THE UNIVERSITY OF OKLAHOMA

GRADUATE COLLEGE

THE ROLE OF THE RETICULOENDOTHELIAL SYSTEM IN THE INDUCTION OF IMMUNE TOLERANCE

A DISSERTATION

SUBMITTED TO THE GRADUATE FACULTY

in partial fulfillment of the requirements for the

degree of

DOCTOR OF PHILOSOPHY

BY

VIOLA M. NEILL

Oklahoma City, Oklahoma

1970

THE ROLE OF THE RETICULOENDOTHELIAL SYSTEM

IN THE INDUCTION OF IMMUNE TOLERANCE

APPROVED BY

Richard M. Hyde

Wallace Friedberg

DISSERTATION COMMITTEE

ACKNOWLEDGMENT

It is with pleasure and gratitude that the author expresses her indebtedness to Dr. Richard M. Hyde for his constant advice and unflagging patience and encouragement throughout the course of this investigation. Appreciation is also expressed to the other members of the Department of Microbiology and Immunology and to the Dissertation Committee for their constructive criticisms and support.

The author is sincerely grateful to Dr. Florence Bannerjee for evaluating the histological sections, to Dr. Donald Parker for the statistical analysis, and to Mrs. Erma James for her capable technical assistance.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF TABLES--Continued

LIST OF ILLUSTRATIONS

LIST OF ILLUSTRATIONS--Continued

THE ROLE OF THE RETICULOENDOTHELIAL SYSTEM IN THE INDUCTION OF IMMUNE TOLERANCE

CHAPTER I

INTRODUCTION AND LITERATURE REVIEW

Origin of the Theory of Phagocytosis

The theory of phagocytosis was born on the shores of the Mediterranean in Messina in 1882. It was there that the brilliant Russian zoologist, Elie Metchnikoff, observed that if he inserted a rose thorn under the skin of a starfish larva, mobile cells of the larva soon migrated to the intruding particle and surrounded it (Metchnikoff, 1907). He suggested the term phagocyte, from the Greek translation of "devouring cells," to describe these mobile cells capable of ingesting foreign materials. Metchnikoff devoted the next 25 years of his life to developing his phagocytic theory and delineating its role in host defense to infectious diseases. The essential role of phagocytic cells in host resistance to disease remains undisputed today.

The Concept of the Reticuloendothelial System

Aschoff (1924) advanced the concept that phagocytic cells constituted a physiological system. This theory was based primarily upon the functional property of these cells of ingesting various vital dyes or other particulate material, rather than upon cyotological criteria.

Aschoff proposed the term, reticuloendothelial, to describe this system of phagocytic cells after observing them as components of both the reticular and endothelial tissues of various organs.

The cells of the reticuloendothelial system (RES) fall into two main anatomical categories, as depicted in Figure 1 (Aschoff, 1924). The sessile macrophages are cells of the reticular network of the spleen, lymphoid tissue and thymus, and the endothelial cells lining the sinusoids of the liver (Kupffer cells), spleen, lymphoid tissues and bone marrow and the capillaries of the adrenal and pituitary glands. These cells are fixed in strategic locations so that they may pick up particles carried to them by the blood and lymph. Also included in the RES are the wandering macrophages (histiocytes or clasmatocytes) found in connective tissue throughout the body and in the blood.

Baillif (1956) believes the corpus luteum of the ovary should be included in the RES as it contains sinusoids lined with actively phagocytic cells. The microglial cells of the central nervous system have also been considered as elements of the RES but some disagreement exists as to the validity of their inclusion (Arteta, 1956).

Measurement of RES Activity by the Carbon Clearance Technique

A classic method of studying the function of a given system has been to surgically remove it from the organism and note the resultant physiological alterations. In some cases it has been possible to maintain the excised system by artificial means and study the effect of various treatments upon its function. The diffuse anatomical nature of the RES has prohibited its study by these methods. However, the ability of

The Reticuloendothelial System

Sessile Macrophages		Wandering Macrophages	
Reticulum Cells	Endothelial Cells	Connective tissue histiocytes (including the lung)	Blood monocytes
Spleen	Spleen sinusoids		
Lymphoid tissue	Liver (Kupffer cells) sinusoids		
Thymus	Lymph sinusoids		
	Bone marrow sinusoids		
	Adrenal capillaries		
	Pituitary capillaries		

Figure 1--Cellular constituents of the reticuloendothelial system.

RE cells to phagocytize foreign materials has led to the development of in vivo methods to evaluate quantitatively RES activity in certain organs. A large number of RE cells are found in the liver (Kupffer cells) and spleen (reticular cells). Since these cells are in contact with the blood, it is possible to measure their phagocytic activity by determining the intravascular clearance rate of various materials such as dyes and colloidal suspensions. Many of the early attempts to evaluate RES activity failed because the materials employed were cytotoxic, impure or had other undesirable qualities. Benacerraf, et al. (1957) and Neukomm, et al. (1957) have outlined the criteria which should be fulfilled by substances utilized in the measurement of phagocytosis.

In recent years, the use of a colloidal suspension of carbon has found favor with many investigators. Halpern, et al. (1953) were among the first to use colloidal carbon to study RES function although Fenn (1921a, 1921b) used it to quantitate the phagocytic activity of leucocytes. The first carbon suspensions employed were found to be toxic to rats at dosages exceeding 16 mg carbon/100 gm body weight. The toxicity was due to shellac which had been added to the suspension as a stabilizer (Halpern, et al., 1953). The shellac caused a release of thromboplastin which depleted the blood of its anticoagulatns resulting in coagulation of fibrin in the circulating blood. Subsequently, a carbon suspension in fish glue, free of shellac, with a small amount of phenol added as a preservative, was found to be non-toxic in doses as high as 64 mg carbon/100 gm body weight (Benacerraf, et al., 1954; Biozzi, et al., 1953) and this preparation is now used routinely in RES studies employing the carbon clearance technique.

Biozzi, et al. (1953) and Benacerraf, et al. (1957) studied, in considerable depth, the kinetics of carbon clearance by the RES in a variety of laboratory animals. They found that the rate of clearance of carbon from the blood was an exponential function of time and could be expressed by the equation, $\frac{\log C_o - \log C}{T} = K$ or $C = C_o 10^{-KT}$, where C_o is the concentration of carbon in the blood immediately after injection and C is the concentration at time T. The constant, K, is a measure of the rate of clearance and is termed the phagocytic index. This index varies inversely with the dose of carbon administered.

RES activity was found to be greatest in the mouse, lower in the rat, still lower in the guinea pig and least in the rabbit. This was explained by the difference in relative size of the livers and spleens, the organs which account for 90-95 percent of the phagocytic activity of the RES. The smaller animals have relatively larger organs and, hence, greater phagocytic activity. When the corrected phagocytic index, α, was calculated from the equation, $\alpha = \frac{W}{Wls} \sqrt[3]{K}$, where W/Wls is the ratio of body weight to liver and spleen weight, the values were quite similar for all animals.

The carbon clearance technique is well standardized, convenient and inexpensive. It has, therefore, been used extensively to investigate the effect of a wide variety of substances, as well as physiological and environmental conditions, on RES function.

RES Depression by Certain Lipids

The alkyl esters of several simple fatty acids have been found to exert a profound depressive effect on RES phagocytic activity as determined by the intravascular clearance of colloidal carbon.

Ethyl Stearate

Inhibition of normal RES function by intravenous injection of the ethyl ester of stearic acid was initially demonstrated by Stuart, et al. (1960). Maximum RES depression in the mice was noted 24 hours after lipid administration with a return to normal function occurring in 3-4 days. Liver and spleen weights were not significatnly altered during this time interval. The duration of RES depression could be extended for some time by repeated injections of lipid. More detailed studies were subsequently made by Stuart and Cooper (1962) on the effect of ethyl stearate in mice. A dose-response effect was seen. A 10 mg dose was not effective but doses of 20 mg and 30 mg produced a three-fold decrease in phagocytic activity and 40 mg resulted in maximum impairment of RES activity. Larger doses were not studied due to their toxicity.

When given intravenously, ethyl stearate exerts its effect primarily on the sessile intravascular phagocytes (Stuart and Cooper, 1962). Histologic studies after ethyl stearate treatment revealed a decrease in the number of splenic and hepatic macrophages containing carbon. This could be interpreted as a decrease in the total number of phagocytic cells or as an impairment of their functional capacity. The former interpretation is supported by electron microscopic studies which demonstrated that Kupffer cells are sometimes severely damaged following ethyl stearate treatment. Thus, it would appear that ethyl stearate exerts its depressive effect on the RES through a direct or indirect cytotoxic mechanism. A similar cytotoxic effect on lymph nodes was noted by Shivas and Fraser (1959) following intraperitoneal injection of

this material.

Phagocytic stimulants, such as triolein and bacterial lipopolysaccharide, have been found to confer some degree of protection upon mice against infection with Diplococcus pneumoniae. Ethyl stearate reversed the RES stimulating and protective effects of these materials (Cooper and Stuart, 1961, 1962).

Cholesterol Oleate

The effect of a sterol ester, cholesterol oleate, on RES function was investigated by Stuart (1962) and Stuart and Davidson (1963). Little change in phagocytic function occurred with a 10 mg dose. However, marked depression lasting for 2-3 days was produced by a 30 mg dose. After a 50 mg dose spleens appeared granular, enlarged and tomato pink in color. The splenic lymphoid follicles were intact but the red pulp was fatty and of poor phagocytic quality. Focal necrosis of the liver was also observed (Stuart, 1962). The growth of mice treated with 90 mg of cholesterol oleate was arrested. Liver and spleen weight were increased and these organs contained large amounts of the ester, suggesting physical saturation of the RE cells as the mechanism of phagocytic depression (Stuart and Davidson, 1963).

