 0.001$) the equation $y = -135.4 + 30.85x - 1.701x^2$; where y = $\log_{10}$ maximum PFC response, and x = $\log_{10}$ (SRC injected). The indirect PFC dose response curve (·····) was found to fit ($F_{2,31}$ = 34.42, $p < 0.001$) the equation $y = -95.25 + 20.75x - 1.089x^2$. Each point represents the mean (± 1 S.E.) of the logarithms of 3 to 10 individual PFC responses; (▲), direct PFC; (x), indirect PFC.

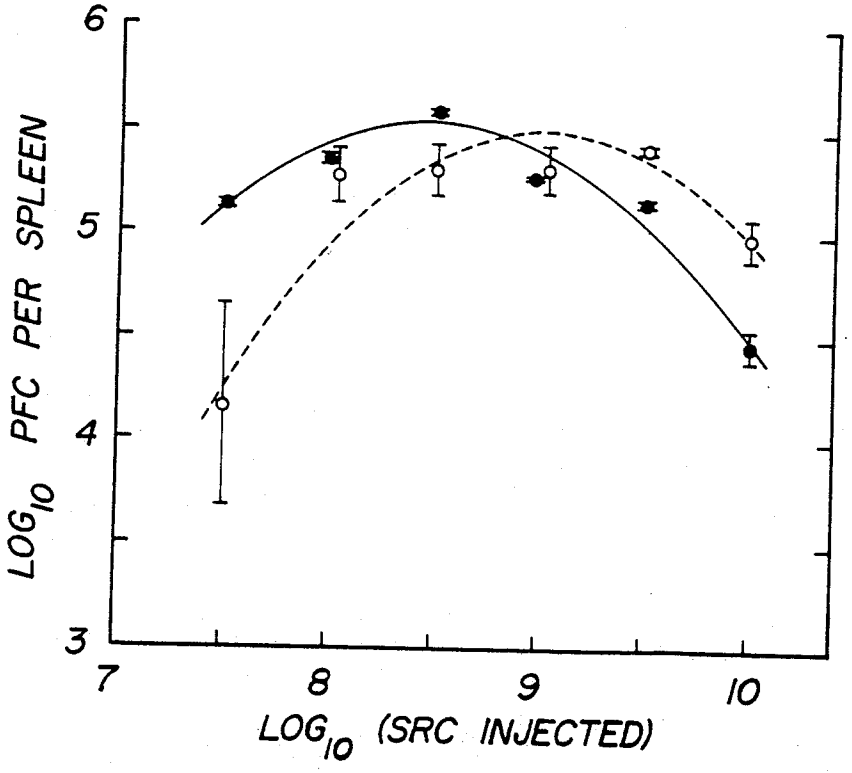


Figure 10:

Maximum anti-SRC PFC responses by 12-week-old C57BL/6 mice. Experimental design was similar to that for Figure 9. The direct PFC dose-response curve (———) was computed by the method of least squares and found to fit ($F_{2,36} = 159.1$, $p < 0.001$) the equation $y = -25.95 + 7.441x - 0.440x^2$; where $y = \log_{10}$ maximum PFC response, and $x = \log_{10}$ (SRC injected). The indirect dose response curve (-----) was found to fit ($F_{2,15} = 7.241$, $p < 0.01$) the equation $y = -38.49 + 9.758x - 0.541x^2$. Each point is based on the analysis of spleen pools with each pool derived from 3 to 6 spleens. The mean (± 1 S.E.) of the logarithms of the PFC determinations at each dose is plotted (●), direct PFC; (○), indirect PFC.

TABLE II

OPTIMUM ANTIGEN DOSES CALCULATED FROM THE COMPUTED DOSE-RESPONSE CURVES PRESENTED IN FIGURES 9 AND 10

Age Group	Optimum Dose	
	IgM	IgG
12-day-old	1.2×10^9	3.4×10^9
12-week-old	2.9×10^8	1×10^9
Ratio: 12 days/12 weeks	4.1	3.4

sensitivity of 3.4-fold. Thus the transition from neonatal to adult life was accompanied by an increase in sensitivity to antigen. This increase was apparent when both the IgM and IgG PFC responses were compared.

When the PFC responses of twelve-day-old and twelve-week-old mice were compared in this way, one factor which had to be evaluated was the difference in blood volumes in the two age groups. Thus for any given dose of antigen administered i.v. or i.p., the effective antigen dose would be expected to be higher in the neonatal mice than in the adult mice due to the smaller blood volume of the former group. In order to compensate for this difference, the ratio of the body weights was used to normalize the data. It was assumed that the blood volume:body weight ratios were comparable for the two groups of mice. The average body weight for the C57BL/6 mice used in these experiments was 5.35 g ± 0.92 (S.D.) for the twelve-day-old group and 20.06 g ± 1.52 for the twelve-week-old group. To determine a value for the effective concentrations of antigen in the twelve-day-old mice, the dose of antigen injected into this group was multiplied by 3.74, the ratio of the body weights. Figure 11 presents a replot of the IgM PFC dose-response curves for the two groups of mice. Actual doses are plotted for the twelve-week-old mice, and normalized doses (actual dose x 3.74) for the twelve-day-old group. Figure 12 expresses the corresponding data for the IgG PFC responses. From a comparison of these two figures it is evident that the introduction of the body weight factor has served to emphasize the difference in sensitivity between the two groups (15-fold for the IgM PFC response and 13-fold for the IgG PFC response).

It had been observed when comparing the dose-response curves for

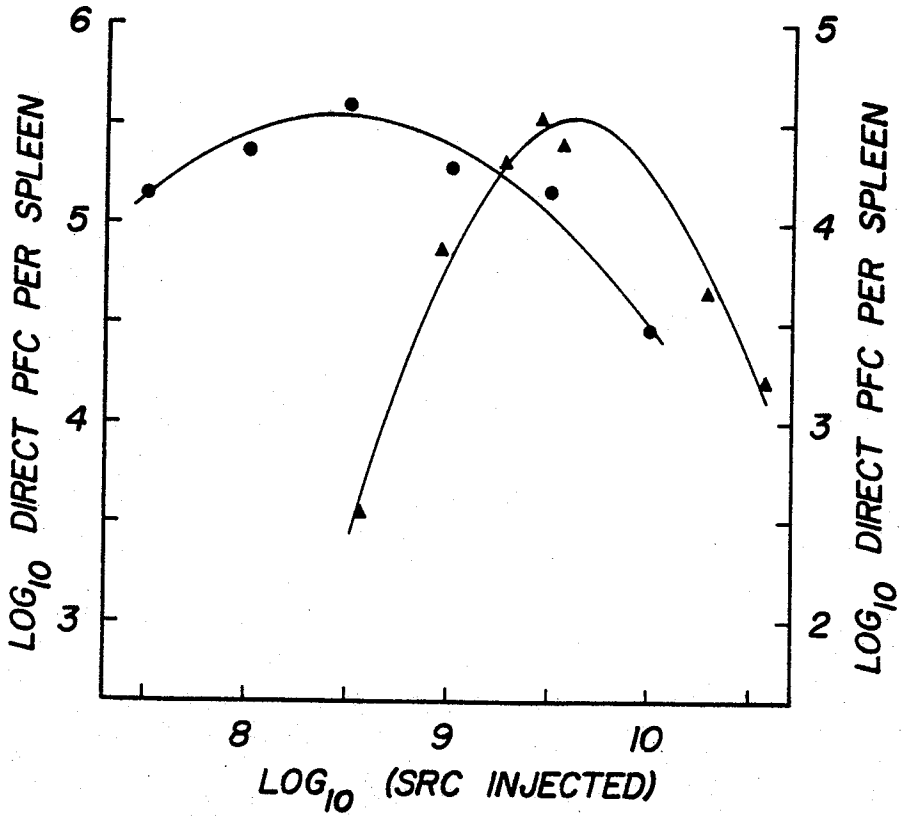


Figure 11:

A comparison of the direct PFC dose-response curves derived from 12-day-old (right scale) and 12-week-old (left scale) C57BL/6 mice. The direct PFC response for 12-week-old animals (●) has been plotted as in Figure 10. The direct PFC dose-response curve for 12-day-old mice (▲) has been normalized by multiplying the actual doses of SRC injected by 3.74 (the body weight factor).

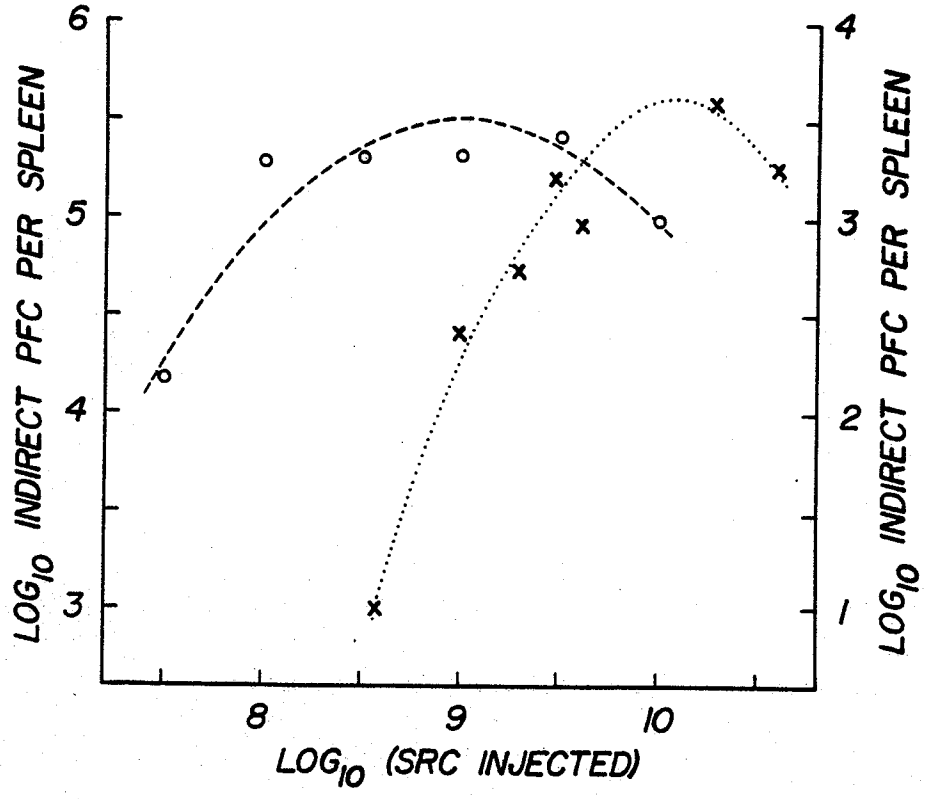


Figure 12:

A comparison of the indirect PFC dose-response curves derived from 12-day-old (right scale) and 12-week-old (left scale) C57BL/6 mice. The indirect PFC response for 12-week-old animals (O) has been plotted as in Figure 10. The indirect dose-response curve for 12-day-old mice (x) has been normalized by multiplying the actual doses of SRC injected by 3.74 (the body weight factor).

the two groups of mice that not only were the optimum antigen doses different but, in addition, the broadness of the curves appeared to be different. The adult mice appeared to be able to respond maximally or nearly so over a wide antigen dose range, whereas the young mice responded maximally only over a relatively restricted antigen dose range. In order to quantitate this observation, the concept of bandwidth (BW) was introduced. BW was defined as the ratio of the two antigen doses which could elicit a PFC response equal to 50% of the maximum response. Since it could be shown that log BW was directly proportional to 1/c, the partial regression coefficient c and its confidence limits were used to assess the BW of the dose response curves.

The c values for the dose-response curves are tabulated in Table III. By applying a two-tailed t-test (using Welch's procedure for unequal variance) it was concluded that: i) the c values (and, therefore, BW) for the younger group differed from the c values (BW) of the older group ($p < 0.05$ for the IgM and $p < 0.15$ for the IgG PFC responses; and ii) there were no significant differences betweed c values (BW) within an age group.

The bandwidths of the dose-response curves were determined by solving the quadratic equations for the values of x when $y = Y_{MAX/2}$. Thus for each dose-response curve, two antigen doses were obtained which elicited 50% of the maximum PFC response. The ratio of these two doses, i.e. the bandwidth by definition, was used to compare neonatal and adult responsiveness. For the IgM responses, the bandwidths were found to increase 6.5-fold with increasing age. Similarly, the bandwidths for the IgG responses increased 2.7-fold.

Table IV presents the IgM PFC responses of seven-day-old C57BL/6

TABLE III

THE COMPUTED VALUES OF THE PARTIAL REGRESSION COEFFICIENT c FOR THE DOSE-RESPONSE CURVES PRESENTED IN FIGURES 9 AND 10

Age Group	c ± 1 S.D.	
	IgM	IgG
12-day-old	-1.701 ± 0.313	-1.089 ± 0.314
12-week-old	-0.440 ± 0.032	-0.541 ± 0.173

TABLE IV

THE IgM PFC RESPONSES OF INTACT 7-DAY-OLD C57BL/6 MICE TO SRC

Dose of SRC	IgM PFC/Spleen[a]
1.0×10^8 (6)[b]	532 ± 137[c]
7.5×10^8 (5)	1750 ± 800[c]
2.0×10^9 (3)	3320 ± 180[d]
6.0×10^9 (3)	595 ± 5[d]

[a]Maximum PFC determined on day 6 or 7 ± 1 S.E.

[b]Numbers in parentheses indicate number of mice in determination.

[c]Determinations made in individual spleens.

[d]Determinations made on pool of spleens.

mice at four antigen doses. Although the data are not sufficiently comprehensive to justify the detailed mathematical analysis used above, the results indicate that seven-day-old mice exhibit an optimal antigen dose at least as high as the twelve-day-old mice, i.e. 1-2 x 10^9. Thus, as with the twelve-day-old mice, seven-day-old mice required more antigen to optimally elicit the PFC responses than did their adult counterparts.

2. Immunization of Neonatal Spleen Cells in Adult Irradiated Recipients

In the preceding section, neonatal and adult mice were compared in terms of their respective dose-response curves. The comparisons were made on the basis of two parameters: a) optimal antigen doses and b) bandwidths. The following series of experiments was designed to establish the extent to which the antigen-processing system was responsible for the differences in optimal antigen doses and bandwidths described earlier.

The basic experimental design consisted of the stimulation of mouse spleen cells by SRC in irradiated syngeneic mice. The spleen cells were taken from two groups of normal mice; a) animals that were 12 or 13 days old and b) animals that were 12 weeks old. Dose-response curves were established for the two groups in the following way. C57BL/6, aged 10-12 weeks were irradiated (770 rads) and 2-4 hours later divided into two groups. Each mouse from group one received 4 x 10^7 spleen cells from 12- to 13-day-old donors; each mouse in the second group received 4 x 10^7 spleen cells from 12-week-old donor mice. Within each group, five different doses of antigen were used. On days 8 and 9 after the injection, spleens were removed from six mice at each antigen dose and assayed for IgM and IgG PFC. It was previously established that the

maximum responses occurred during this period.

As had been found previously using intact mice, the dose-response curves obtained with irradiated recipients were found, over the dose range studied, to be parabolic in nature. In Figure 13 the logarithm of the maximum IgM PFC per spleen attained is expressed as a function of the logarithm of the number of SRC injected which elicited that response. Twelve-day-old and twelve-week-old donor mice are compared. Similarly, Figure 14 presents the corresponding dose response curves for the IgG PFC responses. For all four sets of responses, the data were found to conform very closely ($p < 0.001$) to parabolic functions.

The optimum antigen doses calculated from the computed dose-response curves are presented in Table V. It can be seen that for the IgM PFC response, the optimum antigen doses for the twelve-day and twelve-week donors are essentially identical. This is also apparent for the IgG PFC response. It had previously been observed (Table II) that the ratios of the optimum antigen doses for the two age groups using intact mice had been 4.1 for the IgM and 3.4 for the IgG PFC responses. However, when immunocompetent cells from these two age groups are compared in irradiated recipients, the ratios of the optimum doses approach unity (0.71 for the IgM and 0.70 for the IgG PFC responses). Thus it was concluded that in terms of optimum antigen dose, the use of irradiated recipients effectively eliminated the difference in responsiveness of immunocompetent cells from the two age groups.

When the dose-response curves were compared in terms of the bandwidths, the two donor groups exhibited a difference with the older animals yielding curves with greater bandwidths. Table VI summarizes the partial regression coefficient c derived from the dose-response

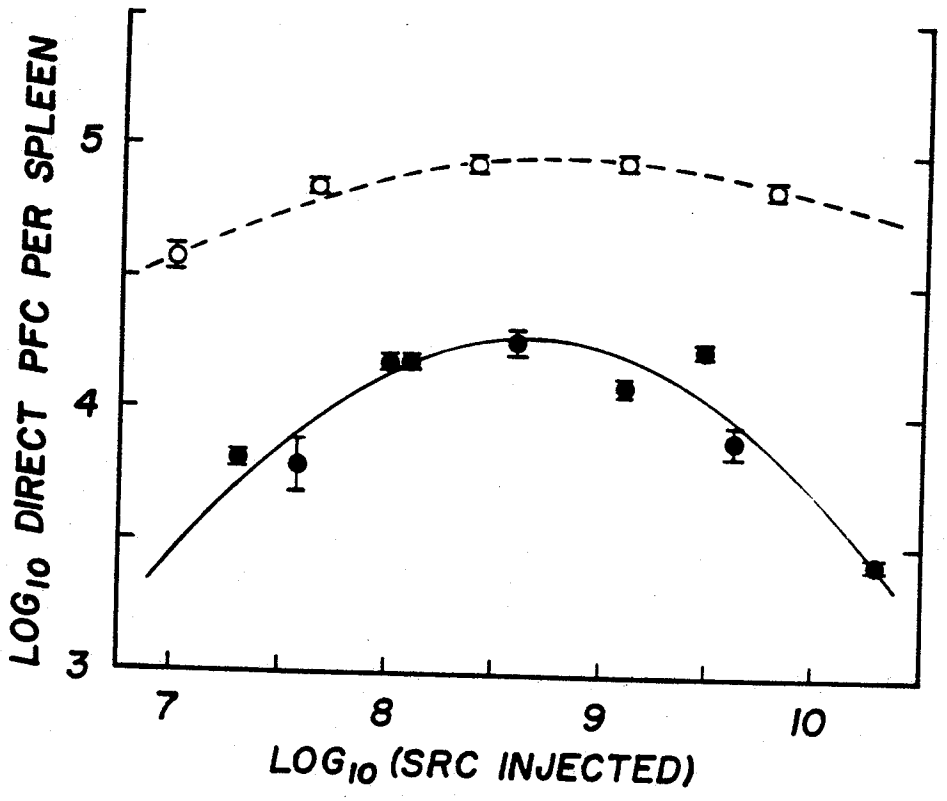


Figure 13:

The direct anti-SRC PFC responses of irradiated C57BL/6 mice reconstituted with normal syngeneic spleen cells from either 12-day-old or adult mice. The dose-response curve obtained by using 12-day-old donors (●) was found to fit ($F_{2,24} = 53.8$, $p < 0.001$) the equation $y = -18.7385 + 5.3102x - 0.3064x^2$ where $y = \log_{10}$ direct PFC/spleen and $x = \log_{10}$ (SRC injected). The dose-response curve obtained by using adult donors (○) was found to fit ($F_{2,11} = 34.8$, $p < 0.001$) the equation $y = -4.2232 + 2.0872x - 0.1184x^2$. Each point is based on the analysis of spleen cell pools with each pool derived from 6 spleens. The mean (± 1 S.E.) of the logarithms of 2 to 4 PFC determinations at each antigen dose is plotted.

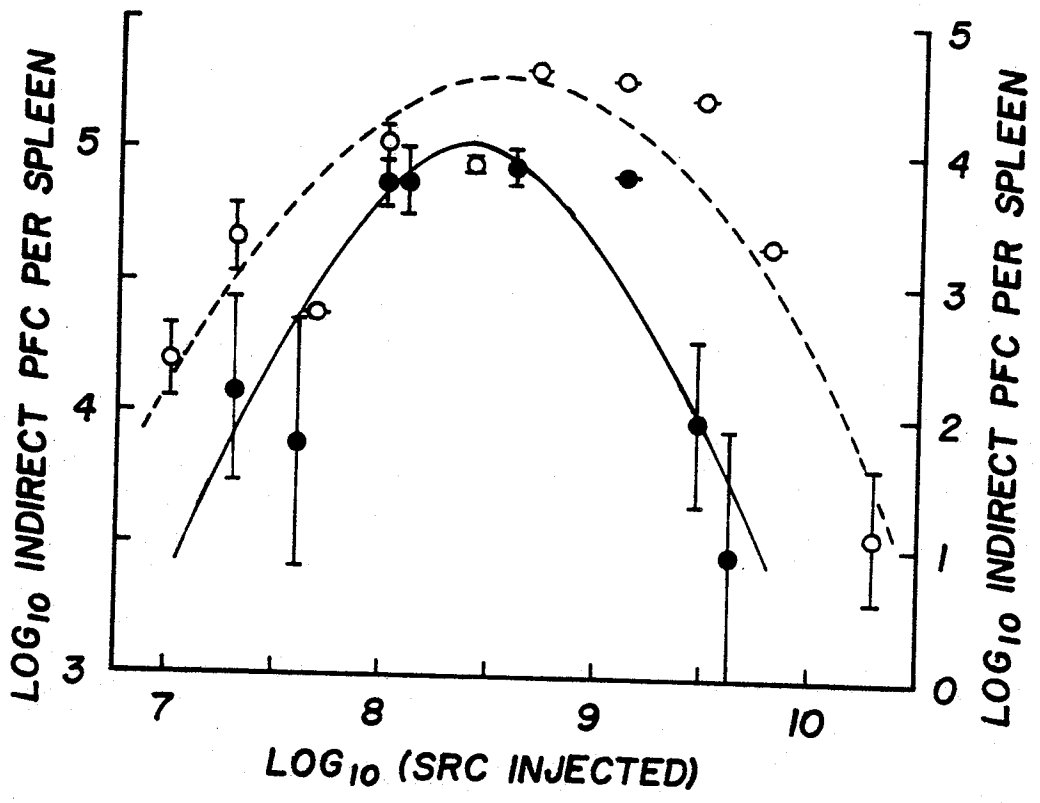


Figure 14:

The indirect anti-SRC PFC responses of irradiated C57BL/6 mice reconstituted with normal syngeneic spleen cells from either 12-day-old or adult mice. The dose-response curve obtained by using 12-day-old donors (●, right scale) was found to fit ($F_{2,30} = 10.2$, $p < 0.001$) the equation $y = -120.6243 + 29.6573x - 1.7651x^2$ where $y = \log_{10}$ indirect PFC/spleen and $x = \log_{10}$ (SRC injected). The dose-response curve obtained by using adult donors (○, left scale) was found to fit ($F_{2,22} = 40.9$, $p < 0.001$) the equation $y = -31.0503 + 8.4944x - 0.4965x^2$. Each point is based on the analysis of spleen pools with each pool derived from 6 spleens. The mean (± 1 S.E.) of the logarithms of 2 to 6 PFC determinations at each antigen dose is plotted.

TABLE V

OPTIMAL ANTIGEN DOSES CALCULATED FROM THE COMPUTED DOSE-RESPONSE CURVES PRESENTED IN FIGURES 13 AND 14

Age of Donors	Optimum Dose	
	IgM	IgG
12 days	4.63×10^8	2.52×10^8
12 weeks	6.52×10^8	3.58×10^8
Ratio: 12 days/12 weeks	0.71	0.70
Ratio: 12 days/12 weeks (using intact mice, Table II)	4.1	3.4

TABLE VI

THE COMPUTED VALUES OF THE PARTIAL REGRESSION COEFFICIENT c FOR THE DOSE-RESPONSE CURVES PRESENTED IN FIGURES 13 AND 14

Age of Donors	c ± 1 S.D.	
	IgM	IgG
12 days	-0.3064 ± 0.0310	-1.7651 ± 0.3997
12 weeks	-0.1184 ± 0.0195	-0.4965 ± 0.0554

curves presented in Figures 13 and 14. By applying a two-tailed t-test it was concluded that for both the IgM and IgG PFC responses, the younger mice exhibited larger c-values (narrower bandwidths) than the adult mice ($p < 0.05$). This conclusion was consistent with the previous observations using intact mice. The increase in bandwidth with age was 16.1-fold for the IgM responses and 5.4-fold for the IgG responses. It was observed, therefore, that immunocompetent cells derived from twelve-day-old mice exhibited narrower bandwidths relative to that obtained with adult immunocompetent cells, regardless of whether they were challenged with antigen *in situ* or in irradiated recipients.

