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by

EDWARD STANLEY RECTOR

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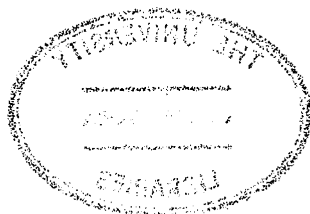

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EDWARD STANLEY RECTOR

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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 reticulo-endothelial 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