Ethyl Palmitate

Ethyl palmitate is an efficient inhibitor of RES phagocytosis as measured by intravascular clearance of colloidal carbon (Stuart, 1960) and human red blood cells (Buchanan and MacGregor, 1964). Early studies in mice by Stuart (1960) revealed widespread splenic destruction, but only a minor amount of liver necrosis, following a single intravenous

injection of 25 mg of lipid. Smaller doses inhibited phagocytic activity without observable splenic necrosis. More recent studies by Prosnitz, et al. (1969) have confirmed Stuart's findings. Single intravenous injections of this ester into mice resulted in a selective and acute splenic necrosis, maximal at 24-72 hours, with regeneration evident in 7-10 days. Within 1 hour after injection both the liver and spleen were extensively infiltrated with fat. Within the next hour, nearly all of the lipid had disappeared from the liver, indicating that ethyl palmitate was rapidly metabolized by this organ.

Repeated injections of ethyl palmitate effected a complete and permanent destruction of the spleen (chemical splenectomy) in most mice, rats and rabbits. Some species, however, reacted differently to ethyl palmitate, e.g., splenic necrosis in guinea pigs was only moderate compared to that seen in mice. Further, random bred Swiss mice had somewhat more marked liver damage and only minimal splenic necrosis (Prosnitz, et al., 1969).

Methyl Palmitate

Wooles and DiLuzio (1963) reported a selective depression of RES function following treatment with methyl palmitate. As with other lipid RES depressants, methyl palmitate was effective only when administered by the intravenous route (DiLuzio and Blickens, 1966). The most profound impairment of phagocytosis occurred with a dose of 35 mg per mouse although doses ranging from 15-40 mg were also effective. RES depression was evident within 5 hours after lipid injection and increased to a maximum at 48 hours. Phagocytic activity remained depressed for 7 days after methyl palmitate administration. Normal function was regained

in 12-17 days.

The mechanism by which methyl palmitate impairs RES function has been studied extensively (Wooles and DiLuzio, 1963; DiLuzio and Riggi, 1964; DiLuzio and Wooles, 1964; Blickens and DiLuzio, 1965; DiLuzio and Blickens, 1966; Saba and DiLuzio, 1968). Nevertheless, although some information is available, the exact mechanism remains uncertain. In contrast to the cytotoxic effects of ethyl stearate (Stuart and Cooper, 1960), ethyl palmitate (Stuart, 1960) and cholesterol oleate (Stuart, 1962), methyl palmitate did not cause any observable damage to either splenic or hepatic cellular elements. Further histological studies revealed a marked decrease in the hepatic uptake of colloidal carbon. DiLuzio and Riggi (1964) observed a similar depression in the splenic and hepatic uptake of an "RE test lipid emulsion" following methyl palmitate administration. These findings, together with the fact that methyl palmitate did not alter hepatic parenchymal cell activity, led DiLuzio and Wooles (1964) to suggest that this lipid exerts its effect exclusively on Kupffer cells by interfering with their phagocytic capability. Blickens and DiLuzio (1965), studying the metabolism of methyl palmitate, concluded that its depressive effect on RES activity was not due to either physical saturation of phagocytic cells or to the formation of methanol resulting from hydrolysis of the lipid.

The possibility that methyl palmitate might decrease the serum opsonic activity of treated mice and, therefore, interfere with phagocystosis of subsequently introduced materials, was investigated by Saba and DiLuzio (1968). They found, however, that oponsic activity was only slightly reduced and was not a significant factor in methyl palmitate-mediated RES depression.

In summary, the available evidence suggests that methyl palmitate depresses the phagocytic ability of the hepatic and splenic macrophages by some mechanism other than physical saturation or necrosis of these cells, the production of methanol or the removal of serum opsonins.

Summary

Ethyl stearate, cholesterol oleate, ethyl palmitate and methyl palmitate have all been shown to depress the phagocytic capacity of the RES. Although direct comparisons have not been reported, methyl palmitate probably exerts the most profound and long lasting depression, followed by ethyl stearate, ethyl palmitate and cholesterol oleate. With the exception of methyl palmitate, all of these lipids have been shown to produce splenic necrosis of varying severity. Necrosis of the liver has also been observed following treatment with ethyl stearate and cholesterol oleate. Thus, it may be that these materials act through a cytotoxic mechanism. The mechanism of action of methyl palmitate is more puzzling, as it has not been shown to be toxic to either hepatic or splenic tissue.

Lipids offer several advantages over the dyes and other inert materials used as RES "blockaders." For example, lipids are specific for the RES and do not cause undesirable side effects. Their effect is evident within a few hours and is of considerably longer duration. Finally, no period of hyperactivity follows when their effect has worn off. Thus, lipids are well suited for studying RES function.

Evidence for RES Participation in the Immune Response

A vast amount of evidence has accumulated over the past 10-15

years implicating the macrophage as an important and essential component of many immune systems. A complete review of the literature would be a Herculean task and is not within the scope of this work. Rather, the various approaches which have been employed in these investigations will be discussed and examples of pertinent results will be presented. The reader is referred to the excellent reviews of Sulitzeanu (1968) and Unanue and Cerottini (1970) for more comprehensive coverage of this complex subject.

Ribonucleic Acid Transfer

One of the first lines of evidence which suggested that the macrophage played a role in the immune response was the observation that antigen fragments were found associated with ribonucleic acid (RNA) extracts of these cells. This phenomenon was first observed by Garvey and Campbell (1957). While studying the retention of bovine serum albumin (BSA) in rabbits following intravenous injection, they found that fragments of BSA complexed to RNA isolated from liver macrophages could be detected after several months. Comparison of the immunogenicity of these complexes and the original BSA revealed the former to be 100 times more effective than the latter in sensitizing guinea pigs as measured by the Schultz-Dale reaction.

Fishman (1959, 1961) and Fishman and Adler (1963) found that cell-free filtrates from rat macrophages, which had been incubated with T2 bacteriophage, evoked the synthesis of specific antibody in cultures of lymph node cells from normal rats. Further study revealed the inducing factor to be species specific, ribonuclease sensitive and present in low molecular weight RNA fractions (Fishman, Hammerstrom and Bond, 1963).

Friedman, et al. (1965), studying Fishman and Adler's system, confirmed and extended their observations. They found that RNA from macrophages incubated with T2 phage, when injected into mice previously immunized with this phage, elicited a secondary antibody response. This finding, plus the additional observation that treatment of macrophage RNA with ribonuclease did not completely eliminate its capacity to elicit antibody synthesis in the lymph node system, led these investigators to search for antigen or antigenic fragments associated with the RNA. Employing a micro method for complement fixation, they detected several T2 phage antigens in the RNA. Further investigation of this system by Fishman and Adler (1967) revealed the presence of two distinct RNA fractions from stimulated macrophages. One fraction, apparently free of antigen, mediated the 19S (IgM) antibody response. The other RNA fraction was complexed with antigen and was responsible for 7S (IgG) antibody formation. Both components of the RNA-antigen complex were found to be essential to its immunogenic function. Although technical limitations prevent proof of the absence of antigen in RNA responsible for the IgM response, several indirect lines of evidence are consistent with this view. For example, the IgM produced as a result of stimulation with this RNA fraction always carried the allotypic markers of the macrophage donor, suggesting a messenger role for the RNA inducing 19S antibody production by cells which received multiple hits with this RNA. If actinomycin D was added to the macrophage-antigen incubation mixture the resultant RNA stimulated IgG production only. This suggested that the RNA involved in the IgM response was newly formed following exposure to antigen, whereas the RNA complexed with antigen and responsible for the IgG response was preformed

within the macrophage. DNA-RNA hybridization studies have also suggested the formation of a new species of RNA following the exposure of macrophages to antigen (Cohen, 1968).

Askonas and Rhodes (1965) reported that murine peritoneal cells degraded the soluble antigen, hemocyanin, into fragments which became complexed with RNA. These complexes were 20 times more immunogenic than hemocyanin alone. RNA preparations obtained from peritoneal cells immediately after the addition of hemacyanin were also immunogenic. They concluded, therefore, that the immunogenicity of the RNA preparations could not be attributed to the formation of a specific new messenger RNA by macrophages following antigen exposure. They stated that although antigen processing enhanced immunogenicity in their system, it was not essential for antibody synthesis. Macrophages may simply serve as antigen storage depots. Complexing with RNA might protect the antigen from enzymatic degradation by lysosomal enzymes.

Recently, Pinchuck, et al. (1968), using a well defined system consisting of inbred animals and synthetic antigens, were able to demonstrate the formation of RNA-antigen complexes. When normal macrophages were incubated with a synthetic polypeptide, an RNA-antigen complex was obtained which evoked an immune response in mice which normally responded poorly, if at all, to the antigen. The response was specific for the polymer. If this RNA extract was incubated with a different synthetic polymer it failed to elicit an antibody response specific for the second antigen.

The recent findings of Roelants and Goodman (1968, 1969) require that experiments of this nature be interpreted with caution. They

have presented evidence which suggests that RNA-antigen complexes may not have a physiologically significant function in the induction of an immune response. This suggestion was based on the failure to demonstrate any correlation between a molecule's immunogenic capacity and its ability to complex with RNA. They further noted that complex formation was non-specific, i.e., antigen complexed as readily with HeLa cell RNA as with macrophage RNA.

Investigations of a different nature, however, have also suggested that RNA plays an active role in eliciting an immune response. Mannick and Egdahl (1962) demonstrated accelerated rejection of skin grafts in rabbits following injection of autologous spleen cells which had been incubated in vitro with homologous RNA extracted from lymph nodes of an animal sensitive to the graft donor. Exposure of the RNA preparation to ribonuclease abolished its sensitizing ability. RNA extracted from rabbits immunized to an unrelated antigen did not display this heightened immunogenicity. Apparently a specific type of RNA is involved in the transfer of transplantation immunity.