In summary, analysis of the dose-response curves of irradiated recipients indicated: 1) that the dose of antigen required to elicit the optimum antibody response was essentially identical for both age groups and 2) the bandwidths obtained using neonatal donors were significantly narrower than those obtained with adult donors.

DISCUSSION

The relative radioresistance of accessory cells (33,35) has facilitated a study of the role of these cells in the anti-SRC response, in particular a definition of their properties in the maturation phenomena which are described in this thesis. By determining dose-response relationships for the two groups of mice of different age (twelve days, twelve weeks) using irradiated recipients, the individual antigen-processing or accessory cell systems were effectively replaced with a single common system, that of the irradiated recipient. Since the individual contributions of the antigen-processing systems had been effectively removed in this adoptive transfer system, any differences remaining between the twelve-day and twelve-week-old groups could be attributed to other factors.

Two aspects of the dose-response curves merit discussion; the antigen optimum dose and the bandwidth.

It was demonstrated that when measured in intact mice, the antigen optimum doses of neonates were higher than those of adult mice (Table II). This was observed for both the IgM and IgG responses. Provision of a common antigen-processing system, in the adoptive transfer system, resulted in the elimination of this difference (Table V). Therefore, on this basis, it was logical to conclude: i) that the antigen optimum dose was a function of the antigen-processing system and ii) that the shift of the antigen optimum doses from relatively high to lower values was the direct result of a maturation of the antigen-processing system which occurred during the period between neonatal and adult life.

The other parameter of the dose-response curves to be discussed is the bandwidth. The bandwidth of such curves can be quantitated in

different ways, such as: the ratio of values of x (antigen dose) which will elicit a response y equal to 50% of Y_{MAX}, or simply as the value of the partial regression coefficient c for the computed parabola; the value of c being inversely proportional to the logarithm of the bandwidth. Since c could be mathematically derived and analyzed, the bandwidths were evaluated in terms of this statistic. Using intact mice it was demonstrated that the c values derived from neonatal dose-response functions were different from those derived from adult mice - the former group exhibiting narrower bandwidths (Table III). In the irradiated recipient system, these differences were maintained, inasmuch as immunocompetent cells from neonatal donors exhibited narrower bandwidths than did their adult counterparts (Table VI). Evidently the cellular basis for the increased bandwidth with increasing age was preserved in the irradiated recipients. Since the provision of a common antigen-processing system did not eliminate the differences observed, it was concluded that, in this operational context, bandwidths, unlike optimum antigen doses, were not related to the state of maturation of the antigen-processing systems. It was concluded, therefore, that the changes observed in bandwidths as a function of age were a reflection of intrinsic changes in the immunocompetent cell populations rather than changes in the antigen-processing systems.

The following Chapter will describe experiments which were designed to further investigate the underlying mechanisms involved in the relative unresponsiveness of neonatal mice. In these experiments particular emphasis was placed on the state of maturation of the antigen-processing system in neonatal mice. Other factors, such as T-cell deficiency and antigenic recruitment of immunocompetent cells will also be considered.

CHAPTER III

INTRODUCTION

In the previous two Chapters, the development of IgM and IgG antibody producing ability was described for neonatal C57BL/6 mice. In Chapter I, it was demonstrated that during the first week following birth, intact mice were able to mount only feeble IgM anti-SRC responses, whereas in the second week of life they demonstrated dramatic increases in both IgM and IgG antibody-forming capabilities. In contrast, when spleen cells from mice of the same age range were challenged in irradiated recipients, a gradual linear increase in responsiveness was observed. In the second Chapter, a second aspect of the immune response of neonatal mice was studied. The sensitivity of 12-day-old mice was compared to that of adults and it was found that neonatal mice were less sensitive to antigen than adult mice and, furthermore, responded optimally over a relatively restricted range of antigen doses.

It was concluded from these findings that the low responsiveness of neonatal C57BL/6 mice to SRC was largely due to a functionally deficient antigen processing system. In this Chapter further analysis of the antigen-processing system of neonatal mice will be described. In addition, other factors such as tissue localization of antigen, cellular recruitment by environmental antigens, and possible T-cell deficiencies were examined.

The experimental approaches to be described will be presented under the following headings:

1) Evaluation of A-Cell Frequencies in Neonatal and Adult Mice;
2) Detection of the A-Cell Compartment Through Antibody-Mediated Suppression;

3) Tissue Distribution of SRC in Neonatal and Adult Mice;

4) Selective Recruitment of Antigen Sensitive Cells by Environmental Antigens;

5) Partial T Cell Deficiencies in Neonatal Mice.

1) Evaluation of A Cell Frequencies in Neonatal and Adult Mice

The results presented in Chapters I and II were consistent with there being a deficiency in the antigen-processing system of neonatal mice. In order to demonstrate such a deficiency in a direct manner, a series of experiments was undertaken to describe the antigen-processing system in more quantitative terms. This was done by utilizing a procedure described by Gorczynski *et al.* (36) in which the antigen-processing system was quantitated in terms of relative A cell frequencies. In utilizing this experimental approach it was reasoned that if neonatal mice possessed a functionally immature antigen-processing system, relatively low A cell frequencies would be expected in the spleens of these mice. In the experiments described below this procedure was used to determine the relative A cell frequencies of neonatal and adult spleens. In its simplest form the A cell assay uses an A cell depleted recipient, designated $B^{+}T^{+}A^{-}$. Into this recipient are transferred the cells of the A cell donor, the lymphocytesof which were previously inactivated by an appropriate treatment, such as by exposure to γ-irradiation, to yield cell populations designated as $B^{-}T^{-}A^{+}$. Thus, by combining the $B^{+}T^{+}A^{-}$ recipient's cells with the $B^{-}T^{-}A^{+}$ cells of the donor, it is possible to vary the A cell source and examine the effect on the antibody response. In practice, it was found necessary to use a lymphocyte and A cell depleted recipient mouse, designated $B^{-}T^{-}A^{-}$. Such mice are completely immunoincompetent. These mice are subsequently repopulated with two functionally distinct populations of cells; the first providing immunocompetent cells but not A cells, i.e. $B^{+}T^{+}A^{-}$, and the second providing A cells but not

immunocompetent cells, i.e. $B^-T^-A^+$. The assay relies on the demonstration that following challenge with SRC, a PFC response is obtained only when all three cell types are present, i.e. $B^+T^+A^+$. Thus, when a common pool of immunocompetent cells ($B^+T^+A^-$) is used, the A cell contents of two different cell populations can be compared by determining the relative magnitudes of the resultant PFC responses.

Gorczynski *et al.* described two procedures for creating an A cell deficiency *in vivo*:

1. Whole body irradiation followed by an elapse of 72 hours before reconstitution with spleen cell suspensions. It was demonstrated that three days after irradiation (950 rads) the spleens of treated mice were functionally A cell deficient. Such mice ($B^-T^-A^-$) could not generate an anti-SRC PFC response when reconstituted with only immunocompetent cells ($B^+T^+A^-$), but did so when a source of A cells ($B^-T^-A^+$) was injected together with the ($B^+T^+A^-$) cells.

2. The injection of large numbers of horse erythrocytes. The same authors also demonstrated that 24 hours following the injection of 2×10^{10} HRC into normal mice, the A cell activity in these mice was much reduced. Such mice were designated horse cell treated or HT-mice. Thus, functionally these mice were considered as $B^+T^+A^-$, and produced very low anti-SRC responses unless a source of A cells was provided.

In the experiments reported here, the production of A cell deficient mice was initially attempted using the irradiation method. Therefore, mice were irradiated (770 rads) and at times ranging from 2 to 72 hours after irradiation, groups of these mice received a single i.v. injection of SRC and spleen cells from HT-mice ($B^+T^+A^-$). The resultant IgM and IgG PFC were enumerated seven days following injec-

tion of SRC and HT-spleen cells, and are plotted in Figure 15. The results were found to concur with those of Gorczynski *et al.* (36) inasmuch as the spleens of irradiated mice gradually lost their A cell function. By 72 hours post-irradiation, both the IgM and IgG responses had fallen to relatively low levels. In addition, the results presented here demonstrated that the IgG response was markedly more dependent on an intact A cell system than the IgM response. For example, during the time span of 2 - 72 hours studied here, the IgG response fell by a factor of 30x, as compared to a 2x drop in the IgM response.

Thus, it was concluded that the irradiation procedure did lead to a loss of splenic A cell activity, however, this method of A cell depletion was not pursued further because of the unacceptably high mortality rates encountered, especially with mice for which the time interval between irradiation and reconstitution was 48 - 72 hours. Consequently, the excess HRC method of depleting A cell function was used in all subsequent experiments.

In order to examine the relative A cell activities of spleen cells taken from 12-day-old and 12-week-old mice, the following experimental protocol was followed. All prospective recipient mice were injected with 2×10^{10} HRC i.p. 24 hours before use to render them $B^+T^+A^-$, and subsequently irradiated (770 rads) two hours prior to reconstitution. Thus, at the time of reconstitution these recipient mice were $B^-T^-A^-$. All $B^-T^-A^-$ mice were then reconstituted with lymphocytes to become $B^+T^+A^-$, and of these mice different groups were injected with varying numbers of $B^-T^-A^+$ cells derived from either 12-day-old or 12-week-old animals. The lymphocytes ($B^+T^+A^-$) were spleen cells from mice injected 24 hours previously with 2×10^{10} HRC, whereas the A cells were normal

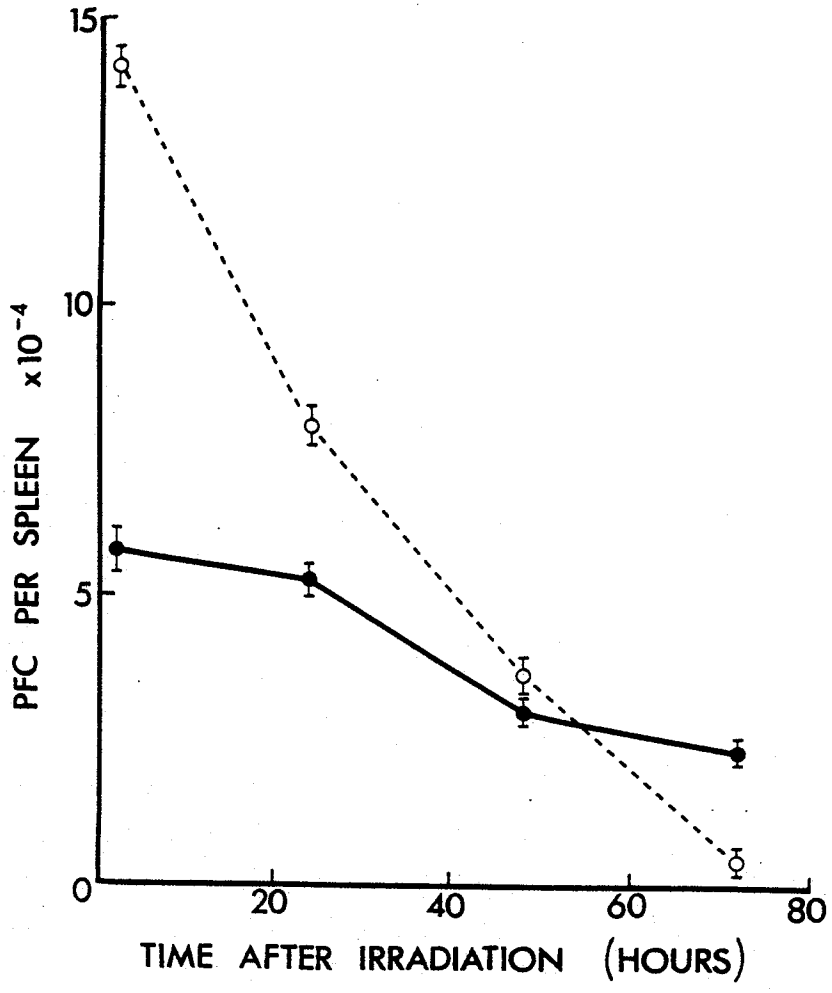


Figure 15:

The loss of A cell activity from the spleens of mice following whole body irradiation. Adult C57BL/6 mice were irradiated (770 rads) and at varying times thereafter were injected i.v. with 4×10^7 HT-spleen cells and 5×10^8 SRC. Seven days following injection, spleen cells from 4-6 recipients were pooled and assayed for direct (●) and indirect (○) PFC. The means (± 1 S.E.) of three PFC determinations are plotted.

spleen cells from either 12-day-old or adult mice irradiated *in vitro* with 1200 rads to eliminate lymphocytes, i.e. $B^-T^-A^+$. Each inoculum of cells contained 4 x 10^7 $B^+T^+A^-$ cells, 5 x 10^8 SRC and varying numbers of $B^-T^-A^+$ cells from either 12-day-old or 12-week-old mice. The IgM and IgG PFC responses were determined, in all cases, seven days after injection.

The results from such experiments are presented in Figures 16 and 17. It was concluded that the response of A cell depleted mice ($B^+T^+A^-$) to SRC was depressed for both the IgM and IgG responses, and that replenishment of the A cell compartment with an appropriate number of $B^-T^-A^+$ cells could result in a partial restoration of the response. This was, of course, fundamental to the validity of the method.

Attempts to restore the IgM response (Figure 16) by the addition of A cells was not consistently successful in that A cells from adult spleens produced only a slight elevation of the response, whereas those from 12-day-old spleens elevated the response, but over only a limited A cell dose range. Consequently, it was concluded that over the dose range studied, no significant difference between the two age groups, in terms of A cell frequency, was revealed in their ability to restore the IgM response.

The pattern of responsiveness observed for the IgG response (Figure 17) was markedly different from that obtained with the IgM response. An examination of the data indicated that over the dose range of A cells studied, an inverse correlation was obtained between the number of A cells injected and the subsequent IgG-PFC response. This inverse relationship was apparent with A cells from either 12-day-old or 12-week-old mice, and as the numbers of A cells were increased, the IgG

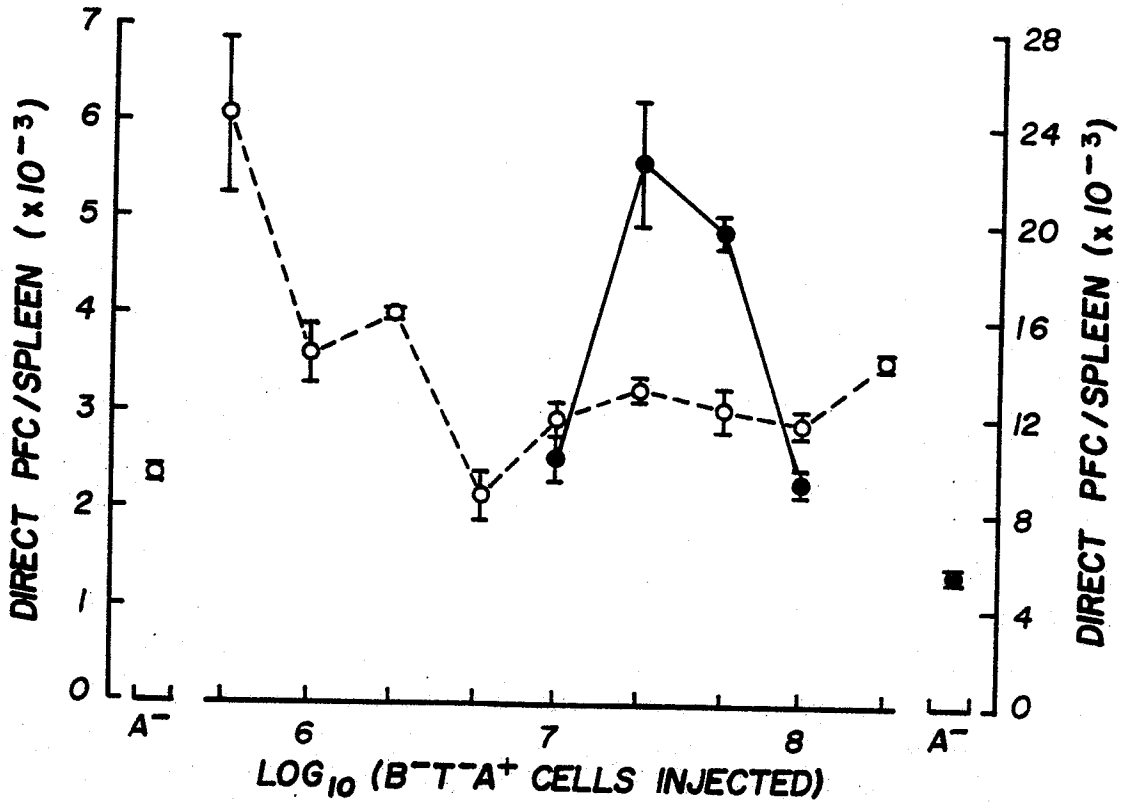


Figure 16:

A comparison of the A cell content of 12-day-old and adult C57BL/6 mouse spleens. Recipient mice were depleted of A cells, irradiated and reconstituted with 4 x 10^7 $B^+T^+A^-$ adult spleen cells along with varying numbers of irradiated syngeneic spleen cells ($B^-T^-A^+$) from either 12-day-old (●, right scale) or adult (○, left scale) mice. PFC responses were determined 7 days later. Each point is based on the analysis of spleen pools, with each pool derived from 6 recipient spleens. The mean (± 1 S.E.) of 6 to 12 PFC determinations is plotted at each concentration of A cells.

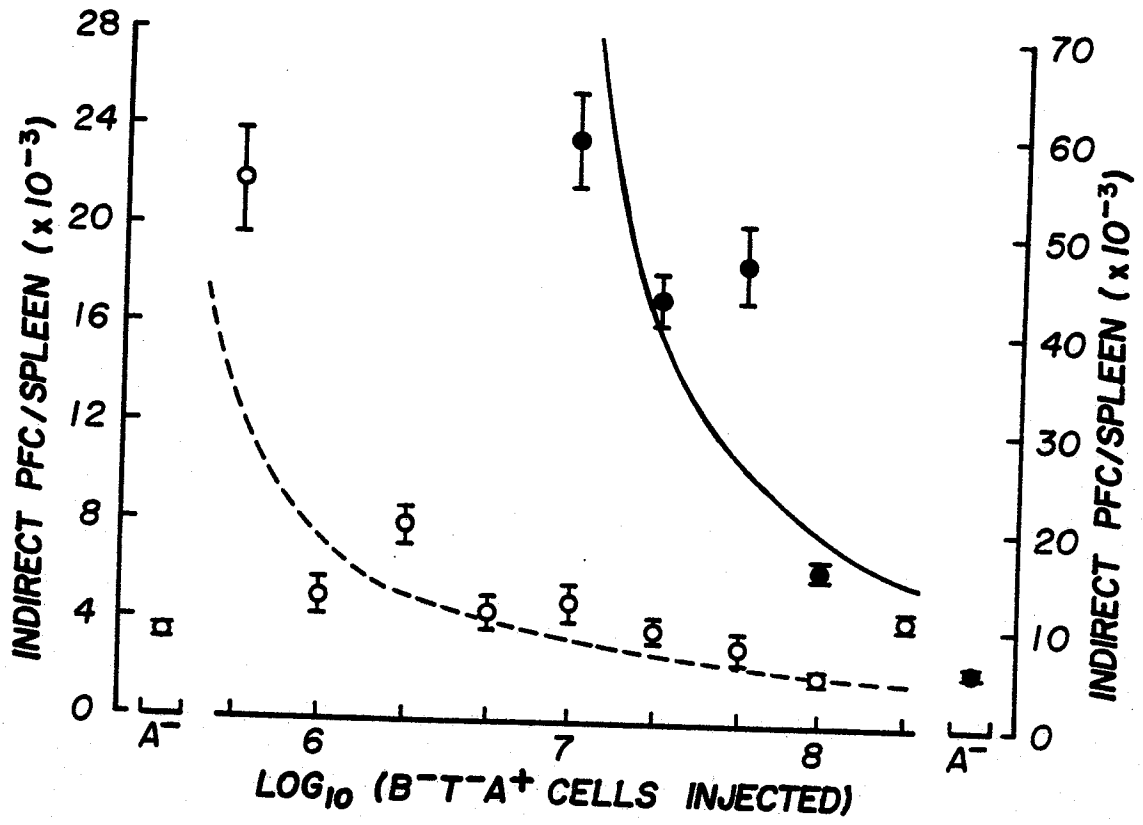


Figure 17:

A comparison of the A cell content of 12-day-old and adult C57BL/6 mouse spleens. Methods as described in Figure 16. The ability of $B^-T^-A^+$ cells from 12-day-old donors (●, right scale) to restore the indirect PFC responses of recipient mice is described by the equation $y = 10^4/(0.4125x - 2.8403)$; ($F_{1,22} = 34.6$, $p < 0.0001$) where y = indirect PFC/spleen and $x = \log_{10}$ ($B^-T^-A^+$ cells injected). The A cell dose-response curve using adult donors (○, left scale) was found to fit ($F_{1,65} = 19.7$, $p < 0.0001$) the equation $y = 10^4/(1.7207x - 8.9562)$. Each point is based on the analysis of spleen pools, with each pool derived from 6 recipient spleens. The mean (± 1 S.E.) of 6 to 12 PFC determinations is plotted at each concentration of A cells.

PFC responses approached background (A^-) levels. However, the two groups of A-cell donors yielded separate and distinct PFC response curves, with the curve derived from 12-day-old donors being shifted to higher cell numbers. In neither case was an optimum level of IgG responsiveness obtained. It was, therefore, concluded that for both 12-day-old and 12-week-old donors, a greater than optimum number of A cells was present, and that the curves obtained were, in fact, the declining portions of possibly bell-shaped curves. It was estimated that the relative positions of the two curves were displaced along the x-axis by a factor of approximately 100. This finding was consistent with there being a 100-fold higher frequency of A cells in adult spleens than in 12-day-old spleens.

A comparison of the results in Figures 16 and 17 indicated that over the dose range studied, $B^-T^-A^+$ cells from adult donors were able to reconstitute the IgG response, but not the IgM response. This may be a reflection of some underlying differences in the mechanisms of IgM and IgG production. In general, evidence which has accumulated from many workers on the mechanism of interaction among B, T and A cells suggests that a) IgM responses are less dependent on T help than are IgG responses (207) and b) thymus dependence of the antibody response is frequently associated with A cell involvement (210-212). On this basis, the relationship between the number of A cells added and the IgM response may not be a reliable indicator of the role of A-cells in the corresponding dose-response curves that are described here.

In summary, an attempt was made to establish the relative numbers of A-cells detectable in 12-day-old and 12-week-old mice. Quantitation of the antigen-processing function using A cell depleted and partially

restored mice indicated that when IgG responses were compared, a 100-fold higher frequency of A-cells was demonstrated in adult spleens as opposed to neonatal spleens.

2) Detection of the A Cell Compartment Through Antibody-Mediated Suppression

During the period of this investigation (1970-1974), a large number of papers were published in the general area of antibody-mediated regulation of immunity. Many different systems were used to study the phenomenon of antibody-mediated suppression of immune responses and, not surprisingly, several different mechanisms were proposed. The theories, attempting to establish the site at which antibody exerted its suppressive effects could be divided into three categories:

a) Suppression at the Level of Antigen.

According to this hypothesis, antibody exerted its suppressive effects by combining with antigen and thereby masking its determinants. Simple determinant masking appeared to be involved in the cyclical antibody response of mice to LPS, described by Britton and Moller (213). Antibody was also postulated to act by facilitating the destruction of antigen or by causing the deviation of antigen to organs not involved in immunoglobulin synthesis (i.e. the liver). For example, the lack of sensitization of Rh^- women to the Rh antigen on ABO incompatible fetal red cells was thought to be mediated by the anti-A and anti-B antibodies, present in the maternal circulation, which caused the uptake of the Rh D^+ red cell by the liver (214). An analogous observation was made by Kappler *et al.* (215) who demonstrated, using TNP-SRC as antigen in the mouse, a suppression by anti-TNP antibodies of both the anti-SRC response as well as the anti-TNP response.

b) Suppression at the Level of Immunocompetent Cells - The Central Effect.