Cohen and Parks (1964) extracted RNA from the spleens of mice immunized with sheep red blood cells (SRBC) and found that it would convert nonimmume isologous spleen cells into antibody-producing cells. Conversion occurred in the presence of trypsin, pronase, both alpha and beta amylase and deoxyribonuclease but was inhibited by chloramphenicol and ribonuclease (Cohen, et al., 1965). Donor RNA and recipient cell crosses between 4 mouse strains revealed a degree of strain specificity. Low doses of 5-fluoro-2-deoxyuridine had an adjuvant effect on this system. Partially degraded RNA from immunized mice exerted a specific

inhibitory effect on antibody induction by intact RNA (Cohen, 1967).

The importance of RNA in the induction of cellular resistance was demonstrated by Fong, *et al.* (1963). Ribosomal RNA extracted from histiocytes of rabbits immunized with *Bacillus Calmette-Guerin* (BCG) was found to initiate development of cellular immunity in normal rabbits.

Whole Cell Transfer Studies

Feldman and Gallily (1967) concluded, from a series of investigations, that both the macrophage and the lymphocyte are essential for antibody production following exposure to antigen. Employing C57BL/6 mice and a *Shigella* antigen system they demonstrated that normal peritoneal macrophages incubated *in vitro* with antigen would elicit an antibody response in sublethally irradiated recipients which could not respond to the antigen alone. Appropriate controls excluded the possibility that the response was due to lymphoid cells contaminating the macrophage preparation or that macrophages themselves might be producing antibody. Cell reconstruction experiments in lethally irradiated recipients added further support to their two cell requirement hypothesis. The only cell recipients which produced a significant titer of agglutinating antibodies were those which had been reconstructed with normal lymphocytes plus macrophages previously incubated with antigen. A further finding was that irradiation of macrophages prior to their reaction with antigen abrogated their capacity to elicit an immune response. Irradiation did not decrease the capacity of macrophages to phagocytize SRBC. Therefore, it appeared that the immunosuppressive effect of irradiation was due to an interference with antigen processing within the macrophage.

Further studies on the macrophage reconstitution of sublethally irradiated mice (Gershon and Feldman, 1968) confirmed the above observations with Shigella paradysenteriae. However, when these experiments were repeated using SRBC in place of the Shigella antigen no such reconstitution of the immune response could be achieved despite lower doses of irradiation and the use of phagocytosis-enhancing incubation media. The investigators suggested two possible explanations for this apparent discrepancy in the function of the immune system. In recent, unpublished work they have shown that when normal thymus explants were added to spleen organ cultures from irradiated animals, previously inoculated with macrophages and Shigella antigen in vitro, an antibody response ensued. Thus, it appeared that lymphoid cells had regenerated and regained immunocompetence following irradiation due to thymic influence. They suggested that a temporal relationship may exist between the return of immune competence following irradiation and the type of antigen under study. On the other hand, a second cell type, more radiosensitive than the macrophage, may be required for the anti-SRBC response but not for the anti-Shigella response. This cell may be similar to, or identical with, the thymus-dependent, antigen reactive cell recently described by Mitchell and Miller (1968) and thought to be involved in the production of hemolysins to SRBC.

Seemingly contradictory results were reported by Argyris and Askonas (1968). Peritoneal cell exudates were obtained from C3H mice following intraperitoneal administration of antigen (formalin-killed penumococci or capsular polysaccharide). Macrophage-rich fractions of these cell exudates failed to elicit a significant antibody response

when transferred to x-irradiated, syngeneic recipients. However, non-irradiated recipients responded with high titers when similarily treated. The conflicting results of this study and those of Gallily and Feldman may be related to the level of irradiation to which the recipients were subjected. In this study the recipients received 700-800 R, whereas in the Gallily and Feldman study they received 550 R. In the latter study the response was significantly decreased in recipient animals irradiated with higher doses (750-900 R). Other differences in the two systems were the antigen and mouse strain employed as well as the method of antigen-macrophage reaction.

Pribnow and Silverman (1967) studied the radiosensitivity of rabbit macrophages and their role in eliciting an antibody response to bovine gamma globulin (BGG). They found that normal peritoneal macrophages, sensitized in vitro with BGG, would initiate antibody synthesis in non-irradiated recipients but not in those exposed to 450 R unless normal lymphoid cells were given simultaneously. Of even greater interest was the finding that macrophages from irradiated donors were incapable of eliciting an immune response in irradiated recipients even in the presence of normal lymphoid cells in the latter. From these data they concluded that antigen processing by macrophages represents the initial radiosensitive phase of antibody formation in their system.

Unanue and Askonas (1968) studied the immunogenicity and radiosensitivity of macrophage-associated Maia squinado hemocyanin (MSH). CBA mice were injected intraperitoneally with a proteose-peptone solution 3 days prior to antigen administration, to augment the macrophage concentration of the cell population. MSH was injected intraperitoneally

and the peritoneal cell exudates subsequently obtained consisted of 80-90 percent macrophages, 75 percent of which contained MSH. Neither the soluble antigen nor the macrophage-associated MSH could elicit a primary response upon transfer to normal recipients. However, both could prime recipients for a secondary response, although the macrophage-associated antigen was much more effective. The macrophage-held MSH was also able to elicit a secondary response in x-irradiated (660-750 R) recipients provided lymphoid cells from normal donors were also transferred. The immunogenicity of the cell-associated MSH persisted for 3 days and was associated with approximately 10 percent of the original MSH taken up by the macrophages which was not rapidly catabolized, therefore allowing sufficient time for reaction with lymphoid cells. A further finding was that x-irradiation (750 R) of normal or protease-peptone stimulated macrophage donors did not impair the immunogenicity of the macrophage-associated hemocyanin. Thus, radiosensitivity of macrophages appears to vary with the immune system.

The same experimental design was employed by Mitchison (1969) to compare the immunogenicity of several protein antigens (bovine serum albumin, human serum albumin, lysozyme and ovalbumin) in free form and after uptake by peritoneal exudate cells (PEC). Mitchison's findings were similar to those of Askonas and Rhodes. In general, macrophage-bound protein antigens induced primary immune responses more readily than the free form of the antigen. Irradiation of PEC donors before, but not after, administration of the antigen inhibited immunization upon transfer to a recipient. PEC from tolerant donors could effectively immunize normal recipients. However, tolerant recipients were not immunized by

PEC from normal donors.

Frisch and Wilson (1969) studied the effect of macrophage reconstitution on the immune response to SRBC in C3H mice which had been immunosuppressed by either sublethal irradiation (550 R) or the drugs, cycloleucine and Cytotoxan. Peritoneal cell exudates were obtained from normal donors 4 days following intraperitoneal injection of thioglycollate medium. The immune response was assayed by the plaque-forming cell technique or by hemagglutination. The transfer of normal macrophages plus SRBC elicited a primary immune response in irradiated recipients, whereas comparable amounts of spleen cell suspensions were ineffective. Macrophage-antigen mixtures from normal or from Cytotoxan-treated donors also elicited a primary response in cycloleucine suppressed recipients. Similar cell preparations from cyloleucine-treated donors as well as normal spleen cell suspensions were not effective, however. Cycloleucine-treated macrophages were no less viable and no less capable of phagocytizing SRBC than were normal macrophages. Therefore, these data support the hypothesis that antigen processing by macrophages is a prerequisite step in the induction of a primary immune response.

Summary

Investigations employing a macrophage transfer system have demonstrated that macrophage-associated antigen is capable of eliciting an immune response in recipient animals. They do not, however, prove that macrophages are essential components of the immune mechanism. Such experiments have demonstrated, however, thatmacrophages are not antibody synthesizing cells. It is difficult to evaluate contradictory findings, such as reported above, because of the variations in experimental design.

Certainly the ability to respond to immunogenic stimuli differs with the animal species and strain employed. Similarly, antigens vary in their immunogenicity and a wide variety have been used in the above experiments (SRBC, bacteria and proteins). In some cases irritants were employed to collect peritoneal cell exudates rich in macrophages. One wonders what effect, if any, these irritants have on the already heterogeneous macrophage population. The time interval between administration of the irritant and collection of the cell exudates varied from a few hours up to several days in the above experiments. This could also be a factor which might influence the subsequent response. Differences in the radiosensitivity of macrophages were also reported. These differences might be explained by variation in dose of x-irradiation or time lapse between irradiation and cell collection. It is also possible that irritants alter macrophage susceptibility to radiation damage and, furthermore, cells may vary in their response to irradiation in vitro as opposed to an in vivo environment. Whatever the exact effect of irradiation on macrophages, it apparently operates at the processing stage rather than at the engulfment stage.

In drawing conclusions from data obtained in this manner, it is implicitly assumed that peritoneal macrophages handle antigen in the same manner as their splenic and hepatic counterparts. This may or may not be so. Peritoneal cells may serve simply to bind antigen and transport it to splenic macrophages. Evidence for such transport has been reported by Roser (1965, 1968). Macrophages labeled with ^{198}Au were traced to the spleens and livers of the intraperitoneally injected recipients.

These investigations have demonstrated that macrophage uptake is an effective means of enhancing the immunogenic potency of many, although not all, antigens. In general, antigens which are normally poorly phagocytized *in vivo* displayed increased immunogenicity following incubation with macrophages. Immunogenic enhancement was not observed when antigens which are readily phagocytized *in vivo* were employed.

In Vitro Cell Interaction

The dependence of antibody formation on the interaction of two cell types was demonstrated by Mosier (1967) in an *in vitro* system. He separated a suspension of mouse spleen cells into macrophage-rich and lymphocyte-rich populations on the basis of the ability of the former cells to adhere to a plastic surface. The incubation of each of these populations with SRBC did not result in hemolysin production which could be detected by the Jerne plaque assay. However, if both cell populations were cultured together in the presence of SRBC a significant immune response was evident.

Roseman (1969), using Mosier's technique to separate splenic lymphocytes and macrophages, confirmed the observation that both cell types were required for the *in vitro* antibody response to SRBC. Further, he demonstrated that the lymphoid cells were highly radiosensitive, whereas macrophages were not affected by doses as high as 1000 R.

Employing a similar cell separation and recombination technique, Pierce (1969) demonstrated the necessity for macrophage-lymphocyte cooperation to elicit a primary response *in vitro*, but not for a secondary response. Subsequently, Pierce and Benacerraf (1969) reported that interaction between antigen-treated macrophages and lymphocytes is

effectively completed within 24 hours incubation *in vitro*. Thereafter, the "activated" lymphocytes develop into antibody synthesizing cells in the absence of native antigen, macrophages and cell clusters.

An *in vitro* culture system was employed by Forbes (1969) to compare the effectiveness of macrophages and lymphocytes from normal and tolerant mice in eliciting a primary immune response. Macrophages from normal and tolerant mice were equally effective in generating plaque-forming cells (PFC). However, splenic lymphocytes from tolerant animals were unable to develop into PFC when incubated with normal macrophages. Thus, it appeared that the tolerance "lesion" was at the level of the lymphocyte in this model. In contrast, Wu and Trice (1967) demonstrated that unresponsiveness to *Klebsiella pneumoniae* capsular antigen could be terminated by passive transfer of peritoneal cells, but not spleen cells, from either normal or unresponsive donor mice. They concluded, therefore, that in their system the tolerance "lesion" resides in the macrophage.

The blastogenic response to antigenic stimulation of glass bead column-purified lymphocytes was markedly reduced when compared to that of unseparated leucocyte suspensions (Hersh and Harris, 1968). Culturing these purified lymphocyte preparations on macrophage monolayers resulted in partial to complete restoration of blastogenesis, providing the two cell populations were not separated by a semi-permeable membrane. Similar results were obtained when blastogenesis was measured by the incorporation of tritiated thymidine into the DNA of the purified lymphocytes.

Using similar techniques, Gordon (1968) demonstrated a

requirement for macrophage interaction in the mixed leucocyte culture reaction. Similar observations that macrophages are required for immunologically induced lymphocyte transformation have been made by Cline and Swett (1968) and Oppenheim, et al. (1968).

Effect of RES Stimulation on the Immune Response

The observation that RES hyperactivity often enhances various immunological phenomena lends further support to the concept of the macrophage as an integral component of the immune mechanism. Zymosan, a powerful RES stimulant, greatly enhanced hemolysin formation in rats when given as early as one month prior to, or as late as two days after antigen administration (Cutler, 1960). Glucan, a highly purified polysaccharide isolated from zymosan (Riggi and DiLuzio, 1961), also serves dually as a stimulant of RES activity and antibody formation against SRBC in mice (Wooles and DiLuzio, 1963; Morrow and DiLuzio, 1965).

Halpern, et al. (1958), observed that mice infected with BCG not only displayed RES hyperactivity but also produced Eberthella typhi anti-H and anti-O agglutinins of much higher titer and over a longer period of time than did control mice. Further, Balner, et al. (1962) found that female mice injected with BCG (to induce RES hyperfunction) demonstrated accelerated rejection of male skin isografts and concluded that this was due to an enhanced immune response.

An association between the RES and the immune mechanism was demonstrated by the failure of homologous and heterologous bone marrow transplants to survive in lethally irradiated mice which had been treated with glucan to induce a hyperfunctional state of the RES prior to irradiation (Wooles and DiLuzio, 1962). The increased mortality of

homologous and heterologous radiation chimeras treated with glucan (Wooles and DiLuzio, 1964) provides further evidence that the RES plays a significant role in the immune response.

Oncolytic activity toward various foreign and experimental tumors has also been observed with glucan (Diller, et al., 1964) and zymosan and BCG (Old, et al., 1960).

Evidence for Lack of RES Participation in Tolerance Induction

Studies with Neonatal Animals

The first observation of tolerance induction to a protein antigen via neonatal injection occurred quite by accident. Hanan and Oyama (1954), intending to study the effect of BSA on gamma globulin levels in young rabbits, repeatedly injected these animals with the protein and discovered that they did not form anti-BSA precipitins, sensitize guinea pigs for passive anaphylaxis or give an Arthus reaction. Throughout the ensuing years it has been demonstrated repeatedly that, within a species and for a given antigen, tolerance can be induced in neonates with considerably greater ease than in adult animals. This could be explained by postulating that the RES of newborn animals has not completely matured by the time of birth if this system is, indeed, an essential and integral part of the immune mechanism. Histiologic and functional studies of the phagocytic phenomenon in foetal and neonatal animals support this view. A functional, although immature, RES has been demonstrated in foetal chicks (Karthigasu, et al., 1965) and foetal rats (Reade and Jenkin, 1965). Sixteen day old foetal rats were shown to ingest both bacteria and carbon particles. The efficiency of

phagocytosis gradually increased up to parturition. It continued to increase after birth up to 3 weeks, then declined in the adult and aged animal (Benacerraf, et al., 1957). The phagocytic cells of foetal chicks and rats were extremely limited in their bactericidal capacity (Karthigasu, et al., 1965; Reade and Jenkin, 1965). Ten day old neonatal rats had developed some bacteriostatic capability but bactericidal activity was not evident before 21 days of age.

Mitchell and Nossal (1966) observed a marked difference in the handling of Salmonella flagellar antigens in newborn and adult rats. In adults the antigens were phagocytized efficiently and were concentrated in the lymphoid follicles. Phagocytosis was never as pronounced in neonates and the antigen was distributed diffusely throughout the lymphoid tissue, including the thymus.

Martin (1966) employed a different approach to evaluate the functional maturity of the phagocytic system of neonates. He found that newborn rabbits, injected with tolerogenic doses of BSA plus peritoneal macrophages from normal, adult rabbits, responded with immunity. Similar findings in newborn mice were reported by Argyris (1968). Day old mice, given adult peritoneal macrophages and sensitized with SRBC 3 days later, developed an enhanced plaque-forming cell response as compared to control mice. The enhancement of immunity in neonates conferred by adult macrophages was not due to a non-specific adjuvant action, as non-viable macrophages were without effect. Further, in Martin's experiment, BSA in Freund's adjuvant did not sensitize rabbits under 5 days of age. Findings such as these strongly support the contention that the ease of tolerance induction in newborn animals is directly related to the lack

of a functional antigen-processing system in the form of mature macrophages.

Physical State of the Antigen

The physical state of antigens influences both the ability to induce tolerance and susceptibility to phagocytosis. In general, soluble antigens tend to be tolerogenic, whereas particulate antigens are usually good immunogens. Similarly, soluble materials are poorly phagocytized, whereas particulate and aggregated substances are phagocytized with ease. Serum proteins have been separated into aggregated and aggregate-free or soluble fractions by a variety of methods including ultracentrifugation, "biological" or animal filtration, fractionation on sephadex and DEAE columns and by Na_2SO_4 precipitation. Dresser (1961, 1962, 1963) initially demonstrated that soluble BGG, separated from the native material by ultracentrifugation, was tolerogenic in mice. Dresser's investigations were confirmed and extended by Claman (1963) and Dietrich and Weigle (1964). Soluble human gamma globulin (HGG), also prepared by ultracentrifugation, has been shown to induce tolerance in mice (Gamble, 1966; Weigle and Golub, 1967; Golub and Weigle, 1967a) and in rabbits (Biro and Garcia, 1965).

Soluble BSA, prepared by "biological filtration" in a rabbit, was tolerogenic in other rabbits (Frei, et al., 1965). However, "biological filtration" was not as effective in removing the immunogenic material as was either ultracentrifugation or sephadex fractionation (Frei, et al., 1968). In some strains of mice, however, biological filtration was more efficient than ultracentrifugation. Golub and Weigle (1969), studied the effect of antigen form and mouse strain on the induction of

unresponsiveness to HGG. BALB/c mice could not be made tolerant to soluble HGG prepared by ultracentrifugation at 105,000 x g for 1 hour. However, if soluble HGG was prepared by biological filtration or by repeated Na_2SO_4 precipitation, it was quite tolerogenic for this mouse strain.

No attempt has been made to review all the literature on this subject. Suffice it to say that of those serum proteins studied, most can be made tolerogenic for most species. That the conditions prerequisite for tolerance induction vary with different antigens, species and even strains within species has been clearly demonstrated by Golub and Weigle (1969).

Effect of Altered RES Activity on Tolerance Induction

It is generally believed that immune tolerance and antibody formation represent antipodal activities of the immune mechanism. A hypothesis, first proposed by Medawar (1963), which has found increasing favor among students of the immune response states that the usual result of direct contact between competent lymphoid cells and antigen may be tolerance, whereas, an antigen that has been phagocytized and appropriately "processed" by macrophages will evoke an immune response. If this hypothesis is correct and if the macrophage is the initiating cell in the immune response, it should be possible to control, at least partially, what type of response will follow antigenic stimulation by manipulating the activity of the macrophage population. In other words, a tolerogenic material may become immunogenic in an animal whose RES has been made sufficiently hyperactive. Conversely, RES depression should render an animal tolerant or hyporesponsive to a normally immunogenic

substance.

RES stimulation. BSA is a relatively poor antigen in rabbits and soluble BSA has been shown to produce an unresponsive state in most rabbits. Pinckard, et al. (1967, 1968) were able to block the induction of unresponsiveness to soluble BSA in rabbits by injecting a heat-killed suspension of Corynebacterium parvum intravenously either 6 days prior to or simultaneously with the soluble BSA. The mechanism of the adjuvant action of this microorganism is not known. Other strains of C. parvum have been observed to cause extensive proliferation of the RES but the strain used in these studies did not. It was noted, however, that the spleen, liver and lungs of the treated animals developed numerous granulomas containing many macrophages but the significance of this is unknown. Two additional interesting observations resulted from these studies. First, C. parvum does not act as an adjuvant for antigens which are, in themselves, good immunogens. Second, it was not possible to block the induction of unresponsiveness in neonatal rabbits. It is possible that neonates do not have enough mature or functional macrophages to be susceptible to adjuvant action.