Evidence that suppression could occur at the level of immunocompetent cells was reported by Feldmann and Diener (216) using polymerized flag-

ellin and SRC as antigens in the mouse. These workers described systems in which immunocompetent cells were rendered specifically unresponsive either *in vivo* or *in vitro*, by the exposure of lymphocytes to appropriate amounts of both antigen and specific antibody. The authors suggested that complexes composed of antigen and antibody formed at the surface of specific immunocompetent cells which then resulted in the generation of a suppressive signal. Sinclair and Chan (217) proposed a similar model also involving antigen, antibody and immunocompetent cells.

c) Suppression at the Level of the Accessory Cell - The Peripheral Theory.

A considerable amount of evidence was reported which suggested that antibody exerted its suppressive effect at sites distinct from those described above. Pierce (218), for example, examined the inhibitory effects of specific antibody on the *in vitro* a-SRC PFC response of mouse spleen cells. In order to establish the site of antibody-mediated suppression, spleen cells were fractionated into adherent (macrophage-enriched) and non-adherent (macrophage-deficient) cell populations. When non-adherent cells were exposed to suppressive antibody, it was found that after washing, these cells could mount a normal PFC response when reconstituted with macrophages. On the other hand, a similar treatment of the adherent cell population, either before or after exposure to antigen, resulted in a marked decrease in the ability of these cells to induce a PFC response in a non-adherent cell population. These results suggested that specific antibody did not act directly on the lymphoid cell population, but was sequestered, most probably, by macrophages and functioned by effectively neutralizing the immunogenic stimulus of these cells.

Ryder and Schwartz (219) reported similar findings using experimental protocols in which the immunological reactivities of cells from normal and passively immunized mice were compared using SRC as antigen. They demonstrated that although passively immunized mice responded only slightly to SRC, spleen cells from such mice, when transferred to syngeneic irradiated recipients, responded normally, thus excluding lymphoid cells as the site of action of immunosuppressive antibody. In a second series of experiments, peritoneal macrophages from normal and passively immunized mice, following phagocytosis of SRC, were compared in terms of their abilities to induce an a-SRC response in normal recipients. They found that although the macrophages from passively immunized mice could engulf SRC to the same extent as their normal counterparts, they were unable to elicit an immune response in normal mice.

Abrahams *et al.* (220) reported that mouse spleen cells which had been treated with specific antibody produced a much reduced response to SRC in relation to the appropriate controls. Spleen or peritoneal cells derived from normal mice were able to reconstitute the inhibited spleen cell population. Such reconstitution could be detected only in systems which were A cell deficient. The reconstituting cell was found to be radiation-resistant, anti-Thy-1 resistant, and exhibited a sedimentation velocity similar to that of A cells. In addition, fractionation of spleen cells which had been exposed to fluorescein-labelled suppressive antibody, yielded a labelled fraction with a sedimentation velocity very similar to that of A cells. These results were consistent with the concept of suppressive antibody binding to A cells in such a way as to prevent the expression of antigen in an immunogenic form.

Thus, it had been demonstrated that in many systems the A cell was

crucially involved in the mechanisms of antibody-mediated suppression. It was reasoned that perhaps the apparent relationship between antibody-mediated suppression and A cells could be utilized for evaluating the antigen-processing systems of neonatal mice. To this end, an experimental protocol was designed to compare neonatal and adult mice in terms of their abilities to exhibit antibody-mediated suppression.

Of particular interest was the reported ability of antibody to be suppressive long after its first administration *in vivo*. Ryder and Schwartz (219) reported that specific antibody injected at birth could effectively suppress the response of mice to SRC injected six weeks later. At this time no passively administered antibody could be detected in the serum of these animals. Haughton and Nash (221) likewise reported the persistence of suppression in mice five weeks after antibody administration. Ryder and Schwartz attributed the suppression which they observed to cell-bound antibody. Evidently antibody in this form was more persistent, presumably isolated from the catabolic events occurring in circulation, and was sequestered at a site where its immunosuppressive capabilities could be expressed, possibly in association with the antigen-processing system.

On the basis of these reported findings, it was reasoned that the ability of mice to sequester and retain specific antibody for an extended period of time would directly reflect the functionality of the antigen-processing system. A study was subsequently undertaken to determine the relative abilities of mice, aged from birth to eight weeks of age, to retain suppressive antibody for an extended period of time.

For the preparation of C57BL/6 mouse anti-SRC antibody, mice were primed with 1×10^7 SRC i.v. and boosted 21 days later with 5×10^8 SRC i.v. Six days later the mice were bled and the γ-globulins isolated

from serum by precipitation with 50% saturated ammonium sulfate. The precipitated γ-globulins were redissolved and dialyzed in PBS, and the concentration adjusted finally to 25 mg/ml.

This antibody preparation was tested first for its ability to agglutinate SRC; a titer of 1024 was obtained. Subsequently, this preparation was tested for its ability to suppress an anti-SRC response. Adult C57BL/6 mice were injected i.p. with 2.5 or 0.25 mg anti-SRC γ-globulins. Two hours later, 2×10^8 SRC were administered i.v. and the subsequent IgM and IgG PFC responses enumerated 5-8 days later. Maximum PFC responses were found to occur on day 6. The results presented in Table VII demonstrate that a high degree of inhibition (90-97%) of both the IgM and IgG responses was obtained with the two doses of γ-globulins tested.

In order to assess the ability of mice of increasing age to retain suppressive antibody for an extended period of time, the following experimental protocol was used. Groups of normal C57BL/6 mice of ages ranging from birth to eight weeks received a single i.p. injection of either anti-SRC γ-globulin (0.25 mg in 0.1 ml of PBS) or 0.1 ml of PBS. Each group consisted of six mice which were of the same age. Six weeks after the injection, all mice received an immunizing dose of 3.2×10^8 SRC i.v. Thus, mice passively immunized at birth were challenged with SRC at six weeks of age; those passively immunized at four weeks of age were challenged at ten weeks of age, and so on. Six days after the injection of SRC, the spleen cells from each group of mice were pooled and assayed for IgM and IgG PFC. The resulting data were subjected to a polynomial regression analysis, and the functions generated by a least squares fit are illustrated in Figure 18. The results demonstrated no inhibition by passively administered antibody when IgM responses were

TABLE VII

INHIBITION OF THE IgM AND IgG ANTI-SRC PFC RESPONSES BY PASSIVELY ADMINISTERED ANTIBODY

Group	Maximum PFC/Spleen[a]	
	IgM	IgG
Normal[b]	316000 (100)	171000 (100)
Passively Immunized:		
2.5 mg	9780 ± 1010 (3.1)	14590 ± 2035 (8.5)
0.25	12290 ± 3390 (3.9)	17490 ± 4390 (10.2)

[a]PFC responses observed on day 6, ±1 S.D.; numbers is parentheses indicate percent of normal response.

[b]Responses derived from Figure 10.

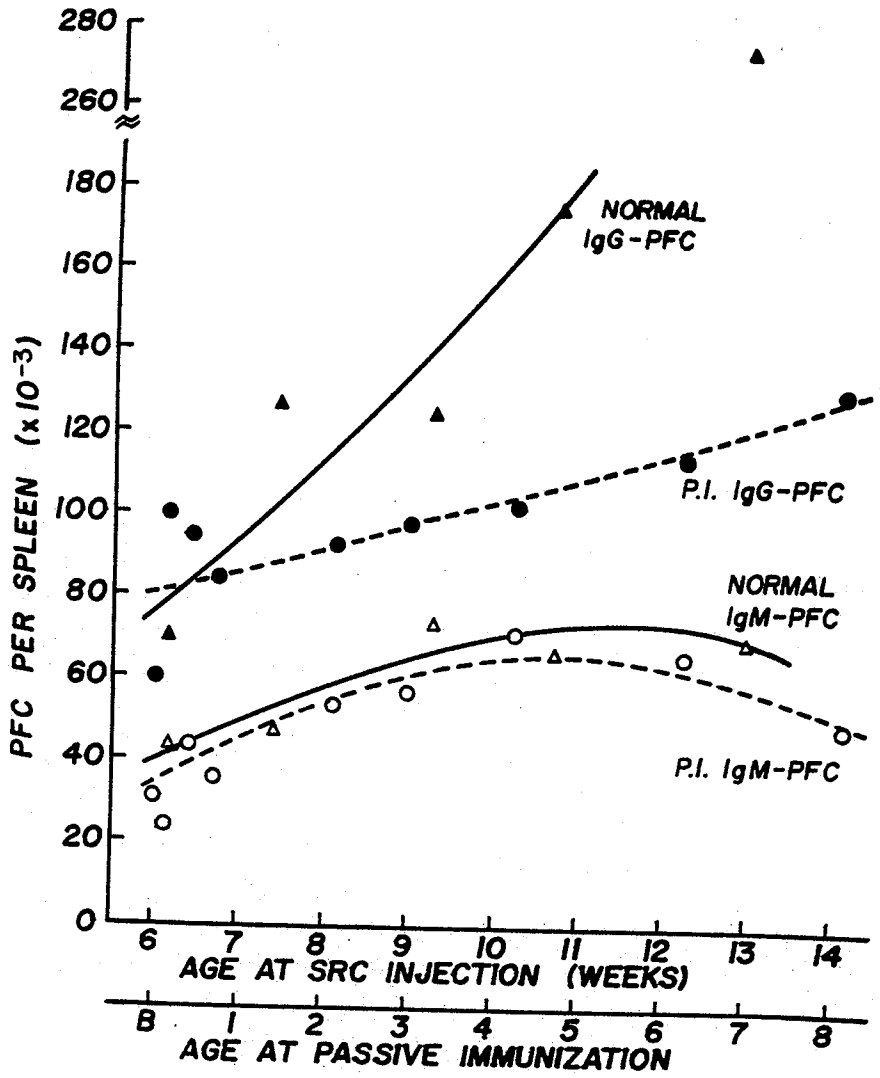


Figure 18:

The anti-SRC PFC response of normal (———) and passively immunized (- - - -) C57BL/6 mice. Mice were injected once i.p. with either PBS or anti-SRC γ-globulin at ages varying from birth to 8 weeks of age. Six weeks later all mice received 3.2×10^8 SRC i.v., and their spleens assayed for IgM (△, ○) and IgG (▲, ●) PFC 6 days later. Each point is based on the analysis of spleen pools, with each pool derived from 4-6 spleens.

compared. In contrast, a marked inhibition was observed for the IgG responses of passively immunized mice.

As a function of age, the IgM responses rose and then declined somewhat. The reasons for this are not clear. It was previously demonstrated in Chapter I (Figure 2) that the kinetics of the IgM PFC response were related to the age of the animal. Whereas relatively young mice respond maximally 6-7 days after challenge, older mice were able to respond 1-2 days earlier. Thus, in the experimental design of Figure 18, the PFC responses could be expected to decline with increasing age as the day of maximum response gradually shifted to times earlier than the time of assay (day 6). It was concluded, therefore, that the shape of the IgM response curves probably reflected a compromise between two opposing factors: (i) the increase in PFC responsiveness as a function of age and (ii) an accompanying shift in the kinetics of the PFC response.

In contrast to the IgM responses, the IgG responses of both normal and passively immunized mice increased throughout the time span of the study. Moreover, a marked difference was observed between the IgG responses of normal mice and those of mice which had been passively immunized. Mice passively immunized at 6-8 weeks of age demonstrated a reduced IgG response, relative to normal mice, when challenged with SRC six weeks later. The reduction was in the order of 50%. On the other hand, mice passively immunized at birth or during the first two weeks of life did not demonstrate any inhibition and responded as did normal mice. The passive immunization of these mice appeared completely ineffectual. However, beginning at approximately two weeks of age, the effect of passive immunization could be observed by subsequent suppression of IgG PFC production. Furthermore, as the age at which mice were passively

immunized increased, a progressively increasing inhibition of the IgG PFC response was observed.

These results could be interpreted in several different ways. One possible explanation for the observed results is related to the difficulties encountered in attempting to inject newborn mice in a reproducible manner. For example, if the passively administered antibodies had leaked out of the peritoneal cavity of the newborn immediately after injection, no subsequent suppression of immunity would be observed. Since this problem would not be expected to occur with older mice, the results in Figure 18 could be rationalized on this trivial basis. The problem of leakage from the peritoneal cavities of newborn mice was in fact encountered early on in this study. To overcome this problem, glass capillaries were drawn out and adapted to disposable 1 ml syringes. Examination under the microscope revealed that the diameters of such glass needles were roughly one-half that of 32 gauge needles. Using these glass needles the success rate for injections was roughly 80%. Any mice which demonstrated leakage subsequent to injection were not included in this study.

A second possible interpretation of the data was that the observed inhibition of the IgG PFC response was simply due to persisting serum antibody. Such antibody, by persisting in circulation for six weeks, could act simply by neutralizing and eliminating antigen. This possibility was tested in the following way. Since it had been reported that the half-life for mouse IgG in circulation was approximately four days (222), the six-week period between passive immunization and challenge with antigen amounted to approximately ten half-lives. An experiment was performed in which the standard inoculum of anti-SRC γ-globulins (0.25 mg/0.1 ml) was subjected to ten doubling dilutions. A 0.1 ml

sample of the diluted antibody solution was then administered passively into 8-9-week-old mice, which were subsequently challenged with 3.2×10^8 SRC two hours later. As can be seen in Table VIII, no suppression of the ensuing antibody responses was evident. Thus, it was concluded that suppressive antibody could not have persisted in circulation in sufficient amounts to cause the inhibition observed in Figure 18. In this system, the passively administered antibody was evidently isolated from the catabolic events occurring in circulation.

A third interpretation of the data presented in Figure 18 is related to the state of maturation of the antigen-processing system. Evidence was cited earlier in this section which strongly implicated the antigen-processing system as the site of action of specific antibody. It was reasoned that in the absence of an antigen-processing system, the effects of specific antibody would not be apparent. Thus, the data presented in Figure 18 are consistent with the conclusion drawn in this thesis, that neonatal mice lack an efficient antigen-processing system.

It could be argued that the effect of antibody in this system was a central one — the direct inhibition of immunocompetent cells. The results could be explained in terms of the relative numbers of immunocompetent cells present in the mouse at the time of passive immunization and at the time of challenge with antigen. When injected at birth, the suppressive antibody would be expected to have little effect on the response elicited six weeks later, since compared to six-week-old mice, the number of immunocompetent cells in the newborn is very low. The response given by six-week-old mice would thus have been given largely by immunocompetent cells which had been newly formed subsequent to passive immunization. Passive immunization at a later age, however, would

TABLE VIII

LACK OF SUPPRESSION BY A 2^{10} DILUTION OF ANTI-SRC ANTIBODIES

Group[b]	Treatment	PFC/Spleen[a]	
		IgM	IgG
Control	0.1 ml PBS	54400 (100)	137000 (100)
Passively Immunized	0.1 ml of 2^{10} dilution	51600 (94.9)	151800 (110)

[a]PFC responses observed on day 6; numbers in parentheses indicate percent of control response.

[b]Five mice per group.

result in the suppression of a significant number of immunocompetent cells, leading to a detectable lowering of the PFC responses. It is difficult to determine whether this central mechanism of suppression is operative in this situation. It should be noted, however, that such systems described in the literature required the addition of antigen to demonstrate the suppressive effects of antibody (216). In the system described here, antigen was not added at the time of passive immunization, although it could be argued that crossreacting environmental antigens were relevant in this regard.

In summary, an experimental protocol involving antibody-mediated suppression was designed in order to evaluate the state of the antigen-processing systems of neonatal mice. It was demonstrated that mice aged 1-14 days could not retain suppressive antibody for an extended period of time, whereas older mice could retain such antibody. Since the antigen-processing system was thought to be a key factor in such long-term retention of antibody, it was concluded that neonatal mice lacked such a system.

3) Tissue Distribution of SRC in Neonatal and Adult Mice

In the previous two Chapters, various aspects of the immune capabilities of neonatal and adult mice were described and compared. It was demonstrated that in relation to adults, mice aged 1-2 weeks either could not respond to SRC or did so rather feebly. In addition, the sensitivity of 12-day-old mice to SRC was considerably lower than that of adult mice. It has been argued that such differences in response characteristics were related to relatively inefficient antigen-processing systems in neonatal mice. The study described in this section was not addressed primarily to an investigation of the functional properties of the antigen-processing system as it related to the production of antibody; but rather, emphasis was placed on an evaluation of those factors which affected the efficient capturing and processing of antigen.

The rationale for this study in the context of the work presented in this thesis stemmed from the possibility that a radical difference in the distribution of antigen in neonatal mice could explain some of the observations related to neonatal responsiveness. If, for example, the reticuloendothelial system of the neonatal mouse caused the destruction of antigen or the deviation of antigen from the spleen to organs not involved in antibody synthesis, relatively low splenic antibody responses would be predicted. A relatively low sensitivity to antigen would also be apparent since proportionally more antigen would be required to result in the capture of a given amount of antigen by the spleen. Consequently, a study was undertaken to determine whether differences in distribution of SRC could be detected in neonatal and adult mice.

For this purpose, radioactive-labelled SRC were prepared. Two different methods for labelling SRC were used. The first method, using

$^{51}CrCl_3$ as the labelling agent, was found to be simple and quite reproducible. However, most if not all of the ^{51}Cr was associated with the SRC hemoglobin. The second method, which was adapted from the surface-labelling technique described by Marchalonis *et al.* (203) was rather more involved, but most of the ^{125}I-label was associated with surface components (see Materials and Methods, page 56). It was decided that the second method of labelling would be more relevant in these studies since the radioactive label was judged more likely to be associated with the antigenic determinants of the SRC.

Two groups of mice aged 12 days and 12 weeks were used in this study. Mice were injected with 2×10^8 ^{125}I-SRC (300,000 - 600,000 cpm) using a tail vein for adult mice and the ocular sinus for 12-day-old mice. At predetermined times following injection, individual mice were bled and then sacrificed. The liver, lungs and spleen were removed and placed separately into TC199 at 0°C. The remainder of the mouse was designated as the carcass. The liver, lungs, carcass and blood sample were counted for radioactivity without further manipulation. The spleen was suspended and subdivided into three fractions: the cellular (C) fraction, the reticular (R) fraction and the cellular supernatant (*C supernatant*). The cellular fraction was defined as those spleen cells which did not sediment at 1 x g in ten minutes. The reticular fraction consisted of the spleen stroma which sedimented at 1 x g in ten minutes. The cellular supernatant consisted of the soluble radioactive material derived from the cellular fraction which could not be sedimented at 600 x g in five minutes.

Table IX presents the data describing the distribution of ^{125}I-SRC in the various organs as a function of time after injection. Neonatal and adult mice are compared. In this Table the uptake in the spleen is

TABLE IX

DISTRIBUTION OF ^{125}I-SRC IN NEONATAL AND ADULT C57BL/6 MICE[1]

Time After Injection (Min.)	Carcass		Liver		Lungs		Blood		Total Spleen		Overall Recovery	
	Neonate	Adult	Neonate	Adult	Neonate	Adult	Neonate	Adult	Neonate	Adult	Neonate	Adult
20	N.D.[2]	16.27 ±4.28	33.57 ±10.61	65.82 ±6.51	12.01 ±2.47	3.67 ±2.43	28.99 ±8.48	2.95 ±0.97	5.40 ±1.29	5.19 ±0.64	79.97 ±16.28	93.90 ±4.52
40	N.D.	17.44 ±2.19	61.87 ±11.81	59.59 ±4.63	7.09 ±0.65	2.32 ±0.19	8.08 ±0.44	3.25 ±0.48	8.49 ±0.06	4.73 ±1.89	85.52 ±11.66	87.34 ±4.58
60	N.D.	37.61 ±17.45	46.67 ±11.47	45.12 ±12.50	3.93 ±0.76	2.00 ±0.99	4.37 ±0.85	4.18 ±0.45	4.22 ±2.99	4.00 ±2.89	59.16 ±9.03	92.91 ±6.40
90	51.78 ±8.15	39.49 ±4.67	42.45 ±7.16	38.90 ±6.40	2.92 ±0.48	1.71 ±1.20	3.68 ±0.58	4.25 ±0.37	1.42 ±0.43	3.15 ±1.24	102.26 ±1.45	92.43 ±8.61
120	61.90 ±2.55	37.22 ±0.69	34.76 ±7.70	25.62 ±5.43	3.23 ±0.06	1.05 ±0.07	4.02 ±0.46	2.98 ±0.42	2.03 ±0.82	2.64 ±0.25	105.92 ±2.37	69.51 ±5.68

[1]Results expressed as percent of injected ^{125}I. Each determination represents the mean ± 1 S.D. derived from 3 mice.

[2]N.D. ≡ not done.

expressed as the total of C, R and *C supernatant* fractions.

The values expressed for the radioactive content of the blood were obtained by multiplying the specific radioactivity of the blood (CPM/ml blood) by the total blood volume of the mouse. The total blood volume of adult mice was determined by injecting ^{51}Cr-labelled syngeneic mouse red cells i.v. and monitoring the level of radioactivity in the blood for two hours. It was found that the radioactivity levels were maintained at a constant level indicating that these cells were not being eliminated from circulation. The blood volume for adult mice was determined as 1.328 ± 0.237 ml (14 determinations). The blood volume of 12-day-old mice was estimated by dividing this volume by the ratio of body weights for 12-day-old and 12-week-old mice. Thus, 1.328/3.74 = 0.355 ml. It can be seen in Table IX that the ^{125}I-SRC were cleared from the circulation of adult mice very rapidly. By 20 minutes only 3-4% of the label was detectable in the blood, with no subsequent change thereafter. In comparison, neonatal mice required about 60 minutes to achieve this level of elimination. However, it was clear that neonatal mice could clear SRC from their circulation to the same extent as adult mice, since by 60 minutes onwards, identical radioactive labels in the blood were observed.

When the radioactivity levels of the liver were examined it was apparent that this organ was responsible for the uptake of a large proportion of the original inoculum of SRC. In adult mice 65% of the injected radioactivity could be detected in the liver after 20 minutes, with this level falling to 25% after two hours. Although the blood volume of the liver was not determined, it is clear that blood-borne radioactivity did not contribute significantly to the values reported here, since during this same time period, <u>total</u> blood levels amounted to only 3-4%. The

results in the neonatal mice followed a slightly different pattern of hepatic uptake inasmuch as a maximum uptake (62%) was observed 40 minutes following injection, as compared to 20 minutes or less for the adult mice. Subsequently the levels of radioactivity decreased to levels which by 120 minutes were comparable to those observed in adults.

The data of Table IX indicate that compared with the liver, the lungs did not exhibit high levels of radioactivity. When the amounts of label in the lungs and the blood were compared, in all cases but one (20 minute samples for adults) the blood contained more radioactivity. In addition, there appeared to be a direct correlation, in the neonates, between these two values. In was concluded, therefore, that the lungs were clearing little, if any, SRC from the circulation and that the radioactivity detected in the lungs could be attributed entirely to the radioactivity of the blood in this tissue.

The total uptake by the spleen in both neonatal and adult mice was found to be quite low. The maximum uptake in adults was 5% at the 20 minute mark, and subsequently declined in a linear manner to approximately 2.6% at 120 minutes. The maximum neonatal uptake in the spleen was 8.49% at 40 minutes after injection, and thereafter declined to 2% at 120 minutes. The kinetics of splenic uptake closely parallelled that of the liver, for both adults and neonates. Thus, it was clear that in terms of total splenic uptake, neonates and adults were comparable.

The levels of radioactivity in the spleen have been corrected for blood-borne radioactivity. In practice, however, this correction was found to be insignificant. The blood volumes of adult mouse spleens were determined by injecting ^{51}Cr-labelled syngeneic mouse red cells into adult mice. Spleens were excised from five to 60 minutes following injection

and the radioactivity determined. Since the levels of activity in the blood and the spleen did not change during this time, it was concluded that the labelled mouse red cells were not being removed from circulation and the label detected in the spleens was due to the presence of radioactive blood. The blood volume of the spleen was obtained by dividing the spleen radioactivity (CPM) by the concentration of label in the blood (CPM/ml), and was determined as 0.014 ± 0.004 ml (14 determinations). Thus, approximately 1% of the total blood supply (1.328 ml) was present in the spleen. The blood volume of neonatal spleens was estimated by dividing 0.014 ml by the ratio of the body weights of adult and 12-day-old mice, i.e. $\frac{0.014}{3.74} = 0.0037$ ml.