A similar adjuvant effect was noted by Mergenhagan, et al. (1967) using lactic dehydrogenase virus (LDV). If LDV was given to BALB/c mice 1 day before or simultaneously with a tolerogenic dose of soluble HGG, the mice responded by producing antibody rather than becoming tolerant. Similar results were obtained with LDV-infected mice injected with soluble rabbit gamma globulin (Howard, et al., 1969). The mechanism by which LDV converts a tolerogenic stimulus into an immunizing one is unknown. However, it was noted that LDV grows and multiplies in

peritoneal macrophages and, thus, it might alter the function of these cells in such a way as to enhance the uptake and/or processing of soluble antigens.

The adjuvant action of bacterial endotoxin on antibody production has long been recognized. Claman (1963) was able to block the induction of tolerance to BGG in adult mice by an intravenous injection of endotoxin 2 hours following antigen treatment.

Golub and Weigle (1967a) investigated the inhibitory effect of endotoxin on the induction of tolerance to soluble HGG in mice. Because endotoxin had previously been shown to stimulate phagocytic activity of the RES, it was presumed that its inhibitory effect on tolerance induction was due to its enhancement of RES function. However, this was found not to be the case. Endotoxin was observed to be an effective inhibitor of tolerance induction regardless of the phagocytic rate of the RES. The effect of endotoxin did, however, depend on the time it was given in relation to antigen administration. Inhibition was observed only when endotoxin was given simultaneously or within 2 days following antigen administration. Moreover, a single dose of endotoxin given within 3 days before antigen abrogated the inhibitory effect of endotoxin given within the 2 day interval following antigen exposure. Other work by these investigators has shown that an induction period of 4 days is required for the establishment of tolerance to HGG (Golub and Weigle, 1967b). Thus, this induction period can be divided into endotoxin sensitive and resistant stages. The investigators speculated that immunocompetent cells are forced to "mature" due to some effect of endotoxin during a critical time interval when antigen is present (Golub and Weigle,

1967a). This does not, however, explain why a prior injection of endotoxin abolishes the effect of an inhibitory dose. These findings are difficult to reconcile with the "macrophage bypass" theory of tolerance induction. Perhaps antigen phagocytosis and processing are requirements for both tolerance induction and the immune response. In any case, the myriad physiological effects of endotoxin preclude anything more than mere speculation at the present time.

Just as Pinckard, et al. (1968) were unable to block tolerance induction to BSA in neonatal rabbits using C. parvum as an adjuvant, Smith (1961) was unable to block tolerance to the same antigen in neonates employing endotoxin as an adjuvant.

RES depression. Cruchaud (1968) studied the effect of RES blockade with various particulate and high molecular weight soluble materials on the immune response of rabbits to BSA. None of the blockading agents employed produced a complete and long lasting RES blockade. However, high doses of colloidal carbon or iron, given once or twice daily for 5 days prior to antigen administration, resulted in a marked inhibition of the primary response in most rabbits and a decreased secondary response in several animals. Specific immune tolerance was observed in a few animals. The significance of this is, however, difficult to evaluate as BSA is not a strong immunogen and approximately 15 percent of the control animals also failed to respond.

The effect of carbon blockade of the RES on both the humoral and cellular responses to SRBC was studied by Sabet, et al. (1968, 1969). An intraperitoneal injection of 10 mg of carbon given 24-48 hours prior to immunization resulted in an 85-95 percent reduction in the number of

plaque-forming cells as compared to the controls. There was also a significant decrease in serum hemagglutinin and hemolysin titers. Further investigation revealed that the carbon-treated mice failed to show a secondary response upon rechallenge with the antigen (Sabet and Friedman, 1969). In fact, the response of these mice closely resembled the primary response of the control animals. Sabet and Friedman hypothesized that a specific immunologic "amnesia" rather than tolerance, resulted from the RES blockade.

Thorotrast, an RES blockading agent, given to mice 15 hours prior to the antigen, suppressed the immune response to aggregated BSA, a material highly immunogenic in non-blockaded mice (Kong and Cheng, 1970).

The tridymite form of silica has been shown to be a specific macrophage toxin (Kessel, et al., 1963). Allison, et al. (1966) have concluded that silica is toxic because, once these particles are ingested, they react with lysosomal membranes effecting an increased permeability which allows lytic enzymes to escape into the cytoplasm and eventually kill the cell. Upon cell death the ingested silica particles are released and can again be phagocytized by other macrophages. Pearsall and Weiser (1968) found that tridymite given to allograft recipients significantly prolonged the graft survival time. Further, they observed that immune macrophages, treated with silica and then incubated with tumor cells, had lost their capacity to suppress tumor growth. Thus, it would appear that the macrophage has a definite role in transplantation and tumor immunity.

The RES depression afforded by various lipids has been discussed

above. A concomitant immunosuppression has been observed in several instances. DiLuzio and Wooles (1964) observed that pretreatment of mice with methyl palmitate drastically suppressed both their primary and secondary responses to heterologous erythrocytes. Similar findings in methyl palmitate-treated rats were reported by Ohbuchi (1968).

Melnick and Friedman (1969) demonstrated the immunosuppressive effect of ethyl stearate. Mice treated with this lipid 1 day prior to immunization with sheep erythrocytes had a markedly reduced number of antibody plaque-forming cells.

Cortisone, also an inhibitor of RES phagocytic activity (Heller, 1955; Conning and Heppleston, 1966; Nicol and Bilbey, 1958; Weiner, et al., 1967), has been shown to suppress antibody formation in rats (Berglund, 1956) and rabbits (Mountain, 1955).

Although crude preparations of anti-macrophage sera (AMS) were prepared as early as 1899 by Metchinikoff, it has only been within the last 4 years that purified preparations have been characterized with regard to their effect on RES activity and the immune response. It is generally agreed that AMS is cytotoxic to macrophages and greatly reduces their phagocytic capacity (Unanue, 1968; Argyris and Plotkin, 1969; Despont and Cruchaud, 1969; Hirsch, et al., 1969; Loewi, et al., 1969; Panijel and Cayeux, 1968). Unanue (1968) studied in some detail the effect of AMS on macrophages. Within minutes after an intraperitoneal injection of AMS, macrophages were observed to be agglutinated into large masses. A few hours later marked cytological changes were evident in the cytoplasm and nuclei of the remaining macrophages. When AMS was given intravenously, marked alterations were noted in the red pulp of

the spleen. The sinuses contained a paucity of cells and were hemorrhagic and dilated. The white pulp was unaltered, as were the liver, lungs, and kidneys. The immunosuppressive effect of AMS is not established. It has been reported that treatment of mice with AMS does not alter their capacity to respond to immunization with SRBC (Loewi, et al., 1969) or to keyhole limpet hemocyanin (Unanue, 1968). On the other hand, Argyris and Plotkin (1969) found that AMS did suppress antibody formation to SRBC in mice but only if the AMS was given 3 days prior to antigen administration and, further, only when low doses of antigen were employed. AMS was ineffective when administered simultaneously with the antigen. A temporal relationship between AMS treatment and immunization, as well as a requirement for low doses of antigen, was also observed by Panijel and Cayeux (1968). They found that AMS would suppress the immune response to bacteriophage in mice, provided the animals were treated daily with AMS from the third day before to the third day after injection of a priming dose of antigen. AMS suppression of immunological memory might be explained, suggested these investigators, by failure of macrophage-associated antigen to persist in an immunogenic form. A similar explanation was proposed by Jennings and Oates (1967) for the immunosuppressive effect of certain normal sera and phytohemagglutinin. These substances accelerated both the phagocytic activity of peritoneal macrophages and the formation of hydrolytic enzymes within these cells. This, then, could prevent antibody formation by enhancing the degradation and clearance of the injected antigen. This might also explain the results reported by Despont and Cruchaud (1969). They found normal rabbit serum to be nearly as immunosuppressive as AMS in mice injected with SRBC or

diphtheria toxoid.

Immunosuppression by antigen-specific antibody has been achieved under certain conditions. Ryder and Schwartz (1969) undertook studies to elucidate the site of action of specific antibody. Mice were passively immunized at birth and challenged 6 weeks later with SRBC. Their immune response was greatly impaired. Binding of the passively administered antibody to the SRBC was ruled out as a possible mechanism of immunosuppression as was any reaction between lymphoid cells and antibody. It appeared that antibody acted at the macrophage level since peritoneal macrophages from passively immunized mice, which had subsequently been injected with SRBC, failed to elicit an immune response in normal recipients.

Statement of the Problem

The problem was an investigation of the role of the RES in tolerance induction. The approach, in brief, was to depress the phagocytic activity of the RES and determine if this would enhance the ease of tolerance induction. Ethyl stearate and methyl palmitate were employed as RES depressants. Phagocytic activity of the RES was determined by measuring the intravascular clearance rate of colloidal carbon. BALB/cJ mice, a strain noted for its resistance to tolerance induction, were employed as test animals and a soluble preparation of HGG was used as the antigen. The presence or absence of tolerance was determined by the passive hemagglutination technique.

CHAPTER II

MATERIALS AND METHODS

Animals

Male BALB/c and C57BL/6 mice, obtained from Jackson Laboratories, Bar Harbor, Maine, were employed throughout this study. They were approximately 5 weeks of age upon receipt and were used experimentally at 6 weeks of age. Their average weight was 22 gm, with a range of 15-26 gm. Prior to and during the experimental period all mice were maintained on Teklad Mouse/Rat Diet and tap water *ad libitum*.

RES Blockade and Measurement

Lipid Emulsions

One gm of ethyl stearate (Eastman Organic Chemicals and K and K Laboratories, Inc.) was added to 5.0 ml of 5.0 percent dextrose water (Cutter Laboratories) containing 0.04 ml tween-20 (donated by Atlas Chemical Industries, Inc.) and heated to 50C. The suspension was then sonified (Branson Sonifier, Model S75) for 10 seconds. The resulting emulsion was maintained at 50C to prevent solidification of the lipid.