In these studies the spleen was subdivided into three fractions. Table X presents the data for the uptake of radioactive label in these fractions. The correction for blood-borne activity was applied to the C fraction, since intact ^{125}I-SRC in the spleen blood would be expected to fall into this fraction. In the adult spleens a trend in radioactive distribution was apparent. At all times, the amounts of label detected in the three fractions followed the order R < C < *C supernatant*. In all these fractions the amount of radioactivity present declined over the time period of study. This finding, coupled with the observation that much of the spleen radioactivity was in a non-cellular soluble form (*C supernatant*), strongly suggested that the membrane components of the SRC were being broken down into lower molecular weight forms and subsequently eliminated from the spleen. The amount of radioactivity associated with the cellualr components of the adult spleens, (R + C), was found to decrease from a total of 2.5% at 20 minutes to 1.2% at 120 minutes. Thus, two hours following the injection of SRC, only about 1% of the total

TABLE X

DISTRIBUTION OF ^{125}I-SRC WITHIN THE SPLEENS OF NEONATAL AND ADULT MICE[1]

Time After Injection (Min.)	R		C		*C Supernatant*		R + C		R + C *C Supernatant*	
	Neonate	Adult	Neonate	Adult	Neonate	Adult	Neonate	Adult	Neonate	Adult
20	0.30 ±0.08	0.83 ±0.18	2.71 ±0.69	1.71 ±0.47	2.39 ±0.68	2.65 ±0.11	3.01 ±0.62	2.55 ±0.55	1.26	0.96
40	0.69 ±0.10	0.99 ±0.55	3.04 ±0.37	1.36 ±0.46	4.76 ±0.33	2.38 ±0.92	3.73 ±0.26	2.35 ±1.00	0.78	0.99
60	0.46 ±0.07	0.69 ±0.44	2.02 ±0.35	1.25 ±0.97	1.74 ±2.71	2.07 ±1.48	2.81 ±0.19	1.94 ±1.41	1.61	0.94
90	0.30 ±0.10	0.67 ±0.34	1.03 ±0.30	0.80 ±0.24	0.09 ±0.02	1.68 ±0.68	1.33 ±0.40	1.47 ±0.57	14.8	0.88
120	0.38 ±0.16	0.52 ±0.03	1.54 ±0.64	0.70 ±0.10	0.11 ±0.05	1.42 ±0.18	1.91 ±0.77	1.22 ±0.08	17.4	0.86

[1]Results expressed as percent of injected ^{125}I. Each determination represents the mean ± S.D. derived from 3 mice.

inoculum could be demonstrated as being associated with cellular components of the adult spleen.

In neonatal spleens, the uptake by the C fraction was higher than that observed in the R fraction for the time period examined. The combination of these cellular fractions (R + C) in neonates was observed to be at least as high as the uptake observed in the corresponding adult spleen fractions; in fact, the total cellular uptakes by neonates, (R + C), appeared to be slightly higher (see R + C column, Table X). It is clear, however, that essentially similar uptakes of radiolabel in these cellular fractions were observed for both neonatal and adult spleens.

When the *C supernatant* fractions of neonatal and adult mice were compared, both groups of mice initially demonstrated substantial amounts of radioactivity. In adults, this radioactivity fell at a constant rate from a high of 2.65% at 20 minutes to 1.42% at 120 minutes. Neonates demonstrated similar uptakes in the *C supernatant* fraction during the first 40 minutes, but starting at about 60 minutes after injection, the levels of radioactivity found in this fraction fell to very low levels, such that at 120 minutes the presence of only 0.11% was observed. Thus, between 90 and 120 minutes, a 13-19 fold difference in this fraction was observed between neonatal and adult mice.

In the last column of Table X, an attempt has been made to distinguish radioactive label in two forms: 1) label associated with cellular fractions, i.e. R + C; and 2) label present in a soluble form (*C supernatant*). The partition of splenic label between these two compartments was expressed as the ratio R + C/*C supernatant*. It was observed that this ratio varied little in adult spleens during the time period of study, with the label being equally distributed between the two forms. In con-

trast, the ratios in neonatal spleens, while approximating those of adult spleens during the early portion of the study, were observed to increase markedly by 90-120 minutes following injection. The possible significance of this finding will be discussed below.

In summary, it was demonstrated that quantitatively, the distribution of ^{125}I-SRC in the various organs of neonatal and adult mice showed considerable similarity. Although the elimination of SRC from the blood was detectably slower in neonates, by 120 minutes comparable levels of radioactivity remained. The lungs of neonatal and adult mice did not appear to play any significant role in the clearance of ^{125}I-SRC from the circulation. The liver was judged to be a major site of SRC uptake and degradation. In both neonatal and adult mice, high levels of uptake (60-65%) were observed in the liver soon after injection. This was then followed by a gradual decrease in the radioactive content of the liver which roughly correlated with a corresponding increase of label in the carcass. These observations are consistent with the interpretation that the liver functioned largely in the capture, degradation and elimination of SRC. The total splenic uptakes for neonatal and adult mice were judged to be comparable. Both age groups demonstrated initial splenic uptakes in the order of 5-8% which subsequently declined to approximately 2%.

The finding that a small percentage of the label is taken up by the spleen suggests that only a small proportion of an immunizing dose of SRC is actually immunogenic. This conclusion is consistent with data obtained using immunosuppressive antibody (221) in which it was demonstrated that high degrees of immunosuppression could be attained with amounts of antibody which were sufficient to neutralize only a small proportion (<1%) of an immunizing dose of antigen.

The gradual decline with time of label in the spleen implied that the spleen, like the liver, was involved in the breakdown and elimination of SRC. This was evidenced by the substantial amount of spleen radioactivity which was present in soluble form and not associated with cells. Although at 120 minutes the total spleen uptakes in adult and neonatal mice were comparable, the distribution of label within the spleen was different for neonatal and adult mice. In the neonates, relatively more label was found associated with the cellular fractions (R + C) than in the soluble fraction (*C supernatant*). This implied that relative to the adult situation, label associated with the cellular fractions in neonatal spleens was possibly more persistent.

In conclusion, the evidence presented in this section has demonstrated that in general, the distribution of ^{125}I-SRC in the various organs of neonatal and adult mice were comparable. No evidence was found to suggest that the relatively low responsiveness of neonatal mice was due merely to the destruction of antigen or the deviation of antigen to organs not involved in antibody synthesis. The uptakes of SRC by neonatal and adult spleens were also similar, suggesting that a simple inability of neonatal spleens to capture antigen was not a significant factor in producing a low antibody response. The processing of this antigen might possibly be different in neonatal spleens since relatively low levels of radioactivity were found in a degraded form (*C supernatant*). This suggested that antigen may be more persistent in the neonatal spleens. Since it has been reported that antigens of a persistent nature tend to result in tolerance rather than immunity (18,21), it could be argued that the relatively low levels of neonatal antibody production are due to a limited degree of

tolerance induction. The relative importance of such a possibility, however, must remain speculative.

4) Possible Selective Recruitment of Antigen-Sensitive Cells by Environmental Antigens

In Chapter II, the relative sensitivities of neonatal and adult mice were compared on the basis of antigen dose-response curves. It was demonstrated that neonatal mice were less sensitive to antigen than adult mice in that the former group required a higher dose of SRC to mount an optimal antibody response (Table II). Furthermore, it was established that the differences seen in the optimum antigen doses were related to the antigen-processing system, since such differences were abolished by the provision of a common antigen-processing system in adult irradiated recipients (Table V).

On the other hand, differences seen in the bandwidths of the dose-response curves derived from intact mice were not attributed to different degrees of maturation of the antigen-processing system in the two age groups, since these differences were preserved in the environment of the irradiated recipient (Table VI). It was concluded that the differences in bandwidths were evidently due to intrinsic differences in the immunocompetent cell populations of the two age groups.

The narrower bandwidths obtained with neonatal mice strongly suggested that the neonatal repertoire of immunocompetent cells was relatively restricted. One possible basis for the observed changes in bandwidth, which was considered, was the selective recruitment of immunocompetent cells by environmental antigens. If one assumed that the changes with increasing age in the responding cell populations were antigen dependent, it followed that the antigen used to define the dose-response curves here (SRC), crossreacted with some environmental antigens.

In order to examine the possibility of such a crossreactivity, a study was undertaken to assess the ability of an antigen prepared from a common enteric bacterium (*E. coli*) to elicit an anti-SRC response. The demonstration of such a crossreactivity would, therefore, provide the theoretical basis for a possible recruitment of antigen-sensitive cells by environmental antigens during the transition from neonatal to adult life.

The lipopolysaccharide cell wall antigen (LPS) was extracted from *E. coli* (ATCC 11303) by the hot phenol-water method described by Staub (205) and subsequently detoxified as described by Britton (206). The LPS was dissolved in saline and injected into adult mice at various dose levels ranging from 1 - 1,000 µg per mouse. The spleens of the injected mice were assayed for IgM PFC four days later using normal SRC as indicator cells. As shown in Table XI, a clear increase above background was observed in the number of SRC-specific PFC in the spleens of treated mice. This suggested the presence of crossreacting antigens on LPS and SRC. An alternative interpretation, however, was that the LPS was acting as a non-specific mitogen.

In order to distinguish between these two possibilities, the specificity of the anti-SRC PFC was examined. Mice were injected with 1.5 mg LPS and their splenic PFC response assayed four days later. The PFC were examined using normal SRC as indicator cells in the presence of varying amounts of LPS. As shown in Figure 19, the PFC which were elicited by LPS and visualized with normal SRC could be inhibited by the addition of LPS. The LPS used in the PFC assay was found not to be generally toxic since the addition of LPS did not significantly reduce the number of PFC elicited by and visualized with normal SRC (data not shown). The lack of inhibition in this latter case suggested that the crossreactivity existing

TABLE XI

PFC RESPONSE ELICITED BY E. COLI LIPOPOLYSACCHARIDE ANTIGEN WITH NORMAL SRC AS INDICATOR

Dose of Antigen Injected	Maximum IgM PFC per Spleen[1] (± 1 S.E.)
µg	
1000	690 ± 30
100	330 ± 24
10	350 ± 45
1	40 ± 10
0	45 ± 5

[1] PFC determined on day 4.

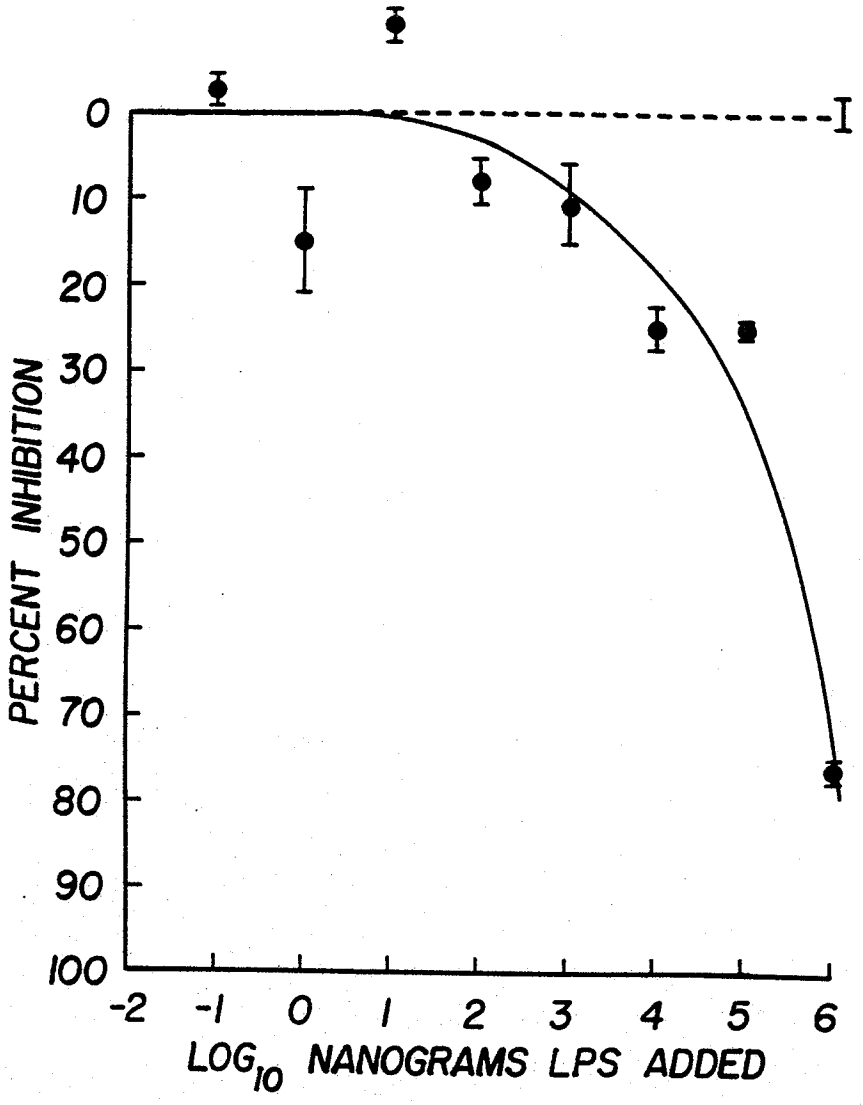


Figure 19:

Specificity of PFC elicited by LPS and visualized by using normal SRC. Spleen cells from mice which had been previously injected with 1.5 mg LPS were plated in the presence of different quantities of LPS. Percent inhibition of PFC (——) is plotted as a function of the log of the weight of LPS added to each plate. (----), no LPS; 1 S.E. is defined for each point by vertical bars.

between LPS and SRC was low.

Thus, it was demonstrated that LPS and SRC crossreacted to a small degree. The question posed was whether LPS or similar environmental antigens could cause, through a process of antigen-driven selection, the increase in bandwidths which was observed to occur with increasing age.

There is strong evidence to indicate that antigen-mediated selection of B cells can occur (223,224), which can be reflected as a change in antibody heterogeneity and affinity (225). If it is assumed that the mice in this study were exposed naturally to antigens structurally similar to SRC, it could be argued that an effect would be evident on their pattern of responsiveness. Thus, the increased bandwidth of the dose-response curves could therefore be interpreted as an increase in the heterogeneity of the responding cell population.

In model studies, small doses of antigen generally have led to an increase in the affinity of specific antibody and a <u>decrease</u> in its heterogeneity (225). However, it could be argued that the apparent increase in heterogeneity of the responding cell populations described in this thesis is an antigen-driven process similar to that envisaged by Cunningham and Pilarski (226,227), since a simple decrease in the heterogeneity of serum antibody does not necessarily imply a decrease in the number of individual antibody species of different affinity which are present (225).

The differences in heterogeneity (bandwidths) between neonatal and adult mice, which have been described in this thesis, were apparent for both the IgM and IgG responses (Tables III and VI). Presumably, if antigen-driven selection was the valid explanation for these changes, it would be necessary to assume that selection by environmental antigens had occurred in both the IgM and IgG B cell populations. Certainly, with respect to the IgG anti-hapten responses in rabbits, the reported increase in anti-

body affinity during a primary response, or in a secondary response, is consistent with the concept of antigen-driven selection of cells bearing specific receptor sites of increasing affinity (228,229).

Wu and Cinader (230) have reported that hapten-specific IgM antibody produced by rabbits exhibits changes in combining properties after primary immunization. Moreover, low doses of antigen were found to elicit IgM antibody of higher affinity than did high doses. However, the fact that little difference was shown in the affinity of the IgM antibody produced after primary and that produced after secondary immunization, suggested that in this response, antigen-mediated selection did not occur in a manner which produced permanent changes in the responding cell population. Baker *et al.* (231), in studying the exclusively IgM antibody response in mice to type III pneumococcal polysaccharide, demonstrated that the average avidity of antibody did not change with time after immunization, suggesting that antigen-driven selection was not occurring. The antibody response to *E. coli* LPS has also been reported as being restricted to the IgM class.

Therefore, if one of the inherent characteristics of the IgM response is an absence or restriction of antigen-mediated selection of receptor sites, it follows that the apparent changes in heterogeneity, here visualized as an increase in bandwidth, cannot be attributed to such selection. Alternatively, it is possible that the nature of the antigen itself may determine the extent of any selection. For example, the polysaccharide antigens may not be capable of any cell selection.

Recently, Marshall-Clarke and Playfair (232) have described age-dependent changes in the relative affinity of anti-DNP antibodies in mice.

These authors demonstrated that the relative affinities of anti-DNP PFC, elicited in mice ranging from 1-24 weeks of age, increased as a function of age. Moreover, the increase in affinities seen during this age span was apparent for both the IgM and IgG PFC responses, was not related to possibly "immature" T cell function in neonatal animals and could be demonstrated in adult irradiated recipients. In addition, they demonstrated a progressive increase of antibody heterogeneity with increasing age. They interpreted their findings as favouring the somatic mutation theories of antibody diversity and implicated the action of "environmental" antigens during ontogenetic development.

In summary, it is possible that environmental antigens can play a role in the recruitment of both IgM- and IgG-producing precursors. It was concluded that crossreacting environmental antigens could be causative agents in the increase, with age, of the dose-response curve bandwidths (Chapter II). The exact nature of these antigens, however, is not known. Because of the low degree of crossreactivity demonstrated between *E. coli* (ATCC 11303) LPS and SRC, it is likely that other antigens are involved in the process of cell selection.

5) Partial T Cell Deficiency in Neonatal Mice

In Chapter I, the responsiveness of neonatal C57BL/6 mice was determined as a function of age. It was demonstrated (Figure 3) that mice less than one week of age could mount only feeble anti-SRC PFC responses, whereas between one and two weeks of age the ability of intact mice to respond increased dramatically. For reasons discussed in Chapter I, this abrupt change in responsiveness was considered due to maturational changes in the antigen-processing system. However, since an apparent T cell deficiency was demonstrated in the spleens of mice aged one to three days (Table I), the possibility existed that the low responsiveness of intact mice during their first week of life was related to this deficiency, and further, the increase in responsiveness following the first week of life was due to a sudden generation of SRC-specific helper T cells. If this was indeed the case, one would predict that at an age when responsiveness was rapidly increasing, the magnitude of the PFC responses obtainable would be strongly dependent on the number of antigen-specific T cells present. The question being addressed in this section is whether a relative T cell deficiency exists in the spleens of neonatal mice; that is, in relation to the number of B cells present in the neonatal spleen, were sufficient numbers of T cells present to facilitate an optimal immune response? Mice 12 days of age were subsequently chosen for this study, since at this age a) their responsiveness was rapidly increasing and b) both IgM and IgG responses could be obtained.

The experimental protocol was as follows. Thymus cells from syngeneic mice were injected i.p. into 8-day-old C57BL/6 mice. Four days later, SRC were injected i.p. and the subsequent IgM and IgG PFC respon-

ses determined 5, 6, 7, 8 and 9 days following antigenic challenge. Mice which received only SRC served as controls. The results presented in Table XII demonstrate that the injection of thymocytes into neonatal mice was apparently ineffectual since neither the magnitude nor the kinetics of the PFC responses were affected. These results are similar to those previously reported by Martin and Miller (233) for the anti-SRC response of adult CBA mice, and suggest that the ratios of antigen-specific B cells to T cells in both adult and 12-day-old mice cannot be distinguished.

Sinclair and Elliott (234) have shown that when compared with normal mice, neonatally thymectomized mice required up to 100-fold higher doses of SRC to produce a primary antibody response. This finding suggested that one manifestation of a relative T cell deficiency was a shift to higher doses of antigen for an optimal antibody response. Since 12-day-old mice were shown to be less sensitive to SRC than adult mice (Chapter II), it could be argued that a relative T cell deficiency existed in the neonatal mice. Although only a single antigen dose was used in the experiment described here, a shift in the antigen sensitivity of neonatal mice injected with thymus cells is unlikely since the magnitude of control and test groups were essentially the same.

In summary, the results described here do not support the concept of a relative T cell deficiency in 12-day-old mice inasmuch as the provision of additional T cells was ineffectual. It was, therefore, concluded that the relatively low responsiveness and sensitivity of 12-day-old C57BL/6 mice are not related to a relative T cell deficiency in these mice.

TABLE XII

EFFECT OF THYMOCYTES ON THE ANTI-SRC PFC RESPONSE OF 12-DAY-OLD C57BL/6 MICE

Experiment	Thymocytes[1]	SRC[2]	Maximum PFC / Spleen[3]	
			IgM	IgG
A	-	2.5×10^8	18200 (7)	3500 (8)
	2×10^7	2.5×10^8	14400 (7)	3810 (8)
B	-	2.5×10^8	11200 (6)	920 (8)
	2×10^7	2.5×10^8	12700 (5)	350 (8)

[1]Injected i.p. into normal 8-day-old mice.

[2]Injected i.p. into mice at 12 days of age.

[3]Each value represents the mean from a pool of three spleens. The numbers in parentheses indicate the day of maximum response.

GENERAL DISCUSSION

During the last ten years, much effort has been expended on the study of the ontogeny of immune responsiveness in an attempt to understand better the mechanisms underlying the many different facets of antibody production, including cellular cooperation and the generation of antibody diversity. Many of these studies have been concerned with the early development of antibody production in the mouse, since this species offers an advantage in that it is born in a developmentally immature state, and allows the possibility of observing and studying events after birth which would have occurred before birth in other species.

The inability of neonatal mice to respond to SRC during early life has been well documented (153, 188-190, 193, 235-237). Moreover, it has been shown that the age of acquisition of this response varies among different strains of mice. Thus, Playfair (189) demonstrated that NZB mice could produce a substantial anti-SRC PFC response when injected at one day of age, whereas BALB/c and C57BL/6 mice could not. The ages at which other strains of mice acquired this responsiveness has been shown to be day 3 for BALB/c (189,193), day 7 for C3H (236), day 10 for C57BL/6 (189,194), day 14 for Swiss-L (235) and day >14,<21 for CBA (237). Playfair (189) proposed that, based on hybrid and backcross experiments, the strain differences found were genetically controlled by at least three genes, however, the cellular mechanisms involved in this phenomenon were not known.

The elucidation of the cellular mechanisms underlying this lack of immunological reactivity was the primary objective of the investigations described in this thesis. In this Discussion, the late development of neonatal responsiveness will be considered in terms of defects in

one or more of the three cellular compartments involved in antibody formation. The relationships between the findings reported in this thesis and those reported by others will be also evaluated.

I. T CELL DEFECT

a) Evidence for a Helper T Cell Deficiency

The concept of a T helper cell deficiency in neonatal mice has been proposed by Spear and Edelman (238). They queried whether the B cell population in unresponsive neonatal mice was fully functional but was unable to undergo maturation to antibody-secreting cells in response to antigen because T cell helper function was absent. They investigated the effects of LPS on neonatal spleen cells since this agent was thought to act on B cells directly and in one sense provide or substitute for T cell function. They demonstrated that the *in vitro* anti-SRC PFC responses of spleen cells taken from 1-3-week-old mice could be enhanced by the addition of LPS to the cultures. This enhancement was apparently quite variable, however. In contrast, similar additions of LPS to spleen cell cultures using donors at least eight weeks of age produced minimal effects. They concluded that B cells from "immunologically immature" neonatal mice could be made capable of responding to antigen providing T cell function was available or enhanced by LPS. The authors described also a PHA-responsive, Con A-responsive T lymphocyte which appeared in the spleens of neonatal mice at about the time humoral responsiveness was attained. Other than this temporal correlation, however, no evidence was presented which implicated this cell as being a limiting factor in the response of neonatal mice to SRC. However, in light of the subse-

quent discovery of suppressor T cells in neonatal spleen cell populations (to be discussed below) and the reports that LPS, under some conditions may induce the differentiation of T cells (239,240), the effects of LPS may be interpreted as the overcoming of suppressor T cell function and not as a reflection of a lack of helper T cells.