The methyl palmitate (Nutritional Biochemical Corporation and Applied Science Laboratories) emulsion was prepared in a similar manner. One gm of the lipid was added to 20 ml dextrose water containing 0.02 ml tween-20, heated and sonified as described above. This emulsion was

also maintained at 50C to prevent solidification.

Both lipid emulsions were prepared immediately prior to use. The mice were anesthetized with ether and injected via a tail vein. To effect vasodilation and facilitate intravenous injections, the tails were immersed in warm water for a brief period. Control animals were given an equal volume of 5 percent dextrose water containing the appropriate amount of tween-20.

Histological Studies

Spleens and livers were removed from mice 1 or 4 days after an intravenous injection of either ethyl stearate, methyl palmitate, or a mixture of these two lipids. Similar organs were obtained from normal mice as well as from animals injected with tween-20 in dextrose water. All tissues were placed in 10 percent formalin and sent to the histochemistry laboratory for section preparation. Conventional histological staining of the tissues with hematoxylin and eosin (H and E) and with a lipid-specific stain was performed on paraffin fixed tissues. Dr. Florence Bannerjee, Department of Pathology, examined and evaluated the sections.

Carbon Clearance

The phagocytic function of the RES was measured by determining the intravascular clearance rate of colloidal carbon (C11-1431a, Guenther Wagner, Pelikan-Werke, Hanover, Germany) as described by Halpern, et al. (1951). The original carbon suspension was centrifuged at 5,000 rpm for 15 minutes to remove the larger carbon particles. The carbon concentration of the supernatant was approximately 90 mg/ml. This suspension was

diluted in a gelatin (Difco or Knox) solution (prepared with distilled water and adjusted to a pH of 7.2 with ammonia) to final concentrations of 8 mg carbon/ml and 1.0 percent gelatin.

All mice were anesthetized with an intraperitoneal injection of sodium pentabarbital (J. T. Baker Chemical Co. and Abbott Laboratories) prior to carbon clearance tests. The anesthetic was prepared by dissolving 10 mg of sodium pentabarbitol in 1 ml of pyrogen-free, 0.8 percent NaCl (saline) (Cutter Laboratories). This solution had to be stored at room temperature to prevent flocculation of the solute. The material was employed at a dose of 0.1 mg/gm body weight.

A carbon dose of 4 mg/100 gm body weight was administered intravenously to each animal. Blood samples were obtained just prior to and at 3, 6, 9 and 12 minutes after carbon injection. The samples were collected from the retro-orbital venous plexus with a 25 µl capillary pipette which had previously been rinsed with a heparin (Fisher Scientific Co.) solution containing 200 units/ml. Each blood sample was added to 2.0 ml of 0.1 percent Na_2CO_3 to lyse the erythrocytes. The quantity of carbon in each sample was determined spectrophotometrically (Bausch and Lomb Spectronic 20) at a wavelength of 650 mµ. The pre-injection blood sample was used as a blank.

The log concentration of carbon in each blood sample was determined from a standard curve in which known quantities of carbon in blood were plotted against optical density. When these concentrations were plotted against time, a straight line was obtained, the slope of which is a constant, K, termed the phagocytic index. The value of K was calculated from the formula derived by Biozzi, et al. (1953), as described

in CHAPTER I. The phagocytic index was converted to intravascular half-time[1] (T/2) by the following formula: $T/2 = -\frac{\log_2}{K}$.

Antigens

Human gamma globulin (7S) was purchased from Immunology, Inc. The same batch (Lot #8691) was used throughout the investigation, both for tolerance induction and as the assay antigen for passive hemagglutination titrations. Human gamma globulin, Fraction II (Lot #J1524), was obtained from Mann Research Laboratories, Inc. This material, after aggregation, was employed as the immunizing antigen. Pentex, Inc. was the supplier of mouse gamma globulin, Fraction II (Lot #9). All antigens were judged pure by immunoelectrophoretic analysis employing the method of Hyde, Bennett and Garb (1967) and an LKB 6800 A apparatus. Two commercial antisera (Hyland) were employed in this analysis; rabbit anti-human gamma globulin and goat anti-human serum. In addition, the sera of normal mice immunized with aggregated human gamma globulin 5 weeks earlier were pooled and assayed against whole human serum and against human gamma globulin.

Soluble Human Gamma Globulin

A soluble preparation of 7S human gamma globulin (HGG) was used as the tolerance inducing material. Lyophilized HGG was dissolved in saline and allowed to stand overnight at 4C. Following centrifugation at 2,000 rpm for 5 minutes to remove the undissolved material, the supernatant was centrifuged for 2.5 or 4 hours at 40,000 x g or 100,000 x g

---

[1] Half-time (T/2) is the time required for the injected carbon to be reduced to one-half its initial concentration in the blood.

in a Beckman Model L2-65B preparative ultracentrifuge with a Type SW 41 swinging bucket rotor. The top one-third of the material from each tube, containing primarily the monomeric form of the antigen, was carefully transferred to a vial with a propipette. This monomeric material was designated sHGG. The protein concentration of each sHGG preparation was determined spectrophotometrically (Beckman DU Spectrophotometer, Model 2400) by the method of Kalckar (1947). The appropriate dilutions for injection were made in saline. Animals were injected with the sHGG as soon as possible after centrifugation, generally within 1 hour.

Heat-Aggregated Human Gamma Globulin

Cohn Fraction II of HGG was dissolved in saline at a concentration of 25 mg/ml. After low speed centrifugation to remove the undissolved material, the supernatant was dialyzed for 12 hours against 0.08 M phosphate buffered saline (PBS) (pH 8.0) made up in distilled water. The dialysate was discarded and a second 12 hour dialysis was performed against 0.08 M PBS (pH 8.0) made up in deionized water. The PBS contained 1.8 gm KH_2PO_4 + 7.6 gm Na_2HPO_4 + 9.0 gm NaCl + 1,000 ml water. The HGG solution was then centrifuged to remove the precipitate. The supernatant was heated at 63C for 20 minutes with occasional stirring, then cooled in an ice bath. The aggregated material was collected by centrifugation at 2,000 rpm, washed three times in 0.08 M PBS (pH 8.0), and resuspended in 20 ml saline. A 1.0 ml aliquot of this suspension was digested in 4.0 ml of 0.1 N NaOH. This suspension cleared in 30 minutes. The protein concentration was determined spectrophotometrically. The aggregated HGG (aggHGG) was diluted in saline to a concentration of 2.0 mg/ml and stored in 4.0 ml aliquots at 4C.

The aggHGG was employed as the immunizing antigen in all experiments. Each animal received a dose of 0.1 mg aggHGG emulsified in an equal volume of incomplete Freund's adjuvant (Difco) and administered subcutaneously in a single site.

Tolerance Induction

Experiment Design

The procedure for inducing tolerance is outlined in Table 1. BALB/c mice were employed in most experiments because they are known to be particularly resistant to the induction of tolerance to protein antigens. C57BL/6 mice were used in one experiment for comparative purposes as they are much more susceptible to tolerance induction.

On the first day of an experiment the test and immunization control mice were given intravenous injections of the ethyl stearate-methyl palmitate emulsion to depress RES activity. Tolerance controls received an equivalent amount of tween-20 in 5 percent dextrose water by the same route. One day later the test and tolerance control animals were injected intraperitoneally with the tolerogenic antigen, sHGG. An equal volume of saline was given to the immunization controls. One week after tolerization all animals were challenged with 0.1 mg aggHGG in incomplete Freund's adjuvant, administered subcutaneously. Mice were first bled 2 weeks after challenge and then at weekly intervals for 3 more weeks. Prior to bleeding, the mice were warmed under a heat lamp for a few minutes. Bleedings were accomplished through a transverse incision in a tail vein. The sera were either titrated immediately or stored at -10C.

TABLE 1

EXPERIMENTAL DESIGN FOR TOLERANCE INDUCTION

BALB/c Mice (Male, 6-7 Weeks)	Experimental	Controls	
		Tolerance	Immunization
RES Modification	ES-MP[a]	Tween-20[d]	ES-MP
↓ 1 day			
Tolerance Induction	sHGG[b]	sHGG	Saline
↓ 7 days			
Tolerance Challenge	aggHGG[c]	aggHGG	aggHGG
↓ 14,21,28,35 days			
Immune Response Assay (passive hemagglutination)			

[a]Ethyl stearate, methyl palmitate

[b]Soluble human gamma globulin

[c]Aggregated human gamma globulin

[d]Some animals were treated with dextrose water or with saline. Immune response was the same, hence the animals are combined into a single tolerance control group.

Immune Response Assay

The immune response was measured by passive hemagglutination, employing sheep red blood cells (SRBC) obtained from the Colorado Serum Co. The cells were preserved in Alsever's solution and were washed 3 times in 0.08 M PBS (pH 7.4) before use.

HGG was coupled to SRBC with bis diazotized benzidine (BDB). The BDB was prepared by dissolving 0.161 gm benzidine dihydrochloride (Lot #127B-2300, Sigma Chemical Co.) in 22.5 ml of 0.25 N HCl at -2C. To this was added 2.5 ml of an aqueous solution of 3.5 percent $NaNO_2$, previously cooled to -2C. The $NaNO_2$ solution was added slowly with stirring over a period of 1 minute. The mixture was allowed to react for 30 minutes at -2C with intermittent stirring. It was then sealed in 1 ml aliquots and stored at -10C.

A modification of the Stavitsky and Arquilla (1955) method was employed to couple the HGG to the SRBC. To 2.0 ml of a 4 mg/ml saline solution of HGG, previously absorbed with SRBC, the following were added: 1.6 ml of 0.85 percent saline, 0.1 ml of a 50 percent SRBC (suspended in 0.08 PBS, pH 7.4), 0.5 ml of 1:15 dilution of BDB (diluted in PBS). This suspension was incubated at room temperature for 15 minutes with intermittent, gentle mixing and then centrifuged at 1,500 rpm for 3 minutes. The supernatant was discarded and the HGG-sensitized SRBC were resuspended and washed twice in 2.0 ml of PBS containing 0.2 percent gelatin (PBSG). Following washing the sensitized cells were resuspended in 2.5 ml of PBSG. This suspension was finally diluted 1:4 in PBS for use in the hemagglutination assay. Control or unsensitized SRBC were prepared in exactly the same manner except that 2.0 ml of PBSG were

substituted for the HGG.