The concept of a relative helper T cell deficiency was addressed by some of the studies reported in this thesis. For example, when spleen cells of mice aged from birth to 16 days of age were stimulated with SRC in adult irradiated recipients, it was found that at a time when the cellularity of the spleen was rapidly increasing (birth to four days of age, Figure 6), no IgG responsiveness was evident, whereas IgM responsiveness increased linearly with age. The difference in IgM and IgG responsiveness during the period of birth to four days was ascribed to a relative helper T cell deficiency since (i) rapid T cell seeding of the spleen was known to be occurring during this time, (ii) a relative helper T cell deficiency was in fact demonstrated in the spleens of 2-3-day-old mice (Table I) and (iii) the IgG response was known to be more dependent on T help than the IgM response (207). A similar helper T cell deficiency in the spleens of mice of this age was reported also by Chison and Golub (153).

However, subsequent to four days of age, the magnitudes of both the IgM and IgG responses, as measured in adult irradiated recipients (Figure 5), increased in a parallel manner. This finding suggested that a relative helper T cell deficiency did not exist in the spleens after four days of age and that the increasing responsiveness seen in intact mice at age 9-10 days (Figure 3) was not due to a sudden change in the helper T cell population. One experiment to demonstrate directly a

helper T cell deficiency in 12-day-old mice was reported in Chapter III. The results (Table XII) indicated that the prior injection of adult thymocytes did not alter the kinetics or the magnitude of the IgM and IgG responses elicited by SRC in 12-day-old mice. Similar results have been reported by Marshall-Clarke and Playfair (232) using neonatal (C57BL/6 x BALB/c)F_1 mice. These authors demonstrated that neither the magnitude nor the restricted nature of the anti-DNP PFC response to DNP-KLH of two-week-old mice was affected by the prior administration of adult thymocytes. Thus, direct attempts to demonstrate a relative T cell deficiency in 1-2-week-old mice have not been successful.

In this connection, Roelants and Askonas (240a) adoptively transferred hapten-primed B cells and carrier-primed T cells together with antigen into irradiated recipients and measured the subsequent anti-hapten response. They found that for a fixed number of B cells, the T cell number could be varied over a 25-fold range without any effect on the magnitude of the antibody response. Apparently an excess of helper T cells did not augment the response of a limited number of B cells. The results presented in this thesis are consistent with the view that at an age when the *in vivo* response of intact neonatal mice is rapidly increasing (Figure 3), helper T cells are not in limiting numbers, and the injection of additional T cells merely increases the T:B cell ratio but does not alter the magnitude of the subsequent antibody response.

b) Studies with T-Dependent and T-Independent Antigens

A neonatal T cell deficiency has also been proposed by Rabinowitz (237) and Hardy *et al.* (241) to explain the apparent differences in mouse neonatal responses to thymus-dependent and thymus-independent antigens.

Rabinowitz (237) has described the mouse spleen cell antigenic responses to the thymus-dependent antigen, SRC, the the thymus-independent antigens, LPS and pneumococcal polysaccharides, Types I and II. These responses were studied both *in vivo* and *in vitro* as a function of age using proliferation (^{3}H-thymidine incorporation) and PFC assays. For the first three weeks of life, primary spleen cell proliferative and PFC responses to SRC were shown to be either absent or meagre in comparison to adult values. Thereafter, the responses rose achieving adult values between 4-8 weeks of age. In contrast, young murine spleen cell proliferative and PFC responses to LPS and Types I and II pneumococcal polysaccharides were approximately the same as adult levels by 6-14 days of life. These data were taken to indicate that T cell responsiveness but not B cell responsiveness was lacking in young mice. Hardy *et al.* (241) reached similar conclusions by comparing the immune response potential of newborn mice to the thymus-dependent and thymus-independent synthetic polypeptides, poly (L-Tyr, L-Glu)-poly L-Pro--poly L-Lys and poly(D-Tyr,D-Glu)-poly-D-Pro--poly D-Lys, respectively.

However, it is possible that the above conclusions concerning neonatal T cell responsiveness are an oversimplification. The antibody responses to thymus-dependent antigens are, by definition, highly dependent on T cells. The presence of accessory cells in such responses is an additional requirement (15,36). On the other hand, the requirement for both A and T cells is much less for the responses to thymus-independent antigens. For example, the response to the thymus-independent antigen DNP-Ficoll has been demonstrated in athymic nude mice (242); furthermore, the lack of a requirement for macrophages in this *in vitro* response was

demonstrated in that non-adherent cells were fully active. However, Lee *et al.* (243) subsequently demonstrated that by stringently eliminating macrophages from the system (removal of carbonyl-iron-ingested cells with a magnetic field), the *in vitro* anti-DNP-Ficoll response of mouse spleen cells could be significantly reduced. This reduction was found also for the responses to two other thymus-independent antigens. Such cultures could be fully restored by the addition of macrophages. Thus, the dependence of these thymus-independent responses on macrophages was shown to be a relative and not an absolute one. Although the removal of macrophages using the adherence technique did not affect the response to DNP-Ficoll, the response to the thymus-dependent antigen SRC was drastically reduced. These observations strongly suggest that the requirements for accessory cells in thymus-dependent and -independent responses are markedly different. Antigens exhibiting a high T cell dependency also exhibit a high accessory cell requirement; and conversely low T cell dependency correlates with low accessory cell involvement. Therefore, interpretations of neonatal responsiveness to thymus-dependent and thymus-independent antigens must be collectively based on the role of T cells and accessory cell function in these responses. Therefore, the delayed development of responsiveness to thymus-dependent antigens described by Rabinowitz (237) and Hardy *et al.* (241) need not necessarily be due to a lack of T cell responsiveness *per se*, since a defect in the antigen-processing system could explain their results equally well.

c) Evidence for a Suppressor T Cell in Neonatal Mice

Mosier and Johnson (51) have described a cell population present in neonatal spleen and thymus which was capable of suppressing the *in vitro*

response of adult spleen cells to SRC. These cells, designated as suppressor T cells, were non-adherent and sensitive to anti-Thy-1 plus complement treatment. More recently, Mosier *et al.* (244) have demonstrated that neonatal suppressor T cells may be found in the outer cortical areas of the thymus and reported their surface antigen phenotype to be Ly-1,2,3$^+$, TL$^+$, with relatively high amounts of Thy-1 and low amounts of H-2 antigens. They proposed that the neonatal suppressor T cells found in the spleens of young mice express the same phenotypic markers, although no direct evidence to support this was given. It is clear, however, that these markers define a population of relatively immature T cells (Figure B), and from this point of view characterize a subpopulation of suppressor T cells unique from the inducible suppressor T cells found in the adult mouse. Durdick and Golub (52) reported that the suppressive effects of neonatal suppressor T cells could not be exerted through cell-impermeable membranes, suggesting the mode of action was by cell-to-cell contact, and did not involve soluble factors.

Mosier and Johnson (51) demonstrated using *in vitro* culture techniques that spleen cells from two-week-old BALB/c mice were unresponsive to SRC. This unresponsiveness, moreover, could not be overcome by the addition of adult T cells. If, however, the neonatal cell population was treated with anti-Thy-1 plus complement, an enhanced anti-SRC response could be obtained providing adult T cells were added to the culture. They concluded that the failure of cells from newborn mice to respond to SRC *in vitro* was due to the presence of suppressor T cells. In addition, they presented evidence which indicated that the suppressive activity of thymocytes in young BALB/c mice decreased in a gradual manner from birth to six weeks of age.

Whether this *in vitro* demonstration of suppressor cells has any bearing on the *in vivo* responses of neonatal mice is not clear at this time. When the responses of newborn to one-week-old C57BL/6 mice (Figure 3) are examined, one could argue that suppressor T cells were causing an active suppression; however, at about age 9-10 days (3-5 days for BALB/c mice [189]), an abrupt decrease in either the numbers or the activity of such cells would be expected since antibody responsiveness is rapidly increasing at this age. The evidence reported by Mosier and Johnson (51) indicates that suppressor cell activity does not change abruptly at this age, but decreases in a gradual manner from birth to six weeks of age. Thus, a direct correlation between *in vitro* suppressor cell activity and *in vivo* responsiveness is not dramatically evident.

Furthermore, since suppressor cells are found in the spleens of neonatal mice, one would expect that their effects would be observed in the adoptive transfer experiments presented in Figure 5. Indeed, when comparing the responses of intact mice and adoptively transferred spleen cells (Figure 8), a constant relationship between these two responses would be predicted if suppressor cells were active in the two systems. It is clear that the results in Figure 8 do not support this prediction.

One could argue also that the abrupt change in *in vivo* responsiveness marked a time in which the neonatal T cell population switched from an overall effect of suppression to one of help. Thus, at this time, the provision of additional helper T cells would be expected to lead to enhanced responsiveness. The results presented in Table XII demonstrated that the injection of adult thymocytes into eight-day-old mice did not alter either the magnitude nor the kinetics of the anti-SRC PFC response when the mice were subsequently challenged with SRC at 12 days of age.

Although the arguments presented above do not favour the role of suppressor T cell function in the lack of responsiveness exhibited by neonatal mice, none of the experiments in this thesis were specifically designed to assess this factor, since neonatal suppressor T cells were discovered after the completion of this work. It is possible that some of the results presented in this thesis could be interpreted in terms of suppressor T cell function. In Chapter I, the responses of neonatal intact mice (Figure 3) were compared with the responses given by spleen cells in adult irradiated recipients (Figure 5). The differences observed between these two sets of results (Figure 8) provided the basis for the proposed defect in the antigen-processing system of neonatal mice. If, however, neonatal suppressor T cells cannot survive adoptive transfer to adult irradiated recipients, or are unable to express their suppressive effects in this environment, the responses seen in the recipients (Figure 5) were elicited in the absence of suppressor T cells. Thus, by transferring spleen cells to irradiated recipients, the negative influence of suppressor T cells would be lost, allowing the appropriate T-B synergism to occur for antibody production. It is not known at this time whether this argument is a valid one, since the effects of adoptive transfer of neonatal suppressor T cells have not been reported. It should be re-emphasized, however, that these cells were discovered in, and are easily demonstrable using *in vitro* culture systems.

d) Suppressive Effects of α-Fetoprotein

Subsequent to the completion of the studies described in this thesis, Murgita and Tomasi described the effects of mouse α-fetoprotein on the *in vivo* (244) and *in vitro* (245) antibody responses in mice. They demonstrated that mice injected with mouse amniotic fluid (containing α-feto-

protein) from birth through young adulthood gave suppressed responses to a primary injection of SRC (244). The IgG and IgA responses were suppressed to a greater degree than the IgM response. Similarly, *in vitro* dose-response experiments utilizing purified α-fetoprotein demonstrated that IgG and IgA responses were suppressed by 100-fold lower concentrations of α-fetoprotein than were IgM responses (245). In addition, it was demonstrated that T cell functions such as allogeneic and mitogen-induced lymphocyte transformation were also suppressed by α-fetoprotein (246). Tomasi and Murgita concluded that the action of α-fetoprotein was consistent with an effect on T cells, although the data did not exclude a participation of macrophages and/or B cells in the suppression.

Since the mechanism of action of α-fetoprotein is not known, it is difficult to evaluate its contribution with regard to the pattern of neonatal responsiveness described in this thesis. Since the concentration of α-fetoprotein falls rapidly during the neonatal period, it could be argued that the sudden development of immunocompetence during this time period was correlated with the removal of this suppressive agent. To date, however, no direct evidence is available which demonstrates that the unresponsiveness of neonatal mice can be altered by the manipulation of α-fetoprotein levels.

Moreover, the suppressive abilities of α-fetoprotein have been recently questioned by Sheppard *et al.* (247). These workers could not demonstrate a significant suppression of the primary *in vitro* anti-SRC PFC response by α-fetoprotein. Using TNP-KLH as antigen *in vitro*, the secondary anti-TNP response was found to be inhibited by amniotic fluid but only at the highest concentrations tested. Normal serum and serum

from tumor-bearing mice, in which the α-fetoprotein levels were elevated, produced similar inhibition despite a 6,000-fold difference in α-fetoprotein concentrations. Also, purified α-fetoprotein was not inhibitory. It was demonstrated also that the *in vivo* PFC response to SRC in mice bearing a transplantable hepatoma was greater than that in normal mice, even though the serum α-fetoprotein concentration of the tumor-bearing mice was 1,000 times higher than normal. The same group of investigators have reported that *in vitro* and *in vivo* experiments in a rat system have failed to identify a consistent immunosuppressive or immunoregulatory role for α-fetoprotein (248). Thus, it appears that the suppressive abilities of α-fetoprotein are, at present, highly controversial.

The experiments reported in this thesis were not designed to evaluate potentially suppressive factors such as α-fetoprotein; however, the data obtained do not support a primary role for α-fetoprotein in the appearance of immunocompetence described here. Firstly, Murgita and Tomasi (245) have reported that IgG responses are inhibited by 100-fold lower concentrations of α-fetoprotein than are IgM responses. It could be argued then that as α-fetoprotein levels fell after birth, IgM and IgG responsiveness would appear at markedly different ages. The results of Figure 3 do not support this, since both responses appear to increase rapidly within 1-2 days of each other. Secondly, at a time when neonatal responsiveness is rapidly increasing (8-12 days of age), one would predict that α-fetoprotein levels would be below levels which were totally suppressive. Since the T cell has been proposed as a target for α-fetoprotein suppression (246), the injection of T cells into mice at an age when this suppressive agent is in apparently limiting amounts would be expected to result in an increased response. The results shown in

Table XII demonstrated that no change in responsiveness was in fact obtained, and are not consistent with α-fetoprotein being a major causative agent in the development of the responsiveness described here.

e) Summary

The possibility of a T cell-related defect being the primary cause in the delayed onset of responsiveness of neonatal C57BL/6 mice to SRC has been discussed from several points of view. The earlier evidence obtained suggesting a deficiency in the numbers of helper T cells (238) were of an indirect nature, and were not supported by evidence reported here (Table XII) and by others (153,232). The suppressive effects of α-fetoprotein and neonatal suppressor T cells have been described, although the *in vivo* relevance of these factors in the developing neonate is still not clear. It still remains to be demonstrated that changes in one or both of these factors during the first two weeks of life directly result in the observed increases in antibody-forming capability. Although the experimental approaches described in this thesis were not specifically designed to investigate these suppressive influences, the results described here do not support the exclusive role of either α-fetoprotein or suppressor T cells in the development of immunocompetence seen in neonatal C57BL/6 mice.

II. A CELL DEFECT

The antigen-processing system of neonatal mice has been the subject of several investigations designed to correlate the function of this system with immune responsiveness. It has been argued here that since

an antigen-processing system is required in the generation of an anti-SRC response, a delay in the maturation of this system in the newborn mouse would consequently result in low antibody responses. Thus, attempts have been made using a variety of approaches to evaluate the antigen-processing systems of neonatal mice.

a) *In vitro* Studies

It has been established that the *in vitro* requirement for an antigen-processing system can be satisfied with macrophages or adherent cells. Fidler *et al.* (249) prepared adherent and non-adherent (macrophage-depleted) cell populations from the spleens of both one-week-old and adult mice and demonstrated that adherent cells from neonatal mouse spleens functioned as well as adult adherent cells in reconstituting the response of a non-adherent (macrophage-depleted) spleen cell population. Similar results were obtained by Mosier and Johnson (51). Rabinowitz (237) demonstrated that adult macrophages, added in concentrations of 2-5% of the total cell number, did not enhance the *in vitro* anti-SRC proliferative or PFC responses of spleen cells derived from unresponsive one-week-old mice. Using these *in vitro* techniques, the conclusions reached were clear and consistent. Macrophages present in the spleens of young mice could function as accessory cells. Thus, these data did not support the concept of a deficiency in the antigen-processing system of young mice, at least not in terms of a complete lack of functional accessory cells.

b) *In vivo Studies*

In contrast, experimental approaches which have attempted to manipulate the *in vivo* antigen-processing systems of neonatal mice, have resulted in conflicting reports. Argyris (190) demonstrated that the

feeble anti-SRC PFC response of intact C3H mice could be enhanced by the injection of adult macrophages. Similar observations have been reported also by others with newborn mice (193) and rabbits (250,251). Thus, it appeared that a lack of functional macrophages could be involved in the low responsiveness of neonatal animals, although the evidence cannot be considered compelling.

Hardy *et al.* (236) compared the ability of peritoneal cells harvested from neonatal and adult mice to enhance the immunogenicity of *Shigella* antigen in sublethally irradiated recipients. Recipients receiving adult peritoneal cells preincubated with *Shigella* antigen were shown to mount higher antibody responses than those receiving *Shigella* antigen alone, or peritoneal cells from four-day-old mice preincubated with *Shigella* antigen. They concluded that the peritoneal cells of newborn mice were somehow incapable of interacting with *Shigella* antigen to lead to antibody formation, and were thus "immunologically immature". Furthermore, this immaturity was manifested in the low responsiveness of neonatal animals. Results not supporting this theory have been reported by Rabinowitz (237). In an attempt to overcome a possible macrophage deficiency or immaturity in neonatal mice, one-week-old "unresponsive" mice were immunized with adult macrophages containing opsonized SRC. Whereas the neonatal mice remained unresponsive, adult mice, serving as controls, mounted an anti-SRC response. Therefore, he concluded the unresponsiveness of neonatal mice was not due to a lack of functional macrophages.

In summary, experiments using *in vitro* techniques have demonstrated that macrophages from neonatal mice can function as accessory cells. Experiments conducted *in vivo* have led to contradictory results, some supporting a macrophage deficiency in neonatal mice, others not. The

experimental results described in this thesis, which have relied on *in vivo* cell cultures in adult irradiated recipients, strongly suggest that the antigen-processing system is lacking in the newborn mouse and, furthermore, the onset of maturation of this system results in an abrupt increase in immune responsiveness at 9-10 days of age.

c) The Concept of a Suitable Microenvironment

Although the antigen-processing system *in vitro* can be supplied by macrophages, it seems likely that the definition of this system *in vivo* is more complex. However, the presence of macrophages in the spleens of young mice may not be the only factor in determining whether or not these animals can process antigen effectively. Another factor which may be of critical importance in antibody responsiveness is the microanatomy of neonatal spleens in relation to bringing together the three cell types necessary for the effective stimulation of B cells. Thus, although neonatal mice may have functional macrophages which can capture and digest antigen, a lack in the ability to efficiently present immunogenic determinants to B and T cells in a suitable microenvironment could result in non-responsiveness.

The exact location in lymphoid tissue of the collaborative events leading to antibody formation is not known (252), however, it seems logical to assume that such a site would be characterized by the presence, at one time or another, of antigen, macrophages, B cells and T cells. The red pulp of the spleen has been shown to contain macrophages, however, it is now generally accepted that these macrophages are involved in the structural degradation of particulate antigens. Whether the digested products are converted to surface-localized immunogenic fragments is

not known. It seems clear, however, that the first histological signs of an immune response are seen in the splenic white pulp. Therefore, if a blood-borne thymus-dependent antigen was processed in the red pulp area of the spleen, the stimulated T cells would have to migrate to the white pulp before expressing the results of such stimulation. However, cell circulation studies suggest T cells in the red pulp are in the process of leaving the spleen, not migrating to areas of T cell proliferation (252,253).

The marginal zone of the spleen has been shown to contain both macrophages and lymphocytes. Intravenously injected antigen is localized in this area and, moreover, antigen associated with marginal zone macrophages appears to be largely extracellular. All lymphocytes entering the spleen from arterial circulation have a high probability of encountering antigen-laden macrophages in the marginal zone. Thus, the marginal zone is a potential site for immune cellular interactions since macrophages, B cells and T cells can be located here. If inductive collaborative processes are in fact occurring at this site, the time required must be short since the level of lymphocyte traffic in this area is high (252).

The periarteriolar lymphocyte sheath and diffuse cortex of the white pulp also contain macrophages, but there is no evidence for extensive antigen uptake by these cells.

The primary and secondary lymphoid follicles of the white pulp appear to be the only sites of long-term extracellular retention of antigen in the spleen. It appears that the dendritic macrophages responsible for antigen retention in this area are not involved in antigen degradation

and processing, so a strict analogy between follicular localization and *in vitro* macrophage-dependent cellular collaboration is not valid. However, since lymphoid follicles are characterized by the presence of (a) both B and T lymphocytes and (b) extracellularly-displayed antigen, they must be considered likely sites for *in vivo* cellular collaboration (252).

Williams and Nossal (191) studied the ontogeny of antigen-capturing structures by determining the distribution of ^{125}I-labelled polymerized flagellin in the newborn rat. At birth, the spleens and lymph nodes were at relatively immature stages of development. The development of structures capable of retaining antigen were first seen during the first 2-3 weeks following birth. In the spleen, a continuous labelling of the marginal zone was seen first at two weeks of age, which was followed, with increasing age, by characteristic follicular localization. Over this same time period, the lymph nodes gradually attained structures recognizable as the cortex and medulla, which were shown to become progressively more efficient in terms of their ability to retain antigen. Between two and six weeks of age, the antigen-retaining ability of lymph nodes, per unit weight, was shown to increase 5-fold. A comparison was subsequently drawn between the progressive increases in antigen retention and antibody-forming capabilities demonstrated by the developing rat (192,252). These studies demonstrated that maturational changes were occurring in the developing animal which allowed for an increased ability to capture and retain antigen.

Similar maturational changes may be responsible for the increase in responsiveness of neonatal mice described in this thesis. The adoptive

transfer system with adult irradiated recipients was extensively used in Chapters I and II in an attempt to define the factor(s) which limited the response of neonatal mice. In Chapter I it was demonstrated that immunocompetent cells could not be effectively stimulated by antigen *in situ* during the first week of life. However, the provision of the antigen-processing system of the adult irradiated recipient enabled these identical cells to respond (Figure 5). In Chapter II the antigen sensitivities of neonatal and adult mice were compared in terms of antigen optimum doses. Whereas intact neonatal mice were shown to be less sensitive to antigen than their adult counterparts, this difference was eliminated when the respective immunocompetent cell populations were challenged in adult irradiated recipients. These results are consistent with the conclusion that adult mice are able to process and present antigen to immunocompetent cells much more efficiently than are neonatal mice. It is proposed that this increased efficiency is likely related to the advanced morphological development of the secondary lymphoid organs of the adult irradiated recipient.

It is clear, however, from a review of the literature, that the morphological development of secondary lymphoid organs is not a parameter which has been routinely incorporated into the definition of the "antigen-processing system". Systems employing *in vitro* dissociated spleen cell cultures cannot possibly evaluate such a factor, whereas the adult irradiated recipient presumably provides the morphological framework necessary for the *in vivo* stimulation of immunocompetent cells — a framework which is apparently poorly developed in the newborn mouse.

Hanna *et al.* (254) reported evidence which supported the concept of an essential role for the reticular network of the spleen in the induction

of antibody formation. These authors compared three immune parameters of young adult mice (12-week-old) and aged mice (two-year-old). They observed that with advancing age, a decrease occurred in (a) antibody production, (b) antigen retention by splenic dendritic cells and (c) germinal centre formation. They demonstrated that in a limited number of aged mice, antibody production could be restored to young adult levels by the surgical implantation into the spleen of reticular stroma obtained from the spleens of young donors. They concluded that the lower immune capability of old mice was due, in part, to a degenerative change in the stromal reticular cells which provided a suitable microenvironment necessary for the stimulation of immunocompetent cells.

d) The Heterogeneity of Antigen-Handling Cells

An understanding of the role of the dendritic cell in the collaborative induction of an antibody response has been hampered by the failure of experimental approaches to isolate these cells *in vitro*. Thus, the *in vitro* manipulations which have led to an understanding of the function of macrophages in the induction of immunity have not so far been applied to dendritic cells. In Chapter III attempts were made to study the functional characteristics of both macrophage and dendritic cell types as they related to the responsiveness of neonatal mice.

The relative A cell frequencies in the spleens of 12-day-old and adult mice were compared in Chapter III-1, utilizing an *in vivo* protocol devised by Gorczynski *et al.* (36). These authors demonstrated that the cell being detected in this *in vivo* assay was likely identical to the adherent cell required in *in vitro* assays. This was shown in experiments using analytical cell separation. Thus, the A cell in this system can be

directly equated to the macrophage described in *in vitro* culture systems. Using this assay system, the A cell frequency in neonatal spleens was shown to be 100-fold lower than that in their adult counterparts (Figure 17).

The conclusions reached in these studies were consistent with the findings of Chapter III-2, in which antibody-mediated suppression had been used to evaluate A cell function. These experiments demonstrated that the suppressive effects of passively-administered antibody could be maintained over a prolonged period of time (six weeks) in adult mice, but not in newborn mice (Figure 18). It was reasoned that the adult mouse was able to sequester such antibody in an immunologically relevant site (possibly the A cell), thus removing it from the catabolic events occurring in circulation. Such a site was apparently not available in the newborn mouse. These data demonstrated that, as the age at which mice were passively immunized increased, from birth through eight weeks of age, the extent of suppression increased. Since suppressive antibody has been shown to affect A cell activity, the extent of suppression in this system was taken to reflect the size of the splenic A cell pool. These findings strongly suggested that the frequency of A cells in the spleens of neonatal mice was relatively low but increased steadily following the neonatal period and extending into early adult life.

In Chapter III-3 experiments were reported which were designed to test whether this lower level of A cell activity in neonatal spleens was reflected in a lowered capability of these spleens to capture circulating antigen. The results (Table IX) demonstrated that the reticuloendothelial system of the 12-day-old mouse was certainly well developed, as measured in terms of its ability to take up antigen, and comparable to that in

adult mice. The overall distribution of the radioactive-labelled antigen among the various body tissues appeared to be the same in both neonatal and adult mice, with the liver accounting for much of the antigen uptake. The splenic uptake for both age groups was low, such that two hours after injection, only 2-3% of the original inoculum could be detected in this organ. In these studies, the spleen was fractionated in an attempt to find differences in antigen localization patterns in the "cellular" and "reticular" fractions of 12-day-old and adult mice (Table X). No obvious differences between the two age groups were found in the levels of activity recovered in these two fractions. It was concluded, therefore, that the low levels of A cell activity present in neonatal spleens was not reflected in either a low level of antigen uptake or an altered distribution of such antigen within the spleen.

Thus, the results from these experiments demonstrated that the lower frequency of A cells found in neonatal mouse spleens was not translated into a lower level of antigen uptake. Therefore, the presence of macrophages, *per se*, in neonatal mouse spleens does not necessarily imply that one of the cellular requirements for antibody production has been met. These findings suggest the existence of a functional heterogeneity of macrophages, all of which may remove and degrade antigen in circulation, but with only a distinct subclass capable of functioning as A cells.

Direct support for this concept has been recently reported by Hodes *et al.* (255). While investigating the cellular and genetic controls of antibody responses *in vitro*, these authors determined the ability of adherent, non-T, non-B, radioresistant mouse spleen cells to function as accessory cells for the response to TNP-KLH and for the Ir gene-controlled response to TNP-(T,G)-A--L. They reported that pretreatment of adherent

cells with appropriate anti-Ia reagents plus complement totally abrogated the accessory function of this cell population for the responses to both antigens. However, while this treated adherent cell population was shown to be devoid of accessory cells, the proportion of phagocytic cells (as determined by latex particle ingestion) following anti-Ia treatment was shown to have actually increased. They concluded that the functionally active Ia^+ accessory cell was either non-phagocytic or represented a subpopulation of latex-ingesting cells. Similar findings and conclusions were reported for the Ia^+ accessory cell involved in Con A-induced T cell proliferation (256).

e) Summary

In conclusion, it is proposed that one reason for the low responsiveness of mice during their first week of life is the relatively low frequencies of A cells resident in their spleens. Since it has been shown that the injection of A cells into mice of this age had minimal (190) or no effect (237) on their ability to respond, it has been concluded that at this stage of development, the spleen is structurally unable to accommodate such cells in a way which can result in the effective stimulation of immunocompetent cells. Perhaps a better anatomical definition of the marginal zone or lymphoid follicles is required in this regard. Moreover, neonatal mice remain relatively unresponsive until such time as the necessary anatomical microenvironment is formed. It is proposed that this maturational event begins to occur in C57BL/6 mice at about ten days of age and results in a marked increase in the ability to produce antibody (Figure 3) and an increase in sensitivity to antigen (Chapter II).

III. B CELL DEFECT

Since the precursors of antibody-forming cells belong to the B cell category, functional deficiencies in this population of cells would directly affect the ability of animals to produce antibody. Thus, if at birth and in early neonatal life, the B cell population of mice is unable to receive and/or process the necessary signals for proliferation and differentiation into antibody-forming cells, the evident lack of responsiveness of neonatal C57BL/6 mice to SRC, described in this thesis, could be readily explained.

In this section, recent data concerning B cell ontogeny will be discussed under two headings. The first will deal with B cell maturation as defined by (i) the relatively high susceptibility of immature B cells to tolerance induction and (ii) the acquisition and proposed function of B cell membrane-associated IgD. Under the second heading, the restricted nature of the B cell repertoire will be discussed. The possible relationships between the findings reported in this thesis and these recent reports will be considered.

a) B Cell Maturation

i) Susceptibility of immature B cells to tolerance induction.

During the last two decades, it had been generally accepted that animals exhibited a higher susceptibility to tolerance induction during their fetal and neonatal periods as compared with adult life (257,258); however, the cellular mechanisms underlying this difference was not clear. It was speculated that lymphocytes passed through a stage of differentiation during which time they were highly susceptible to tolerance induction by antigen (259). This viewpoint was particularly attractive in the

context of theories dealing with the acquisition of self-tolerance during embryonic development. Moreover, the relatively low responsiveness of neonatal animals could be explained on this basis, with exposure to antigen predominantly resulting in tolerance as opposed to immunity.

More recently, Nossal and Pike (260) proposed that prior to maturity, B cells might be highly sensitive to an antigen-specific elimination by what has been termed a clonal abortion mechanism. This proposal was based on the findings that far lower concentrations of DNP-HGG were required to induce hapten-specific tolerance *in vitro* in adult bone marrow cells than in adult spleen cells.

Cambier *et al.* (261-263) reported similar findings when the susceptibilities of neonatal and adult B cells to tolerance induction were compared. In their experimental protocol, spleen cells from either adult or 6-10-day-old normal BDF_1 mice were treated with anti-brain-associated Thy-1 antiserum plus complement to remove T cells. Subsequently, these B cell populations were independently cultured *in vitro* for 24 hours with varying concentrations of TNP_{17}-HGG, washed, reconstituted with SRC-specific helper T cells, and cultured *in vitro* for an additional four days with TNP-SRC. At the end of this time, the anti-TNP and anti-SRC PFC responses were determined. The anti-SRC PFC responses, which served as controls, were not suppressed by the treatment of either age group of B cells with TNP_{17}-HGG. However, the anti-TNP PFC responses of both neonatal and adult B cells were found to be inhibited by the TNP_{17}-HGG tolerogen pretreatment; the degree of inhibition observed being dependent on the concentration of tolerogen used. Moreover, it was observed that the concentration of tolerogen required for 50% suppression of the anti-TNP res-

ponses was 1,000x lower for neonatal cells than for adult B cells. In addition, evidence was obtained which suggested the tolerogenic effect to be on B cells directly. Thus, the tolerogen dose requirements of neonatal and adult cells were not affected by the presence of T cells (cells susceptible to anti-brain-associated Thy-1 antiserum plus complement treatment) or the variation of accessory cell numbers during tolerance induction. Furthermore, removal of $Ly\text{-}2^+$ cells (possibly suppressor T cells) from the helper T cell population did not alter the effects of the tolerogen. Taken collectively, these data were interpreted as supporting the concept of functional clonal abortion of B cells as the mechanism for producing tolerance to self antigens.

However, a high susceptibility to tolerance induction certainly cannot be regarded as a general property of immature B cells with respect to all antigens. In 1962, Dresser (18) reported no difference in the dose of deaggregated BGG required to induce tolerance in neonatal and adult mice. Szewczuk and Siskind (264) have recently confirmed this finding in that with ultracentrifuged BGG, B cells from 17-day fetal, 8-day-old and adult mice were equivalent with respect to ease of tolerance induction both *in vivo* and *in vitro*. However, as reported by others (260-263), they observed that B cells from neonatal donors exhibited higher susceptibilities to tolerance induction than B cells from adult mice when polyvalent tolerogens were used, e.g. DNP_6-D-GL and deaggregated DNP_{22}-BGG. Howard and Hale (265) studied the susceptibility of mouse B cells to tolerance induction using two thymus-independent polysaccharide antigens and reported that no difference between neonatal and adult B cells could be demonstrated.

Thus, it appears from the information currently available, enhanced susceptibility of neonatal B cells to tolerance induction can be demonstrated using only hapten-carrier conjugates as tolerogens. Multivalency may be an important variable in this phenomenon but, clearly, it is not the only one since polysaccharide antigens with repeating structural determinants, do not make evident an enhanced susceptibility of neonatal B cells. T dependence of the response also does not appear to be a critical factor since both BGG and DNP-BGG are T dependent, but exhibit different tolerogenic properties. These considerations, therefore, make it likely that several different mechanisms may lead to B cell unresponsiveness and that immature B cells may differ from mature B cells in some of these mechanisms, but not in others.

The heightened susceptibility of immature B cells, as demonstrated with some antigens, might be due to improper membrane receptor modulation. For example, it has been demonstrated that immature B cells are unable to re-express surface Ig after stripping by exposure to anti-Ig, antigen or pronase (266-268), in contrast to their more mature counterparts. Alternatively, the acquisition of other cell membrane components such as IgD or CR may be relevant in the transition of B lymphocytes from an immature, tolerance-prone stage to a more mature state of immunocompetence.

Although the demonstration of susceptibility to tolerance induction has provided an insight into the concept of self-tolerance and B cell differentiation, as a whole, it remains to be seen whether this phenomenon has been reflected in the observations reported in this thesis. For example, it could be argued that the lack of responsiveness of intact C57BL/6 mice to SRC during the first week of life (Figure 3) was caused

by the preferential induction of tolerance as opposed to immunity. This, however, would not be consistent with the finding that spleen cells from mice of this age were perfectly able to respond when transferred to irradiated recipients (Figure 5). Furthermore, if neonatal mice were very susceptible to tolerance induction, one would predict that if they were to respond following challenge with antigen, they would do so at relatively low antigen doses. However, when the antigen sensitivities of neonatal and adult mice were compared, the exact opposite was found in that young mice required more antigen to respond optimally than did their adult counterparts (Figures 9-12). In conclusion, these findings strongly suggest that the relatively low responsiveness and sensitivity of neonatal C57BL/6 mice to SRC is not related to a heightened susceptibility to tolerance induction in B cells.

ii) Role of IgD

IgD was first identified as a separate Ig class by Rowe and Fahey (269) on the basis of studies of an unusual human myeloma protein. This Ig was found to lack various biological properties such as complement fixation and reactivity with skin, mast cells or neutrophils (270), and was present in serum in relatively low concentrations (20-50 μg/ml); very few IgD-containing plasma cells were reported in normal human tissues (271).

In contrast to the scarcity of IgD both in plasma cells and in circulation, IgD, in association with IgM, has been recently found on a high proportion of human B lymphocytes (272,273). Due to this preponderance of IgD on B cell surfaces and its relative absence in circulation, it was suggested that this Ig served a unique specialized function as a cellular receptor for antigen. The general applicability of this concept has re-

ceived support by the finding of "IgD-like" immunoglobulins in mice (274, 275), rats (276), and possibly in rabbits (277), monkeys (278), chickens (279) and tortoises (280). However, a strict homology between these "IgD-like" molecules and human IgD still remains to be formally demonstrated.

Much of the information presently available concerning the function of membrane-bound IgD, particularly as it relates to the ontogenic development of B cells, has been derived using the mouse. Abney and Parkhouse (274) and Melcher *et al.* (275) were the first to demonstrate the presence of a "non-μ, non-γ" heavy chain on mouse B cells, a component which was thought to be the murine analogue of the human δ chain. It was subsequently demonstrated that the great majority (80-95%) of B cells in the spleens, lymph nodes and Peyer's patches of adult mice were found to express IgD as well as IgM (281). Moreover, IgD was the predominant membrane Ig, accounting for 60-70% and 85-95% of the radioiodinatable surface Ig on cells from the spleen (184) and Peyer's patches (187), respectively.

In marked contrast, a substantial proportion of B cells from adult bone marrow or neonatal spleen and lymph node was found to be IgD^- and expressed only IgM. Thus, Vitetta *et al.* (184) demonstrated that during the first week after birth, virtually all of the Ig^+ spleen cells of BALB/c mice were $\mu^+\delta^-$. Cells detected as $\mu^+\delta^+$ began to appear first at nine days of age, increased in frequency thereafter, and achieved adult levels at three months of age, at which time 50-60% of the Ig^+ cells were $\mu^+\delta^+$. Since $\mu^+\delta^-$ cells appeared at a very early stage in ontogeny, it was concluded that they were the immediate precursors of $\mu^+\delta^+$ cells. This conclusion was further substantiated by the findings of Goding (282), who

reported that following the injection of stem cells into lethally irradiated C57BL/6 mice, the first B cells to appear 12 days following reconstitution, were $\mu^+\delta^-$. Cells which were $\mu^+\delta^+$ appeared four days later (day 16), and by day 24 accounted for 80-90% of the total B cell pool.

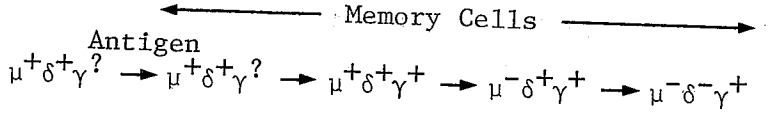
Some B cells, particularly those found in Peyer's patches, express membrane Ig which is predominantly, if not entirely, IgD. This has led to the proposal of a $\mu^-\delta^+$ cell by Vitetta and Uhr (283) and Abney *et al.* (284). However, the existence of significant numbers of $\mu^-\delta^+$ cells has been recently challenged by Goding *et al.* (285) based on technical considerations involving the fluorescent staining techniques used in these studies. Goding *et al.* (285) found very few cells which were $\mu^-\delta^+$ and point out that the frequencies of light chain-positive cells have always been shown to be very similar to that of μ^+ cells (286-288), regardless of the age of the cell donor or the organ examined. Furthermore, recent evidence indicated that B cells lost their surface IgD following activation (289,290). Therefore, these findings suggest the presence and amount of membrane-associated IgD to be a direct indicator of immunological cell maturity and that the normal antigen independent sequence of maturation in terms of IgM and IgD is:

$$\mu^-\delta^- \longrightarrow \mu^+\delta^- \longrightarrow \mu^+\delta^+$$

Subsequent to antigenic stimulation, it is evident that most of the responding B memory cells express IgG (291,292); only a fraction of these IgG-bearing memory cells express also IgM and IgD. Recently, Black *et al.* (293) reported evidence to suggest that the loss of surface IgD from B cells is an indication of the state of maturation of memory cells. Thus, spleen cells from either unprimed (normal) or hapten-primed mice were separated into IgD^+ and IgD^- fractions using a fluorescence-activated cell

sorter. These fractionated cells were adoptively transferred into irradiated recipients along with T cells and antigen, and the subsequent PFC responses determined. They observed that with unprimed cells, all the B cells capable of responding to a thymus-dependent antigen (burro red cells) were IgD^+; no responding B cells were found in the IgD^- fraction. In contrast, both IgD^+ and IgD^- cells from hapten-primed mice, when supplemented with carrier-primed cells, generated substantial IgG PFC responses when challenged with hapten-carrier conjugates. Moreover, the proportion of memory cells found in the IgD^- fraction apparently increased with time after the original exposure to antigen. In addition, using the plaque-inhibition technique, they demonstrated that high avidity antibody producers were primarily derived from the IgD^- cell population, whereas the low avidity producers were from the IgD^+ cell fraction.

An attempt to summarize these and other findings is presented in the scheme illustrated below:



However, it should be emphasized that the relationships between isotype expression and the state of differentiation is still a subject of wide study and controversy. One example is the antigen-independent acquisition of IgG on B cells proposed by Parkhouse and Cooper (185) and contested by Goding *et al.* (285).

One conclusion which can be drawn from the available evidence is that IgD is found on both unprimed and memory cells in conjunction with at least one other isotype; however, the definition of its exact role remains elusive. Recent attempts to define the function of surface IgD

have studied the reaction of mouse spleen cells with anti-μ or anti-δ (294). The findings indicated that, for thymus-dependent antigens, both IgM and IgD receptors were required for the activation of B cells, since pretreatment of cells with either anti-μ or anti-δ abrogated the capacity to produce antibodies. In contrast, only anti-μ was effective in inhibiting the responsiveness to a thymus-independent antigen suggesting that for this class of antigens, IgM but not IgD receptors were required.

However, this apparent correlation between IgD expression and thymus-dependency cannot be considered completely general. For example, the thymus-dependent responses of a significant subpopulation of memory cells are thought to be induced in IgD^- precursors (293). Moreover, thymus-independent antigens can be classified into two categories: (a) ones such as TNP-LPS and TNP-*Brucella abortus* which trigger B cells with IgM as the predominant isotype and (b) antigens such as TNP-Ficoll and TNP-Dextran which trigger B cells with IgD as the predominant isotype (295).

Perhaps the role of IgD in B cell activation is related to some intrinsic ability to modulate surface Ig in an appropriate manner subsequent to interaction with antigen. Thus, Cambier *et al.* (296) have demonstrated that preferential cleavage of surface IgD from cells results in an enhanced susceptibility to tolerance induction. However, it is not at all clear why modulation of surface IgD would lead to more effective triggering than other isotypes, unless there is a yet still undefined unique interaction with other membrane components (e.g. Ia, FcR or CR).

Regardless of the mechanism of action, surface IgD is required on B cells for a primary response to SRC (294). Therefore, it is likely that the development of responsiveness seen in neonatal spleen cells following transfer to irradiated recipients (Figure 5) is directly re-

lated to the increasing numbers of SRC-specific $\mu^+\delta^+$ cells derived from their $\mu^+\delta^-$ precursors. However, the results presented in Figures 3 and 8 demonstrated that these immunocompetent B cells cannot express their normal function in the intact neonatal environment. It follows, therefore, that the apparent lack of immunocompetence seen in intact 1-2-week-old C57BL/6 mice cannot be solely attributed to a lack of functional $\mu^+\delta^+$ antibody-forming cell precursors. Furthermore, these results suggest that the acquisition of functional B cells is not the "rate-limiting" step determining the responsiveness of intact mice.

A more direct analysis of this topic might be obtained by comparing the ages at which $\mu^+\delta^+$ cells and immune responsiveness to SRC are acquired. The available data, however, is still rather limited. Vitetta *et al.* (184) and Goding *et al.* (285) have determined the number of $\mu^+\delta^+$ B cells appearing in the spleens of BALB/c and C57BL/6 mice, respectively, as a function of time after birth. When the percentage of B cells which are $\mu^+\delta^+$ is expressed as a function of age, their combined results indicate that BALB/c and C57BL/6 mice acquire $\mu^+\delta^+$ cells at about the same rate, achieving 50% of their adult proportions at 2-3 weeks after birth. It should be noted, however, that Playfair (189) observed BALB/c mice to acquire responsiveness to SRC almost one week before C57BL/6 mice. Kearney *et al.* (297), using CBA mice, demonstrated that $\mu^+\delta^+$ cells achieve 50% of their adult proportions by 5-6 days of age, while Rabinowitz (237) reported that mice of this strain are late responders, first acquiring responsiveness to SRC between two and three weeks of age. This late responsiveness described by Rabinowitz, however, is likely to be a rather pessimistic assessment since anti-SRC IgM PFC responses were determined only on day 4 after immunization, a time certainly too early for neonatal responses (see

Figures 1 and 2). With the limited data available, it is not presently possible to correlate the increase in $\mu^+\delta^+$ cells and immune responsiveness, suggesting the acquisition of these cells is not the last maturational step in the development of antibody-forming capacity in newborn mice.

b) The Restricted Nature of the Neonatal B Cell Repertoire

In Chapter II, the anti-SRC responses of 12-day-old and 12-week-old mice were compared in terms of antigen dose-response curves. Two parameters of such curves were considered; the antigen optimum dose and the bandwidth. It was demonstrated that with intact mice, the antigen optimum doses for neonatal mice were 3- 4-fold higher than those observed in adult mice (Table II). When the spleen cells from such mice were analyzed in adult irradiated recipients using the dose-response analysis, the differences in the antigen optimum doses were no longer apparent (Table V). This was observed for both the IgM and IgG responses. Thus, it was concluded that the antigen optimum dose was a function of the antigen-processing system, and that such a system was relatively inefficient in the neonatal mouse since much higher antigen concentrations were required to effectively stimulate the available immunocompetent cell compartment.

When the bandwidths of the dose-response curves derived from intact 12-day-old and adult mice were compared (Figures 11, 12 and Table III), it was concluded that the neonatal mice tended to respond over a narrower antigen dose range than did their adult counterparts. This difference in responsiveness was apparent in both the IgM and IgG responses. Wortis *et al.* (298) described the anti-SRC PFC responses of CBA mice to varying doses of SRC administered i.v. or i.p. These workers found that the two

routes of immunization yielded identical PFC responses at optimal or supra-optimal doses of antigen. At low doses, however, i.p. administration yielded lower responses than the i.v. route. Thus, the possibility existed that the differences in bandwidths demonstrated in intact neonatal and adult mice were due to the different routes of immunization employed since neonatal mice were injected i.p. and adult mice i.v. This potentially trivial explanation for the differences in bandwidths was ruled out when immunocompetent cells from neonatal and adult mice were challenged with varying doses of antigen in adult irradiated recipients. In this system, the immunocompetent cells from either age group and antigen were administered i.v. The results demonstrated that the differences in bandwidths observed in intact mice (Table III) were maintained in the adoptive transfer system (Table VI). It was, therefore, concluded that the bandwidth was a reflection of some intrinsic property of the immunocompetent cell compartment and was not a parameter of the route of immunization. This finding eliminated also the antigen-processing system as a contributing factor in the determination of bandwidths.

i) Evaluation of the number of responding cell clones.

The cellular basis for the bandwidths described in this thesis is not known, however, it seems likely that the relatively narrow bandwidths observed in neonatal mice are a reflection of relatively restricted B cell repertoires in these mice. Klinman *et al.* (299) have estimated the total number of B cell specificities in neonatal and adult mice. They reported that neonatal mice can express at least 10^4 B cell specificities whereas in adult mice the number has been estimated to exceed 10^7. In the studies reported here, the multideterminant SRC has been used as antigen. It is conceivable that the neonatal mouse does not possess or express sufficient numbers of B cell specificities to react with

all the SRC determinants, thereby leading to a PFC response which is restricted to a limited number of SRC determinants. This lack of responsiveness to all of the determinants of the SRC would be consistent with the findings of Silverstein and Prendergast (161), who reported that there is an ordered sequential appearance of the capacity of developing young animals to respond to various antigens. Such a restriction could be translated into a narrow dose-response curve, since compared to adults, a relatively low number of cells would be responding.

Furthermore, the response of neonates to a given antigenic determinant may be restricted to a very limited number of clones. Montgomery and Williamson (300) investigated the degree of heterogeneity of anti-DNP antibodies produced by neonatal rabbits. They demonstrated that the antibodies produced by individual neonates could at times be very homogeneous, both in terms of antigen binding and the number of bands observed in isoelectric spectra. Thus, compared to adults, neonatal rabbits mounted restricted antibody responses.

Klinman *et al.* (299) have studied the response of neonatal BALB/c mice to the DNP and TNP haptenic determinants. These authors reported that using the *in vitro* splenic focus technique, three DNP- and three TNP-specific clonotypes could be identified in BALB/c splenic B cell populations during the first few days after birth. These specificities, which were expressed repeatedly among neonatal mice of the BALB/c population during the first few days after birth, accounted for approximately 90% of the DNP- or TNP-specific precursor cell populations, and were therefore designated predominant clonotypes. Although the neonatal population as a whole repeatedly expressed three DNP- or TNP-specific clonotypes, individual mice were found to express only one of these three predominant

clonotypes. Thus, at birth, the response of BALB/c mice to the DNP and TNP determinants was shown to be very restricted.