Disposable, microtiter "V" plates (Cooke Engineering Co. and Linbro Chemical Co.) were utilized for the passive hemagglutination assays. Each serum was titrated in two diluents, PBSG and a 5 percent aqueous solution of bovine serum albumin (BSA) (Mann Research Laboratories, Inc.).

The stepwise procedure was as follows:

1. 25 µl of diluent was added to each well.
2. 25 µl of mouse serum was added to the first well in each row.
3. Serial, twofold dilutions of the serum were made up to, but not including, the last well in the row.
4. 25 µl of HGG-sensitized SRBC were added to each well including the last one in the row. The last well, therefore, served as a control for non-specific hemagglutination of the sensitized cells.
5. A 1:100 dilution of rabbit anti-HGG serum (Hyland) was also titrated as a positive control.
6. This procedure was repeated using unsensitized SRBC as a control for non-specific reactions between the serum and SRBC.
7. The plates were incubated at room temperature and read at 4 and 12 hours.

All titers were expressed as $\log_2$ units of the reciprocal of the highest dilution giving any indication of hemagglutination. A thin, uniform lawn of SRBC covering the entire bottom surface of the well was designated as complete hemagglutination. An irregular lawn of cells was interpreted as incomplete hemagglutination. Negative hemagglutination was evidenced by a round, compact button of SRBC in the center of the well surface. Figure 2 shows a sample titration of eight sera. Each serum was serially diluted in the horizontal rows, i.e., wells one through eleven. The $\log_2$ titers were read as follows: serum #1=3;

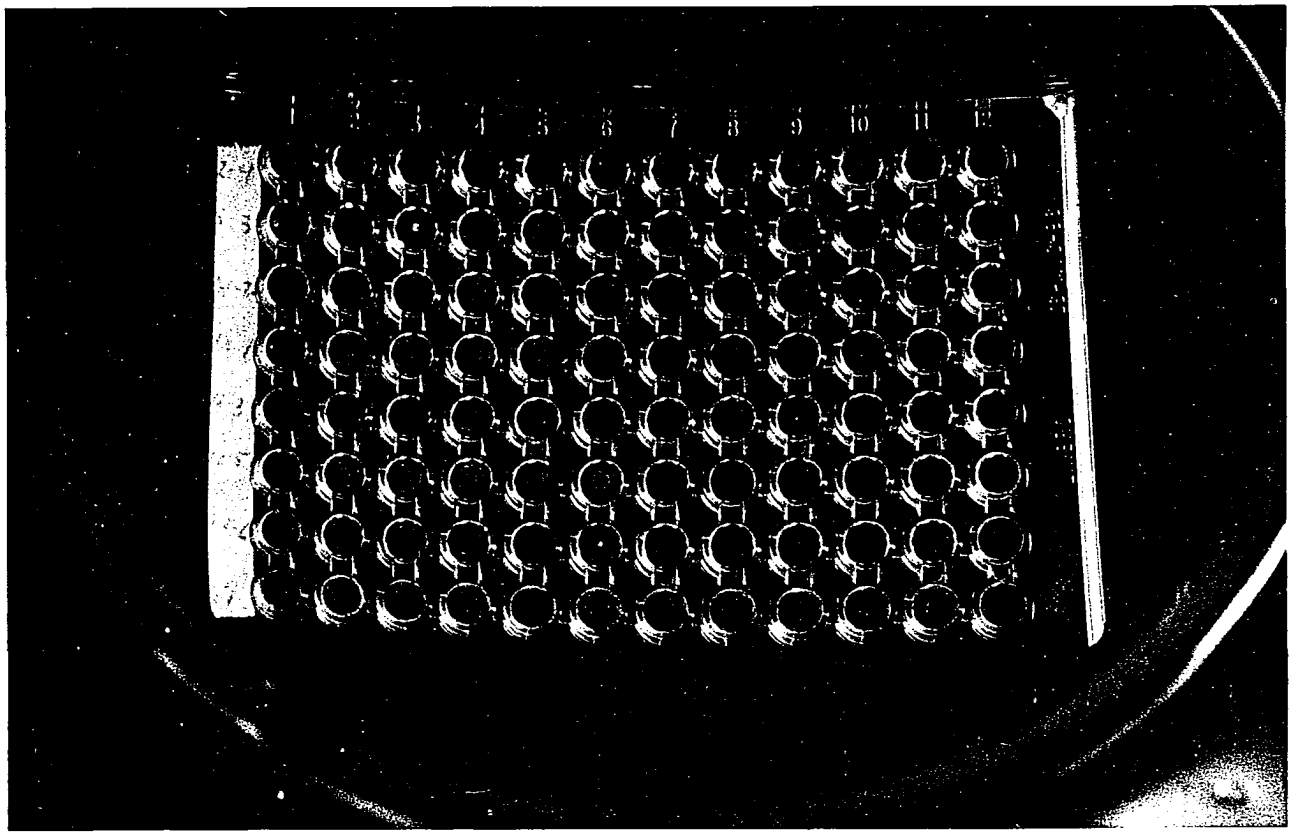

Figure 2--Passive hemagglutination assays of eight mouse sera.

serum #2=1; serum #3=4; serum #4=6; serum #5=5; serum #6=6; serum #7=4; serum #8=5. Passive hemagglutination assays were done in duplicate whenever the volume of serum permitted and titers reported are the average of these duplicate titrations.

CHAPTER III

RESULTS

Effect of Anesthesia on RES Function

Carbon clearance tests can be carried out with greater ease, rapidity and accuracy in anesthetized animals. Sodium pentabarbital is the anesthetic agent of choice for mice when a relatively long duration of anesthesia is required (Taber and Irwin, 1969). However, virtually no studies have been done to evaluate the effect of anesthesia on physiological function in mice. Therefore, it was necessary to determine what effect, if any, sodium pentabarbital had on normal carbon clearance rates. Two test groups, done on different days, received 0.1 ml (2.0 mg) of sodium pentabarbital intraperitoneally. The control group was injected with an equal volume of sterile, physiological saline by the same route. The results are given in Table 2 and clearly demonstrate no significant difference in the clearance rates of the 3 groups of animals.

Effect of Lipids on RES Function

Ethyl Stearate

The effect of ethyl stearate on phagocytic activity was compared using ethyl stearate from two suppliers. Carbon clearance rates were determined 24 hours after administration of the lipid. Tables 3

TABLE 2

EFFECT OF ANESTHESIA ON THE CARBON CLEARANCE RATES OF NORMAL BALB/c MICE

Anesthesia	Number of Animals	Carbon T/2[a] (min.)	Range
Yes	4	15.4 ± 3.1	9.8 - 23.7
Yes	5	11.6 ± 2.5	6.5 - 19.7
No[b]	7	14.7 ± 1.0	11.4 - 18.3

[a]Half-time. Values are means ± standard error.

[b]Unanesthetized animals received an equal volume of saline.

TABLE 3

EFFECT OF ETHYL STEARATE OBTAINED FROM K AND K LABORATORIES, INC. ON RES FUNCTION

Ethyl Stearate Dose (mg)	Number of Animals	Carbon T/2[a] (min.)	Range
40	7	72.5 ± 14.7	36.3 - 150.5
60	7	109.1 ± 27.7	44.9 - 231.5
80	6	74.7 ± 7.1	56.8 - 100.3
120	5	145.4 ± 40.8	64.0 - 301.0
Controls	8	15.8 ± 1.8	10.9 - 27.4

[a]Half-time. Values are means ± standard error.

and 4 show the effect of ethyl stearate obtained from K and K Laboratories, Inc. and Eastman Organic Chemicals, respectively. The data contained in Table 4 were compiled from experiments done on different days. Therefore, the effect of slight differences in the daily preparation of the individual lipid emulsions and carbon suspensions is obviated.

The ethyl stearate preparation obtained from K and K Laboratories, Inc., appeared to be slightly more depressive to the RES, but it is doubtful that there is any real difference in the effectiveness of the two preparations. Also, it appeared that doses from 20 to 80 mg were of approximately equal effectiveness. Doses of 80 mg and greater had a decidedly toxic effect on the mice (Table 5). Considering the relative toxic and RES blockading effects, it was decided that a dose of 60 mg would be used in subsequent experiments. Also, the ethyl stearate preparation from Eastman Organic Chemicals was employed in all subsequent work.

Studies were carried out to determine the duration of the depressive effect of ethyl stearate on RES phagocytic activity. Mice were injected with 60 mg of ethyl stearate and divided into five groups. Carbon clearance tests were done at 1, 2, 4, 7, and 10 days following administration of the lipid. A few of the lipid-treated animals exhibited exceptionally prolonged half-times which suggested some reaction in this small group not observed in the majority of the population. These exceptional animals are discussed for each experiment in which they were observed. For statistical purposes, the comparisons of the control group means vs test group means did not include these animals. In this experiment, and in all subsequent studies on the duration of lipid effect,

TABLE 4

EFFECT OF ETHYL STEARATE OBTAINED FROM EASTMAN ORGANIC CHEMICALS ON RES FUNCTION

Ethyl Stearate Dose (mg)	Number of Animals	Carbon T/2[a] (min.)	Range
20	6	68.6 ± 5.1	44.9 - 81.3
40	5	55.7 ± 5.1	44.9 - 75.2
Control	5	16.0 ± 2.5	9.5 - 21.0
60	8	52.8 ± 7.9	26.6 - 100.3
Control	11	21.3 ± 3.2	12.2 44.9
80	3	89.7 ± 21.8	56.8 - 130.9
120	1	81.3 ± 0	-
Control	3	13.2 ± 1.4	10.6 - 15.3
160	1	231.5 ± 0	-
Control	5	16.6 ± 2.9	11.1 - 24.5

[a]Half-time. Values are means ± standard error.