In contrast, adult mice appeared capable of expressing 1,500 - 7,000 distinct clonotypes specific for the DNP determinant (299). A rapid increase in diversity was demonstrated in early neonatal life as a shift away from the expression of predominant clonotypes to the expression of clonotypes designated as sporadic, since repeats were not identified. Whereas shortly after birth 90% of the clonotypes were of the predominant type, by nine days of age >90% were of the sporadic type.

It is possible that such an increase in the number of clonotypes is reflected further by an increased heterogeneity in the antigen sensitivity of the responding B cell population, since each clonotype is characterized by a unique Ig receptor with a correspondingly unique affinity for antigen. Such an increase in heterogeneity would be evident as an increase in the bandwidth of the antigen dose-response curves, with cells bearing high affinity receptors responding to low antigen doses and cells bearing low affinity receptors responding to high antigen doses. Thus, an increasing array of clonotypes expressed in the mouse following birth is likely responsible for the increase in bandwidths seen as the mouse matures with age.

ii) Evaluation at the cellular level of the heterogeneity of antibody affinities.

Goidl and Siskind (301) and, more recently, Marshall-Clarke and Playfair (232) have used a different approach in order to evaluate the restricted repertoire of neonatal mice. These studies have involved the determination of the relative affinities of antibodies at the cellular level utilizing the PFC inhibition technique. It was demonstrated that

when the anti-DNP PFC responses of adult and neonatal mice were compared, neonatal mice distinguished themselves by producing PFC which could be inhibited only by high concentrations of free hapten, i.e. PFC secreting antibody of relatively low affinity. Whereas PFC from adult mice were highly heterogeneous with regard to affinity, 1-2-week-old neonates produced PFC which, in contrast, were homogeneous in terms of affinity. This inability of neonatal mice to express an adult type of affinity heterogeneity was analyzed both *in vivo* (232) and in adult irradiated recipients (301), and was judged not to be related to a possible functional inadequacy of T cells or the antigen-processing system, but rather due to intrinsic properties of the antibody-forming cell precursors (B cells). This maturational aspect of the immune response was studied as a function of age. The results of Goidl and Siskind (301), using LAF_1 mice, demonstrated that by two weeks of age, neonatal mice were indistinguishable from adults with regard to the spectrum of affinities which could be elicited. The transition to increased heterogeneity occurred between 7-10 days of age. Marshall-Clarke and Playfair (232), on the other hand, reported that the PFC responses of (C57BL/6 x BALB/c)F_1 mice were still of the restricted type at two weeks of age. Thus, the transition of a PFC response from the restricted neonatal type to the more heterogeneous adult type can occur rapidly; however, the rate at which this occurs may be genetically determined as has been reported by Cohen *et al.* (302).

The results discussed above (232, 299-302) support the concept that one of the maturational events associated with the development of an antibody response in neonatal animals is a shift from a clonally restricted response to a response which is more heterogeneous — as evidenced by an increase in the number of distinct clonotypes expressed and the spectrum

of affinities observed. The results presented in this thesis related to the bandwidths of the dose-response curves are interpreted as a further manifestation of the restricted nature of the neonatal response. These results are consistent with there being an expansion in the responding immunocompetent B cell compartment during the development of the neonatal mouse, and that at least part of this process occurs subsequent to 12 days of age in the C57BL/6 strain of mice.

The mechanism(s) involved in such an expansion or generation of antibody diversity is presently a subject of considerable investigation and discussion. Two general theories have been proposed. Germ-line theories propose that all the necessary genetic information for the entire catalogue of antibodies is present in every B lymphocyte. Individual cells during gestation are rendered specific by virtue of a differentiational event which causes the suppression of all but one or a very limited number of genes. Such an event would be selected for by evolutionary pressures. On the other hand, somatic theories propose a limited germ-line inheritance from which the antibody repertoire is generated via subsequent somatic events. In this case, the nature of the selective pressures involved in the generation of specificities is largely unknown, although antigen-driven selection may be a factor.

The results presented in this thesis do not allow for the differentiation of these theories. It can be argued that the increase in bandwidths of the dose-response curves is a reflection of an antigen-driven mutational process since the mice used in this study were not germ-free and an antigen isolated from at least one commonly-occurring enteric bacterium was shown to crossreact with SRC (Chapter III-4). However, the possible role of such environmental antigens in the determination of bandwidths was not assessed directly.

On the other hand, Goidl and Siskind (301) have reported that germ-free mice produce an antibody response which is indistinguishable from conventionally reared mice with respect to heterogeneity of affinity. If one assumes that this latter property is directly related to the heterogeneity demonstrated by the bandwidths of the dose-response curves, then a justifiable conclusion would be that antigen-driven somatic mutation does not play a role in the increase in heterogeneity of antigen sensitivity reported in this thesis.

IV. SUMMARY AND CONCLUSION

In this Discussion, the ontogeny of antibody formation in mice has been considered in terms of maturational development in three cellular compartments. The maturation of the B cell compartment was discussed in terms of recent reports by other investigators relating to (i) the relative susceptibility of B cells from neonatal mice to tolerance induction, (ii) the acquisition of surface IgD by B cells and (iii) the generation of diversity in the B cell repertoire. When the results reported in this thesis were interpreted in light of these recent findings, several conclusions were made.

Firstly, the relative susceptibility of B cells from neonatal mice to *in vitro* tolerance induction did not appear to be a major factor in the *in vivo* responsiveness of neonatal mice. It was argued that, under conditions which preferentially led to tolerance induction, the very feeble responses of neonatal mice would be expected to be elicited by relatively low doses of antigen. This prediction was not, in fact, borne out by the data obtained, since both 7-day-old and 12-day-old C57BL/6

mice exhibited 3- 4- fold higher optimum antigen doses than did their adult counterparts (Tables II and IV).

Secondly, evidence was discussed which emphasized the importance of surface IgD in the triggering of B cells. Although the exact role of this membrane-bound Ig is not presently known, it has been shown to be essential in primary responses to thymus-dependent antigens. Since this surface component appears relatively late in B cell ontogeny, it is possible that the acquisition of IgD, perhaps concomitantly with other markers such as Ia and CR, represents the last major differentiative step in the formation of immunocompetent B cells. However, it is apparent from data presented in this thesis that immunocompetent B cells can be detected in the spleens of neonatal mice (Figure 5) before the intact animal develops responsiveness. Therefore, it was concluded that although the acquisition of IgD and other membrane components may control the rate of development of immunocompetent B cells, there was at least one other factor which served to limit or restrict the development of responsiveness of the intact animal.

Thirdly, it became apparent, when the antigen dose-response curves of neonatal and adult mice were compared, that the former group responded over a relatively restricted dose range. It was concluded that this observation reflected a relatively restricted B cell repertoire in neonatal mice — a finding consistent with the reports of others.

The possibility of a helper T cell deficiency in the spleens of neonatal mice was considered as a factor in the delayed onset of immune responsiveness, particularly to thymus-dependent antigens. Based on indirect evidence, such as the ability of LPS (a putative T cell-replacing factor) to activate neonatal B cells (238) and the observations that responses

to thymus-independent antigens precede, during ontogeny, those to thymus-dependent antigens (237,241), a neonatal helper T cell deficiency was proposed. However, evidence of a more direct nature is decidedly lacking. Experiments reported in this thesis which addressed this point demonstrated that, although a relative T cell deficiency could be detected in the spleens of two-day-old mice (Table I), such a deficiency was not apparent by twelve days of age (Table XII). These findings were consistent with those of others, who reported the absence in newborn spleens of cells capable of synergizing with adult B cells in an adoptive transfer system; however, such cells could readily be detected at four days of age (153). Therefore, it was concluded that a helper T cell deficiency was not a primary factor in the abrupt development of responsiveness seen in intact neonatal mice.

The relatively recent discovery of suppressor T cells in the thymus and spleen of the neonatal mouse has led to a re-evaluation of the immunological status of neonatal T cell populations. Up till now, the description of the suppressive capabilities of such cells has been confined to *in vitro* culture systems, whereas the *in vivo* properties of neonatal suppressor T cells are largely unknown. In more specific terms, it is not known whether changes in either the numbers of, or the intrinsic properties of suppressor T cells occur at the time when the responsiveness of neonatal mice is seen to be rapidly increasing. Nor is it known what effects this cell type would have on the overall sensitivity of the intact mouse to antigen. Clearly, more information is required in this general area. Nevertheless, if the delayed onset of immune responsiveness and the relatively low sensitivity to SRC of neonatal mice is ascribed to the presence of suppressor T cells, the apparent lack of expression of these cells in the adoptive transfer system using irradiated recipients

remains to be explained. This possibility would be difficult to reconcile with the ability of these cells to express their suppressive effects in *in vitro* cultures.

Thus, the available data on neonatal suppressor T cells do not allow for a definitive description of their effects *in vivo*. Although the experimental approaches described in this thesis were not designed to assess this aspect, the results obtained do not favour the neonatal suppressor T cell as a primary agent in controlling neonatal responsiveness.

In contrast, maturational changes in the antigen-processing system were implicated in the development of responsiveness. Thus, at an age when intact mice were unable to respond to an antigenic stimulus, spleen cells from such unresponsive mice, following transfer to adult irradiated recipients, could respond to SRC (Figures 3 and 5). The interpretation drawn from these observations was that the adult irradiated recipient was providing an essential component necessary for the induction of antibody responses to SRC. This contribution, which was apparently radiation-resistant and absent in newborn mice, was defined as the antigen-processing system.

When the responsiveness of neonatal mice was further defined in terms of antigen dose-response curves, it was found that intact 12-day-old C57BL/6 mice were 3- 4- fold less sensitive to SRC than their adult counterparts (Table II). Furthermore, this difference in sensitivity was not seen when spleen cells from neonatal and adult donors were analyzed in adult irradiated recipients (Table V). It was concluded that sensitivity to antigen, as defined by the antigen optimum dose, was a parameter of the antigen-processing system and that this system was relatively inefficient in neonatal mice.

Analysis of the antigen-processing system by means of A cell assays demonstrated that the A cell frequency in 12-day-old spleens was 100-fold lower than in the spleens of adults (Figure 17). Furthermore, the A cell population, as judged by the property of prolonged retention of specific suppressive antibody, was also shown to be relatively deficient in young mice (Figure 18). However, these functional deficiencies in neonatal A cell populations were not reflected in an assessment of the reticulo-endothelial system, since the rate of clearance and tissue distribution patterns of radioactive-labelled SRC were essentially the same in neonatal and adult mice. Collectively, these observations suggested the existence of a functional heterogeneity among antigen-handling cells, with A cells defining a specialized subpopulation.

Finally, the functional deficiency of the neonatal antigen-processing system is not simply due to a total lack of A cells, since neonatal spleen cells exhibiting this activity have been reported here and elsewhere. Furthermore, other investigators have demonstrated that the injection of adult macrophages (containing A cells) resulted in, at best, a minimal increase in antibody-forming capacity. Therefore, it is proposed that the neonatal spleen lacks the appropriate anatomic microenvironment necessary for the effective accommodation of A cells. This, in turn, results in a relatively inefficient presentation of antigenic determinants to the immunocompetent cell pool. It is further proposed that changes in the splenic architecture conducive to cellular collaboration first occur 9-10 days following birth and represent a major factor in the increased immune responsiveness seen in intact neonatal mice.

LIST OF REFERENCES

1. Ehrlich, P.
Proc. R. Soc. London, Ser. B. 66, 424 (1900)

2. Landsteiner, K. (1936) The Specificity of Serological Reactions, Springhill, Ill., Thomas.

3. Pauling, L.
J. Am. Chem. Soc. 62, 2643 (1940)

4. Jerne, N.K.
Proc. Natl. Acad. Sci. 41, 849 (1955)

5. Burnet, F.M. (1959) The Clonal Selection Theory of Immunity. Nashville, Tenn., Vanderbilt University Press.

6. Good, R.A. and A.E. Gabrielson (ed.) (1964) The Thymus in Immunobiology, New York, Harper and Row.

7. Miller, J.F.A.P., A.H.E. Marshall and R.G. White.
Adv. Immunol. 2, 111 (1962)

8. Glick, B., T.S. Chang and R.G. Jaap.
Poult. Sci. 35, 224 (1956)

9. Cooper, M.D., R.D.A. Peterson, M.A. South and R.A. Good.
J. Exp. Med. 123, 75 (1966)

10. Cooper, M.D., W.A. Cain, P.J. Van Alten and R.A. Good.
Int. Arch. Allergy Appl. Immunol. 35, 242 (1969)

11. Claman, H.N., E.A. Chaperon. and R.F. Triplett.
Proc. Soc. Exp. Biol. Med. 122, 1167 (1966)

12. Davies, A.J.S., E. Leuchars, V. Wallis, R. Marchant and E.V. Elliott.
Transplantation 5, 222 (1967)

13. Mitchell, F.T. and J.F.A.P. Miller.
Proc. Natl. Acad. Sci. U.S.A. 59, 296 (1968)

14. Nossal, G.J.V., A. Cunningham, G.F. Mitchell and J.F.A.P. Miller.
J. Exp. Med. 128, 839 (1968)

15. Mosier, D.E.
Science 158, 1573 (1967)

16. Mosier, D.E. and L.W. Coppleson.
Proc. Natl. Acad. Sci. U.S.A. 61, 542 (1968)

17. Mosier, D.E., F.W. Fitch, D.A. Rowley and A.J.S. Davies.
Nature (London) 225, 276 (1970)

18. Dresser, D.W.
Immunology 5, 378 (1962)

19. Claman, H.N.
J. Immunol. 91, 833 (1963)

20. Battisto, J.R. and J. Miller.
Proc. Soc. Exptl. Biol. Med. 111, 111 (1962)

21. Frei, P.C., B. Benacerraf and G.J. Thorbecke.
Proc. Natl. Acad. Sci. 53, 20 (1965)

22. Benacerraf, B., B.H. Halpern, C. Stiffel, C. Cruchard and G. Biozzi.
Ann. Inst. Pasteur 89, 601 (1955)

23. Thorbecke, G.J., P.H. Maurer and B. Benacerraf.
Brit. J. Exptl. Path. 41, 190 (1960)

24. Unanue, E.R. and B.A. Askonas.
Immunology 15, 287 (1968)

25. Unanue, E.R. and B.A. Askonas.
J. Exp. Med. 127, 915 (1968)

26. Gallily, R. and M. Feldmann.
Immunology 12, 197 (1967)

27. Mitchison, N.A.
Immunology 16, 1 (1969)

28. Spitznagel, J.K. and A.C. Allison.
J. Immunol. 104, 128 (1970)

29. Schmidtke, J.R. and E.R. Unanue.
J. Immunol. 107, 331 (1971)

30. Unanue, E.R.
Adv. Immunology 15, 95 (1972)

31. Katz, D.H. and E.R. Unanue.
J. Exp. Med. 137, 967 (1973)

32. Roseman, J.
Science 165, 1125 (1969)

33. Haskill, J.S., P. Byrt and J. Marbrook.
J. Exp. Med. 131, 57 (1970)

34. Shortman, K., E. Diener, P. Russell and W.D. Armstrong.
J. Exp. Med. 131, 461 (1970)

35. Osoba, D.
J. Exp. Med. 132, 368 (1970)

36. Gorczynski, R.M., R.G. Miller and R.A. Phillips.
J. Exp. Med. 134, 1201 (1971)

37. Mitchison, N.A. (1969) in "Immunological Tolerance" (M. Landy and W. Braun, eds.), p. 149, Academic Press, New York.

38. Ovary, Z. and B. Benacerraf.
Proc. Soc. Exp. Biol. Med. 14, 72 (1963)

39. Mitchison, N.A.
Eur. J. Immunol. 1, 18 (1971)

40. Raff, M.C.
Nature (London) 226, 1257 (1970)

41. Gershon, R.K. and K. Kondo.
Immunology 18, 723 (1970)

42. Gershon, R.K. and K. Kondo.
Immunology 21, 903 (1971)

43. Baker, P.J., P.W. Stashak, D.F. Amsbaugh, B. Prescott and R.F. Barth.
J. Immunol. 105, 1581 (1970)

44. Takemori, T. and T. Tada.
J. Exp. Med. 142, 1241 (1975)

45. Kapp, J.A., C.W. Pierce and B. Benacerraf.
J. Exp. Med. 145, 828 (1977)

46. Okumura, K. and T. Tada.
J. Immunol. 106, 1019 (1971)

47. Okumura, K. and T. Tada.
J. Immunol. 107, 1682 (1971)

48. Herzenberg, L.A., K. Okumura and C.M. Metzler.
Transplant. Rev. 27, 57 (1975)

49. Eichmann, K.
Eur. J. Immunol. 5, 511 (1975)

50. Waldmann, T.A., S. Broder, M. Durm, M. Blackman and B. Meade (1975) in "Immune Depression and Cancer" (G.W. Siskind, C.I. Christian and S.D. Litwin, eds.), p. 20, Grune & Stratton, New York.

51. Mosier, D.E. and B.M. Johnson.
J. Exp. Med. 141, 216 (1975)

52. Durdick, J. and E.S. Golub, quoted in "Lymphocyte Differentiation, Recognition and Regulation" by D.H. Katz, p. 119, Academic Press, New York, 1977.

53. Benacerraf, B.
Ann. Inst. Pasteur 125, 143 (1974)

54. Kantor, F.S., A. Ojeda and B. Benacerraf.
J. Exp. Med. 117, 55 (1963)

55. Levine, B.B., A. Ojeda and B. Benacerraf.
J. Exp. Med. 118, 953 (1963a)

56. Levine, B.B. and B. Benacerraf.
Science 147, 517 (1965)

57. Levine, B.B., A. Ojeda and B. Benacerraf.
Nature 200, 544 (1963b)

58. Green, I., W.E. Paul and B. Benacerraf.
J. Exp. Med. 123, 859 (1966)

59. McDevitt, H.O. and M. Sela.
J. Exp. Med. 122, 517 (1965)

60. McDevitt, H.O. and A. Chinitz.
Science 163, 1207 (1969)

61. McDevitt, H.O., B.D. Deak, D.C. Shreffler, J. Klein, J.H. Stimpfling and G.D. Snell.
J. Exp. Med. 135, 1259 (1972)

62. Shreffler, D.C. and C.S. David.
Adv. Immunol. 20, 125 (1975)

63. Shevach, E.M., D.L. Rosenthal and I. Green.
Transplantation 16,126 (1973)

64. Dorf, M.E., H. Balner and B. Benacerraf.
J. Exp. Med. 142, 673 (1975)

65. Berzofski, J.A. and D.J. Killion.
Abstract , 4th Ir Gene Workshop, Annapolis, Maryland, May 22, 1978.

66. Okuda, K., S.S. Twining, M.Z. Atassi and C.S. David.
Abstract, 4th Ir Gene Workshop, Annapolis, Maryland, May 22, 1978.

67. Dorf, M.E. and B. Benacerraf.
Proc. Nat. Acad. Sci. 72, 3671 (1975)

68. Cantor, H. and E.A. Boyse.
J. Exp. Med. 141, 1376 (1975)

69. Feldmann, M., P.C.L. Beverley, M. Dunkley and S. Kontiainen.
Nature (London) 262, 495 (1975)

70. Benacerraf, B. and M. Dorf. (1976) in "The Role of Products of the Histocompatibility Gene Complex in Immune Responses" (D.H. Katz and B. Benacerraf, eds.), p. 225, Academic Press, New York.

71. Benacerraf, B. and M.E. Dorf.
Cold Spring Harbor Symp. Quant. Biol. 41, 465 (1976)

72. David, C.S.
Transplant. Rev. 30, 299 (1976b)

73. Hämmerling, G.J., B.D. Deak, G. Mauve, V. Hämmerling and H.O. McDevitt.
Immunogenetics 1, 68 (1974)

74. Hämmerling, G.J., G. Mauve, E. Goldberg and H.O. McDevitt.
Immunogenetics 1, 428 (1975).

75. Dorf, M. E. and Unanue , E.R. (1977) in "Ir Genes and Ia Antigens", Proc. Third Ir Gene Workshop (H.O. McDevitt, ed.), Academic Press, New York.

76. Taussig, M.J., A.J. Munro, R. Campbell, C.S. David and N. Staines.
J. Exp. Med. 142, 694 (1975)

77. Mozes, E. (1976) in "The Role of Products of the Histocompatibility Complex in Immune Responses" (D.H. Katz and B. Benacerraf, eds.), p. 485, Academic Press, New York.

78. Murphy, D.B., L.A. Herzenberg, K. Okumura and H.O. McDevitt.
J. Exp. Med. 144, 699 (1976)

79. Tada, T., M. Taniguchi and C.S. Takemori.
J. Exp. Med. 144, 713 (1976)

80. Greene, M.I., A. Pierres, M.E. Dorf and B. Benacerraf.
J. Exp. Med. 146, 293 (1977)

81. Thèze, J., C. Waltenbaugh, M.E. Dorf and B. Benacerraf.
J. Exp. Med. 146, 287 (1977a)

82. Rosenthal, A.S. and E.M. Shevach.
J. Exp. Med. 138, 1194 (1973)

83. Shevach, E.M., W.E. Paul and I. Green.
J. Exp. Med. 136, 1207 (1972)

84. Shevach, E.M.
J. Immunol. 116, 1482 (1976)

85. Shevach, E.M., M.L. Lindquist, H.F. Geczy and B.D. Schwartz.
J. Exp. Med. 146, 561 (1977)

86. Erb, P. and M. Feldmann.
J. Exp. Med. 142, 460 (1975)

87. Pierce, C.W., J.A. Kapp and B. Benacerraf.
J. Exp. Med. 144, 371 (1976)

88. Benacerraf, B. and R.N. Germain.
Immunol. Rev. 38, 70 (1978)

89. Pierres, M. and R.N. Germain.
J. Immunol. 121, 1306 (1978)

90. Katz, D.H., T. Hamaoka, M.E. Dorf, P.H. Maurer and B. Benacerraf.
J. Exp. Med. 138, 734 (1973c)

91. Katz, D.H., N. Chiorazzi, J. McDonald and L.R. Katz.
J. Immunol. 117, 1853 (1976a)

92. Waltenbaugh, C., J. Thèze, J.A. Kapp and B. Benacerraf.
J. Exp. Med. 146, 970 (1977b)

93. Katz, D.H., T. Hamaoka, M.E. Dorf and B. Benacerraf.
Proc. Natl. Acad. Sci. U.S.A. 70, 2624 (1973)

94. Katz, D.H., M.E. Dorf and B. Benacerraf.
J. Exp. Med. 140, 290 (1974)

95. Katz, D.H., W.E. Paul, E.A. Goidl and B. Benacerraf.
J. Exp. Med. 133, 169 (1971)

96. Amerding, D., D.H. Sachs and D.H. Katz.
J. Exp. Med. 140, 1717 (1974)

97. Feldmann, M. and A. Basten.
Nature (London), New Biol. 237, 13 (1972)

98. Feldmann, M. and A. Basten.
J. Exp. Med. 136, 49 (1972)

99. Feldmann, M. and A. Basten.
J. Exp. Med. 136, 737 (1972)

100. Cantor, H. and E.A. Boyse.
J. Exp. Med. 141, 1376 (1975)

101. Lance, E.M. S. Cooper and E.A. Boyse.
Cell. Immunol. 1, 536 (1970)

102. Schlesinger, M. and V.K. Golalai.
Science 155, 1114 (1967)

103. Andersson, B. and H. Blomgren.
Cell. Immunol. 1, 362 (1970)

104. Raff, M.C. and Cantor, H.
Prog. Immunol., Int. Congr. Immunol., 1st. Vol. 1, p. 83 (1971)

105. Takemori, T. and T. Tada.
J. Exp. Med. 140, 253 (1974)

106. Okumura, K., T. Takemori, T. Tokuhisa and T. Tada.
J. Exp. Med. 146, 1234 (1977)

107. Tada, T., M. Taniguchi and C.S. David.
Cold Spring Harbor Symp. Quant. Biol. 41, 119 (1976)

108. Tada, T., M. Taniguchi and K. Okumura (1977) in "Progress in Immunology", Vol. III, 369.

109. Tada, T., T. Takemori, K. Okumura, M. Nonaka and T. Tokuhisa.
J. Exp. Med. 147, 446 (1978)

110. Feldmann, M., P. Erb, S. Kontiainen and M. Dunkley (1975) in "Membrane Receptors of Lymphocytes", (M. Seligmann, J.L. Preud'homme and F.M. Kourilsky, eds.), North Holland Publishing Co., Amsterdam.

111. Erb, P. and M. Feldmann.
Cell. Immunol. 19, 356 (1975)

112. Erb. P., P. Vogt, B. Meier and M. Feldmann.
J. Immunol. 119, 206 (1977)

113. Kontiainen, S. and M. Feldmann.
Eur. J. Immunol. 6, 296 (1976)

114. Feldmann, M. and S. Kontiainen.
Eur. J. Immunol. 6, 302 (1976)

115. Kontiainen, S. and M. Feldmann.
J. Exp. Med. 147, 110 (1978)

116. Eardley, D.D., J. Hugenberger, L. McVay-Boudreau, F.W. Shen, R.K. Gershon and H. Cantor.
J. Exp. Med. 147, 1106 (1978)

117. Cantor, H., L. McVay-Boudreau, J. Hugenberger, K. Naidorf, F.W. Shen and R.K. Gershon.
J. Exp. Med. 147, 1116 (1978)

118. Moore, M.A.S. and J.J.T. Owen.
J. Exp. Med. 126, 715 (1967)

119. Moore, M.A.S. and J.J.T. Owen.
Lancet 2, 658 (1967)

120. Owen, J.J.T. and M.A. Ritter.
J. Exp. Med. 129, 431 (1969)

121. Ford, C.E.
Thymus: Exp. Clin. Stud. Cib. Found. Symp., p. 131 (1965)

122. Strominger, J.L., P. Cresswell, H. Grey, R.H. Humphreys, D. Mann, J. McCune, P. Parham, R. Robb, A.R. Sanderson, T.A. Springer, C. Terhorst and M.J. Turner.
Transplant. Rev. 21, 126 (1974)

123. Stutman, O. (1975) in "Biological Activity of Thymic Hormones", p. 87, Kooyker Sci. Publ., Rotterdam.

124. Nossal, G.J.V. and B.L. Pike.
Immunology 25, 33 (1973)

125. Owen, J.J.T., M.C. Raff and M.D. Cooper.
Eur. J. Immunol. 5, 468 (1975)

126. Katz, D.H. (1977) in "Lymphocyte Differentiation, Recognition and Regulation", Academic Press, New York.

127. Dalmasso, A.O., C. Martinez, K. Sjodin and R.A. Good.
J. Exp. Med. 118, 1089 (1963)

128. Basch, R.S. and G. Goldstein.
Proc. Natl. Acad. Sci. U.S.A. 71, 1474 (1974)

129. Gershwin, M.E., A. Ahmed, A.D. Steinberg, G.B. Thurman and A.L. Goldstein.
J. Immunol. 113, 1068 (1974)

130. Boyse, E.A. and J. Abbott.
Fed. Proc. 34, 24 (1975)

131. Komuro, K. and E.A. Boyse.
Lancet 1, 740 (1973)

132. Komuro, K. and E.A. Boyse.
J. Exp. Med. 138, 479 (1973)

133. Scheid, M.P., G. Goldstein and E.A. Boyse.
Science 190, 1211 (1975)

134. Scher, I., A.D. Steinberg, A.K. Berning and W.E. Paul.
J. Exp. Med. 142, 637 (1975)

135. Storrie, B., G. Goldstein, E.A. Boyse and U. Hämmerling.
J. Immunol. 116, 1358 (1976)

136. Basch, R.S. and G. Goldstein.
Cell. Immunol. 20, 218 (1975)

137. Raff, M.C.
Transplant. Rev. 6, 52 (1971)

138. Boyse, E.A. and D. Bennett (1974) in "Cellular Selection and Regulation in the Immune Responses" (G.M. Edelman, ed.), p. 155, Raven Press, New York.

139. Schlesinger, M.
J. Immunol. 93, 255 (1964)

140. Lance, E. M. and S. Cooper.
Hormones and the Immune Response Ciba Found. Study Group 36, 73 (1970)

141. Raff, M. C.
Nature (London), New Biol. 229, 182 (1971)

142. Leckband, E. and E.A. Boyse.
Science 172, 1258 (1971)

143. Miller, J.F.A.P.
Proc. R. Soc. London, Ser. B. 156, 415 (1962)

144. Harris, J.E. and C.E. Ford.
Anat. Rec. 150, 113 (1964)

145. Nossal, G.J.V.
Ann. N.Y. Acad. Sci. 120, 171 (1964)

146. Weissman, I.
J. Exp. Med. 126, 291 (1967)

147. Stobo, J.D. and W.E. Paul.
Cell. Immunol. 4, 367 (1972)

148. Mosier, D.E.
J. Immunol. 112,305 (1974)

149. Miller, J.F.A.P.
Lancet 2, 748 (1961)

150. Taylor, R.B.
Nature 208, 1334 (1965)

151. Raff, M.C. and J.J.T. Owen.
Eur. J. Immunol. 1, 27 (1971)

152. Miller, J.F.A.P. and D. Osoba.
Physiol. Rev. 47, 437 (1967)

153. Chiscon, M.O. and E.S. Golub.
J. Immunol. 108, 1379 (1972)

154. Cooper, M.D. and A.R. Lawton.
Contemp. Top. Immunobiol. 1, 49 (1973)

155. Moore, M.A.S. and J.J.T. Owen.
Dev. Biol. 14, 40 (1966)

155a. Moore, M.A.S. and J.J.T. Owen.
Nature (London) 215, 1081 (1967)

156. Kincade, P.W. and M.D. Cooper.
J. Immunol. 106, 371 (1971)

157. Lawton, A.R., P.W. Kincade and M.D. Cooper.
Fed. Proc. 34, 33 (1975)

158. Durkin, H.G., G.A. Theis and G.J. Thorbecke (1971) in "Morphological and Functional Aspects of Immunity" (D. Lindahl-Kiessling, G. Alm and M.G. Hanna, eds.), p. 119, Plenum Press, New York.

159. Choi, Y.S. and R.A. Good.
J. Immunol. 111, 1485 (1973)

160. Dent, P.B. and R.A. Good.
Nature (London) 207, 491 (1965)

161. Silverstein, A.M. and R.A. Prendergast (1970) in "Developmental Aspects of Antibody Formation and Structure" (J. Sterzl and I. Riha, eds.), p. 69, Academic Press, New York.

162. Owen, J.J.T. and M.D. Cooper.
Nature (London) 249, 361 (1974)

163. Wu, A.M., J.E. Till, L. Simonovitch and E.A. McCulloch.
J. Cell. Physiol. 69, 177 (1967)

164. Trentin, J., N. Wolf, V. Cheng, W. Fahlberg, D. Weiss and R. Bonhag.
J. Immunol. 98, 1326 (1967)

165. Yung, L.L.L., T.C. Wyn-Evans and E. Diener.
Eur. J. Immunol. 3, 224 (1973)

166. Abramson, S., R.G. Miller and R.A. Phillips.
J. Exp. Med. 145, 1567 (1977)

167. Lafleur, L., R.G. Miller and R.A. Phillips.
J. Exp. Med. 135, 1363 (1972)

168. Lafleur, L., R.G. Miller and R.A. Phillips.
J. Exp. Med. 137, 954 (1973)

169. Lau, C., R.G. Miller and R.A. Phillips, quoted in Prog. Immunol. III, 155 (1977)

170. Phillips, R.A. and F. Melchers.
J. Immunol. 117, 1099 (1976)

171. Melchers, F., J. Andersson and R.A. Phillips.
Cold Spring Harbor Symp. Quant. Biol. 41, 147 (1976)

172. Osmond, D.G. and N.B. Everett.
Blood 23, 1 (1964)

173. Yoffey, J.M., G. Hudson and D.G. Osmond.
J. Anat. 99, 841 (1965)

174. Everett, N.B. and R.W. Caffrey (1967) in "The Lymphocyte in Immunology and Haemopoiesis" (J.M. Yoffey and E. Arnold, eds.), London.

175. Rosse, C.
Nature 227, 73 (1970)

176. Rosse, C.
Blood 38, 372 (1971)

177. Yoshida, Y. and D.G. Osmond.
Blood 37, 73 (1971)

178. Osmond, D.G. (1972) in "Proceedings of the Sixth Leucocyte Culture Conference" (M.R. Schwartz, ed.), Academic Press, New York.

178a. Osmond, D.G. and N.B. Everett.
Nature 196, 488 (1962)

179. Röpke, C. and N.B. Everett.
Cell Tissue Kinet. 6, 499 (1973)

179a. Osmond, D.G. and Nossal, G.J.V.
Cell. Immunol. 13, 132 (1974)

180. Miller, S.C. and D.G. Osmond.
Cell Tissue Kinet. 6, 259 (1973)

181. Ryser, J.E. and P. Vassalli.
J. Immunol. 113, 719 (1974)

181a. Raff. M.C., M. Megson, J.J.T. Owen and M.D. Cooper.
Nature 259, 224 (1975)

182. Gelfand, M.C., R. Asofsky and W.E. Paul.
Cell. Immunol. 14, 460 (1974)

182a. Hämmerling, V., A.F. Chin and J. Abbott.
Proc. Natl. Acad. Sci. 73, 2008 (1976)

182b. Kearney, J.F., M.D. Cooper, J. Klein, E.R. Abney, R.M.E. Parkhouse and A.R. Lawton.
J. Exp. Med. 146, 297 (1977)

183. Sidman, C.L. and E.R. Unanue.
J. Immunol. 114, 1730 (1975)

184. Vitetta, E.S., U. Melcher, M. McWilliams, M.E. Lamm, J.M. Phillips-Quagliata and J.W. Uhr.
J. Exp. Med. 141, 206 (1975)

185. Parkhouse, R.M.E. and M.D. Cooper.
Immunol. Rev. 37, 105 (1977)

186. Urban Jr., J.F. and K. Ishizaka.
J. Immunol. 121, 199 (1978)

187. Vitetta, E.S., M. McWilliams, J.M. Phillips-Quagliata, M.E. Lamm and J.W. Uhr.
J. Immunol. 115, 603 (1975)

188. Bosma, M.J., T. Makinodan and H. Walburg.
J. Immunol. 99, 420 (1967)

189. Playfair, J.H.L.
Immunology 15, 35 (1968)

190. Argyris, B.F.
J. Exp. Med. 128, 459 (1968)

191. Williams, G.M. and G.J.V. Nossal.
J. Exp. Med. 124, 47 (1966)

192. Williams, G.M.
J. Exp. Med. 124, 57 (1966)

193. Bendinelli, M., S. Senesi and G. Falcone.
J. Immunol. 106, 1681 (1971)

194. Carter, B.G. and E.S. Rector.
J. Immunol. 109, 1345 (1972)

195. Rector, E.S. and B.G. Carter.
J. Immunol. 110, 1591 (1973)

196. Carter, B.G. and E.S. Rector.
J. Immunol. 116, 218 (1976)

197. Jerne, N.K., A.H. Nordin and C. Henry (1963) in "Cell Bound Antibodies" (B. Amos and H. Koprowski, eds.), p. 109, Wistar Institute Press, Philadelphia.

198. Keckwick, R.A.
Biochem. J. 34, 1248 (1940)

199. Potter, N.
Methods Cancer Res. 2, 105 (1967)

200. Chou, C.-T., B. Cinader and S. Dubiski.
Fed. Eur. Biochem. Soc. Symp. 15, 133 (1969)

201. Phillips, D.R. and M. Morrison.
Biochem. and Biophys. Res. Commun. 40, 284 (1970)

202. Vitetta, E.S. and J.W. Uhr.
J. Exp. Med. 136, 676 (1972)

203. Marchalonis, J.J., R.E. Cone and V. Santer.
Biochem. J. 124, 921 (1971)

204. Dodge, J.T., C. Mitchell and D.J. Hanahan.
Arch. Biochem. Biophys. 100, 119 (1963)

205. Staub, A.M. (1967) in "Methods in Immunology and Immunochemistry" (C.A. Williams and M.W. Chase, eds.), Vol. I, p. 29, Academic Press, New York.

206. Britton, S.
Immunology 16, 513 (1969)

207. Taylor, R.B. and H.H. Wortis.
Nature 220, 937 (1968)

208. Evans, R.
J. Reticuloendothel. Soc. 8, 571 (1970)

209. Price, G.B. and T. Makinodan.
J. Immunol. 108, 403 (1972)

210. Feldmann, M.
J. Exp. Med. 135, 735 (1972)

211. Lachmann, P.J.
Proc. R. Soc., Ser. B. 176, 425 (1971)

212. Basten, A. and J.F. Howard.
Contemp. Top. Immunobiol. 2, 265 (1973)

213. Britton, S. and G. Moller.
J. Immunol. 100, 1326 (1968)

214. Woodrow, J.C. (1970) in "Rh Immunization and its Prevention", Series Haematologica 3, No. 3, p. 27.

215. Kappler, J.W., M. Hoffman and R.W. Dutton.
J. Exp. Med. 134, 577 (1971)

216. Feldmann, M. and E. Diener.
J. Exp. Med. 131, 247 (1970)

217. Sinclair, N.R. St. C. and P.L. Chan.
Adv. Exp. Med. Biol. 12, 609 (1971)

218. Pierce, C.W.
J. Exp. Med. 130, 365 (1969)

219. Ryder, R.J.W. and R.S. Schwartz.
J. Immunol. 103, 970 (1969)

220. Abrahams, S., R.A. Phillips and R.G. Miller.
J. Exp. Med. 137, 870 (1973)

221. Haughton, G. and D.R. Nash.
Transplantation Proc. 1, 616 (1969)

222. Fahey, J.L. and S. Sell.
J. Exp. Med. 122, 41 (1965)

223. Davie, J.M. and W.E. Paul.
J. Exp. Med. 135, 643 (1972)

224. Andersson, B.
J. Exp. Med. 135, 312 (1972)

225. Werblin, T.P. and G.W. Siskind.
Transplant. Rev. 8, 104 (1972)

226. Cunningham, A.J. and L.M. Pilarski.
Scand. J. Immunol. 3, 5 (1974)

227. Cunningham, A.J. and L.M. Pilarski (1974) in "The Immune System" (E. Sercarz, A.R. Williamson and C.F. Fox, eds.), p. 367, Academic Press, New York.

228. Eisen, H.N.
"The Harvey Lectures", Series 60, 1 (1964)

229. Siskind, G.W., P. Dunn and J.G. Walker.
J. Exp. Med. 127, 55 (1968)

230. Wu, C.-Y. and B. Cinader.
Eur. J. Immunol. 2, 398 (1972)

231. Baker, P.J., B. Prescott, P.W. Stashak and D.F. Amsbaugh.
J. Immunol. 107, 719 (1971)

232. Marshall-Clarke, S. and J.H.L. Playfair.
Immunology 29, 477 (1975)

233. Martin, W.J. and J.F.A.P. Miller.
J. Exp. Med. 128, 855 (1968)

234. Sinclair, N.R. St. C. and E.V. Elliott.
Immunology 15, 325 (1968)

235. Spear, P.G., A. Wang, U. Rutishauser and G. Edelman.
J. Exp. Med. 138, 557 (1973)

236. Hardy, B., A. Globerson and D. Danon.
Cell. Immunol. 9, 282 (1973)

237. Rabinowitz, S.G.
Cell. Immunol. 21, 201 (1976)

238. Spear, P.G. and G.M. Edelman.
J. Exp. Med. 139, 243 (1974)

239. Scheid, M.P., M.K. Hoffman, K. Komuro, V. Hämmerling, J. Abbott, E.A. Boyse, G.H. Cohen, J.A. Hooper, R.S. Schulof and A.L. Goldstein.
J. Exp. Med. 138, 1027 (1973)

240. Armerding, D. and D.H. Katz.
J. Exp. Med. 139, 24 (1974)

240a. Roelants, G.E. and B.A. Askonas.
Eur. J. Immunol. 1, 151 (1971)

241. Hardy, B., E. Mozes and D. Danon.
Immunology 30, 261 (1976)

242. Mosier, D.E., B.M. Johnson, W.E. Paul and P.R.B. McMaster.
J. Exp. Med. 139, 1354 (1974)

243. Lee, K.-C., C. Shiozawa, A. Shaw and E. Diener.
Eur. J. Immunol. 6, 63 (1976)

244. Orga, S.S., R.A. Murgita and T.B. Tomasi Jr.
Immunol. Commun. 3, 497 (1974)

245. Murgita, R.A. and T.B. Tomasi Jr.
J. Exp. Med. 141, 269 (1975)

246. Murgita, R.A. and T.B. Tomasi Jr.
J. Exp. Med. 141, 440 (1975)

247. Sheppard, Jr., H.W., S. Sell, P. Trefts and R. Bahu.
J. Immunol. 119, 91 (1977)

248. Sell, S., H.W. Sheppard Jr. and M. Poler.
J. Immunol. 119, 98 (1977)

249. Fidler, J.M., M.O. Chiscon and E.S. Golub.
J. Immunol. 109, 136 (1972)

250. Murczynska, W., J. Andrzejewski and A. Bogunowicz.
Nature 227, 721 (1970)

251. Martin, W.J.
Aust. J. Exp. Biol. Med. Sci. 44, 605 (1966)

252. Nossal, G.J.V. and G.L. Ada (1971) "Antigens, Lymphoid Cells and the Immune Response", Academic Press, New York.

253. Nelson, D.S. (1969) "Macrophages and Immunity" in "Frontiers of Biology" (A. Newberger and E.L. Tatum, eds.), North Holland Publ., London.

254. Hanna, Jr., M.G., P. Nettesheim and L.C. Peters.
Nature (New Biol.) 232, 204 (1971)

255. Hodes, R.J., G.B. Ahmann, K.S. Hathcock, H.B. Dickler and A. Singer.
J. Immunol. 121, 1501 (1978)

256. Ahmann, G.B., D.H. Sacks and R.J. Hodes.
J. Immunol. 121, 1981 (1978)

257. Smith, R.T.
Adv. Immunol. 1, 67 (1961)

258. Chiller, J.M., C.G. Romball and W.O. Weigle.
Cell. Immunol. 8, 28 (1973)

259. Lederberg, J.
Science 129, 1649 (1959)

260. Nossal, G.J.V. and B.L. Pike.
J. Exp. Med. 141, 904 (1975)

261. Cambier, J.C., J.R. Kettman, E.S. Vitetta and J.W. Uhr.
J. Exp. Med. 144, 293 (1976)

262. Cambier, J.C., E.S. Vitetta, J.W. Uhr and J.R. Kettman.
J. Exp. Med. 145, 778 (1977)

263. Cambier, J.C., J.W. Uhr, J.R. Kettman and E.S. Vitetta.
J. Immunol. 119, 2054 (1977)

264. Szewczuk, M.R. and G.W. Siskind.
J. Exp. Med. 145, 1590 (1977)

265. Howard, J.G. and C. Hale.
Eur. J. Immunol. 6, 486 (1976)

266. Raff, M.C., J.J.T. Owen, M.D. Cooper, A.R. Lawton III, M. Megson and W.E. Gathings.
J. Exp. Med. 142, 1052 (1975)

267. Sidman, C.L. and E.R. Unanue.
Nature 257, 149 (1975)

268. Bruyns, C., G. Urbain-Vansanten, C. Planard, C. DeVosCloetens and J. Urbain.
Proc. Natl. Acad. Sci. 73, 2462 (1976)

269. Rowe, D.S. and J.L. Fahey.
J. Exp. Med. 121, 171 (1965)

270. Henney, C.S., H.D. Welscher, W.D. Terry and D.S. Rowe.
Immunochemistry 6, 445 (1969)

271. Pernis, B., G. Chiappino and D.S. Rowe.
Nature 211, 424 (1966)

272. Knapp, W., R.L.M. Bolhuis, J. Radl and W. Hijmans.
J. Immunol. 111, 1295 (1973)

273. Rowe, D.S., K. Hug, L. Forni and B. Pernis.
J. Exp. Med. 138, 965 (1973)

274. Abney, E.R. and R.M.E. Parkhouse.
Nature 252, 600 (1974)

275. Melcher, U., E.S. Vitetta, M. McWilliams, M.E. Lamm, J.M. Phillips-Quagliata and J.W. Uhr.
J. Exp. Med. 140, 1427 (1974)

276. Ruddick, J.H. and G.A. Leslie.
J. Immunol. 118, 1025 (1977)

277. Pernis, B., L. Forni and K.L. Knight (1975) in "Membrane Receptors of Lymphocytes" (M. Seligman, J.L. Preud'homme and F.M. Kourilsky, eds.), p. 57, North Holland, Amsterdam-Oxford.

278. Pernis, B. ibid., p. 25.

279. Fraser and M.D. Cooper - quoted in: R.M.E. Parkhouse and M.D. Cooper, Immunological Rev. 37, 105 (1977)

280. Fiebig, H. and M. Ambrosius (1976) in "Phylogeny of Thymus and Bone Marrow-Bursa Cells" (R.K. Wright and E.L. Cooper, eds.), p. 195, Elsevier (North Holland Biomedical Press), Amsterdam.

281. Goding, J.W. and J.E. Layton.
J. Exp. Med. 144, 852 (1976)

282. Goding, J.W.
Contemp. Top. Immunobiol. 8, (1977)

283. Vitetta, E.S. and J.W. Uhr.
Science 189, 964 (1975)

284. Abney, E.R., J.R. Hunter and R.M.E. Parkhouse.
Nature 259, 404 (1976)

285. Goding, J.W., D.W. Scott and J.E. Layton.
Immunol. Rev. 37, 152 (1977)

286. Herzenberg, L.A., L.A. Herzenberg, S.J. Black, M.R. Loken, K. Okumura, W. Van der Loo, B.A. Osborne, D. Hewgill, J.W. Goding, G.A. Gutman and N.W. Warner.
Cold Spring Harbor Symp. Quant. Biol. 41, 33 (1976)

287. Mason, S.W.
J. Exp. Med. 143, 1122 (1976)

288. Scher, I., S.O. Sharnow, R. Wistar, R. Asofsky and W.E. Paul.
J. Exp. Med. 144, 494 (1976)

289. Bourgois, A., K. Kitajima, I.R. Hunter and B.A. Askonas.
Eur. J. Immunol. 7, 151 (1977)

290. Preud'homme, J.L.
Eur. J. Immunol. 7, 191 (1977)

291. Coffman, R.L. and M. Cohn.
J. Immunol. 118, 1806 (1977)

292. Zan-bar, I., S. Strober and E.S. Vitetta.
J. Exp. Med. 145, 1188 (1977)

293. Black, S.J., W. Van der Loo, M.R. Loken and L.A. Herzenberg.
J. Exp. Med. 147, 984 (1978)

294. Cambier, J.C., F.S. Ligler, J.W. Uhr, J.R. Kettman and E.S. Vitetta.
Proc. Natl. Acad. Sci. 75, 432 (1978)

295. Mosier, D.E., I.M. Zitron, J.J. Mond, A. Ahmed, I. Scher and W.E. Paul.
Immunol. Rev. 37, 89 (1977)

296. Cambier, J.C., E.S. Vitetta, J.R. Kettman, G. Wetzel and J.W. Uhr.
J. Exp. Med. 146, 107 (1977)

297. Kearney, J.F., M.D. Cooper, J. Klein, E.R. Abney, R.M.E. Parkhouse and A.R. Lawton.
J. Exp. Med. 146, 297 (1977)

298. Wortis, H.H., R.B. Taylor and D.W. Dresser.
Immunology 11, 603 (1966)

299. Klinman, N.R., J.L. Press, N.H. Sigal and P.J. Gearhart (1976) in "The Generation of Antibody Diversity: A New Look" (A.J. Cunningham, ed.), p. 127, Academic Press, New York.

300. Montgomery, P.C. and A.R. Williamson.
J. Immunol. 109, 1036 (1972)

301. Goidl, E.A. and G.W. Siskind.
J. Exp. Med. 140, 1285 (1974)

302. Cohen, J.E., P. D'Eustachio and G.M. Edelman.
J. Exp. Med. 146, 394 (1977)