TABLE 5

TOXICITY OF ETHYL STEARATE FOR MICE

Dose (mg)	Survival Rate[a] Source No. 1[b]	Source No. 2[c]
20	6/6	
40	8/8	7/7
60	8/8	8/8
80	3/5	6/8
120	2/5	5/10
160	1/5	
Controls	26/26	8/8

[a]Number of animals surviving/number of animals treated.

[b]K and K Laboratories, Inc.

[c]Eastman Organic Chemicals.

the calculated F values for testing control group means vs test group means at each time interval were determined by the procedure for testing simple effects in an unweighted mean analysis for a two-way classification as given by Winer (1962). This technique was selected since it offered a convenient computational procedure for solving the problem of unequal cell size and required assumptions no more stringent than those of the usual Student's t test where the estimate of error is obtained by pooling. The statistic employed, F value, with one degree of freedom for the numerator is, of course, equivalent to the "t" test for difference of two means in that the square root of the F value is the t value. This analysis allowed the pooling of information in all cells in the estimation of experimental error. The robustness of analysis of variance procedures relative to departures from the normality and homogeneity of variance assumptions (Box, 1954) was also an influencing factor in the choice of this technique. Although the individual comparison could have been made using a non-parametric technique, such as the Mann-Whitney U test, there is a loss of power of differentiation with this procedure as compared to the selected parametric method.

All animals had significantly depressed phagocytic function 1 day following treatment with ethyl stearate (Table 6). At 2 days, one animal, not shown in the table, was completely suppressed (T/2 - ∞), six were markedly depressed and one displayed a normal clearance rate (T/2 - 17.7 minutes). At 4 days, 9 of 11 animals were marginally depressed. By 7 days, half of the animals had regained normal function and half were still depressed, 2 quite severely (T/2 - ∞ and 231.5 minutes). All animals except one had regained normal or nearly normal

TABLE 6

DURATION OF RES DEPRESSION FOLLOWING A 60 mg DOSE OF ETHYL STEARATE

Days After Ethyl Stearate Treatment	Animal Group	Number of Animals	Carbon T/2[a] (min.)	Range	F Value
1	Test	4	67.5 ± 11.0	52.8 - 100.3	10.19[b]
1	Control	7	16.1 ± 1.3	12.2 - 22.0	
2	Test	7	60.9 ± 14.9	17.7 - 130.9	8.84[b]
2	Control	7	12.9 ± 2.0	7.2 - 23.1	
4	Test	11	46.3 ± 3.5	30.1 - 64.0	1.74
4	Control	6	25.0 ± 2.4	20.5 - 34.6	
7	Test	7	65.4 ± 29.6	13.5 - 231.5	7.62[b]
7	Control	6	20.8 ± 3.3	10.6 - 35.0	
10	Test	7	27.4 ± 6.0	12.1 - 60.2	0.92
10	Control	8	11.9 ± 2.0	6.8 - 21.5	

[a]Half-time. Values are means ± standard error.

[b]Significant at 0.05 level in test of difference between control and test group means.

function by day 10.

Methyl Palmitate

The effect of methyl palmitate on phagocytic function was compared using methyl palmitate from two sources. The effect of 20 mg and 40 mg doses of lipid obtained from Nutritional Biochemicals Corp., on phagocytic function 24 hours after administration is shown in Table 7. Two animals, not shown in Table 7, were completely blockaded (T/2 - ∞) by the 20 mg dose, 2 were significantly depressed (T/2 - 143.3 and 88.5 minutes) and 2 were not depressed (T/2 - 16.4 and 16.1 minutes). The 40 mg dose depressed 3 of the 4 mice to a marked degree. The toxicity of this material is evident from an inspection of the data presented in Table 8. Approximately one-third of the treated animals did not survive.

In contrast to the Nutritional Biochemicals Corporation preparation of methyl palmitate, that supplied by Applied Science Laboratories was considerably less toxic (Table 8). The purity of the latter preparation was listed as 99+ percent and this has been confirmed by DiLuzio and Wooles (1964). Seventy-three percent of the mice survived treatment with a 45 mg dose and 79.0 percent survived a 35 mg dose. The effect of various doses of this preparation on phagocytic activity is shown on Table 9. None of the mice was depressed by the 15 mg dose. The 25 mg dose depressed 9 of the 15 animals but 2 of these had T/2 values approaching the upper limit of carbon half-times of control animals (T/2 - 56.8 minutes). Approximately one-half of the animals receiving the 35 mg dose were depressed. The 45 mg dose exhibited the greatest depressive effect as well as the most toxicity. All animals were severely depressed by this dose of methyl palmitate.

TABLE 7

EFFECT OF METHYL PALMITATE OBTAINED FROM NUTRITIONAL BIOCHEMICALS CORPORATION ON RES FUNCTION 24 HOURS AFTER TREATMENT

Methyl Palmitate Dose (mg)	Number of Animals	Carbon T/2[a] (min.)	Range
20	4	66.1 ± 30.9	16.1 - 143.3
Controls	9	19.8 ± 2.0	11.3 - 29.2
40	4	55.4 ± 11.8	28.1 - 75.2
Controls	3	25.9 ± 2.3	22.0 - 30.1

[a]Half-time. Values are means ± standard error.

TABLE 8

TOXICITY OF METHYL PALMITATE FOR MICE

Dose (mg)	Survival Rate[a] Source No. 1[b]	Source No. 2[c]
15		4/5
20	6/9	
25		15/15
35		11/14
40	10/32	
45		41/56
Control	14/14	15/15

[a]Number of animals surviving/number of animals treated.

[b]Nutritional Biochemicals Corporation.

[c]Applied Science Laboratories.

TABLE 9

EFFECT OF METHYL PALMITATE OBTAINED FROM APPLIED SCIENCE LABORATORIES ON RES FUNCTION 24 HOURS AFTER TREATMENT

Methyl Palmitate Dose (mg)	Number of Animals	Carbon T/2[a] (min.)	Range
15	4	24.8 ± 4.1	18.2 - 36.3
25	15	73.6 ± 9.9	26.6 - 150.5
35	11	68.2 ± 12.6	13.9 - 177.1
45	4	120.5 ± 12.2	100.3 - 150.5
Controls	15	17.4 ± 3.6	4.6 - 52.8

[a]Half-time. Values are means ± standard error.

The duration of the depressive effect of both preparations of methyl palmitate was determined. The results of the experiment using methyl palmitate obtained from Nutritional Biochemicals Corp. are shown in Table 10. Two animals, not shown in the table, were completely depressed, 2 were significantly depressed and 2 were only slightly affected 1 day following treatment. At 4 days all treated animals had prolonged clearance times. At 7 days 1 animal was quite depressed (T/2 - 273.6 minutes), 1 had regained normal function and the remaining 3 had clearance rates approaching those of the controls. By 10 days all animals had normal or nearly normal clearance rates.

The results of a similar experiment, using a 45 mg dose of methyl palmitate obtained from Applied Science Laboratories, are shown in Table 11. At day 1 all animals were greatly depressed. One animal, not shown in the table, was exceedingly depressed (T/2 - 1,003.3 minutes). Again, at day 2, all animals exhibited decreased RES activity but the depression was not as marked as at day 1. The results shown for day 4 are equivocal. They represent the averages of experiments done on three separate occasions. In 2 of these experiments several of the control animals had considerably longer half-times than were usually found. Further, in one case, 3 of 4 test animals were not suppressed at all. Combining all three groups, as was done in Table 11, shows that 11 of the 14 test animals were depressed to varying degrees but so were 9 of the 21 controls. At day 7 two-thirds of the test group had normal clearance rates and by day 10 all but 1 test animal had normal or nearly normal half-times.

It was thought that two successive treatments with methyl

TABLE 10

DURATION OF RES DEPRESSION FOLLOWING A 20 mg DOSE OF METHYL PALMITATE OBTAINED FROM NUTRITIONAL BIOCHEMICALS CORPORATION

Days After Methyl Palmitate Treatment	Animal Group	Number of Animals	Carbon T/2[a] (min.)	Range	F Value
1	Test	4	66.1 ± 30.9	16.1 - 143.3	2.87
1	Control	9	19.8 ± 2.0	11.3 - 29.2	
4	Test	4	68.4 ± 5.9	56.8 - 81.3	3.83
4	Control	5	14.9 ± 2.0	10.7 - 21.5	
7	Test	5	73.7 ± 50.0	17.5 - 273.6	5.38[b]
7	Control	5	10.4 ± 1.3	8.1 - 17.7	
10	Test	5	24.2 ± 4.1	16.4 - 39.1	0.12
10	Control	5	14.7 ± 3.3	6.3 - 26.6	

[a]Half-time. Values are means ± standard error.

[b]Significant at 0.05 level in test of difference between control and test group means.

TABLE 11

DURATION OF RES DEPRESSION FOLLOWING A 45 mg DOSE OF METHYL PALMITATE OBTAINED FROM APPLIED SCIENCE LABORATORIES

Days After Methyl Palmitate Treatment	Animal Group	Number of Animals	Carbon T/2[a] (min.)	Range	F Value
1	Test	4	120.5 ± 12.3	100.3 - 150.5	166.58[b]
1	Control	10	16.8 ± 4.1	9.1 - 52.8	
2	Test	6	64.2 ± 10.4	37.6 - 100.3	30.76[b]
2	Control	6	19.6 ± 1.0	17.7 - 24.5	
4	Test	14	44.5 ± 5.9	13.5 - 94.1	5.23[b]
4	Control	21	26.1 ± 3.1	9.9 - 52.8	
7	Test	12	30.8 ± 3.5	14.1 - 50.2	4.30[b]
7	Control	13	14.2 ± 1.6	7.5 - 25.0	
10	Test	4	32.2 ± 5.7	22.1 - 48.5	5.82[b]
10	Control	5	12.8 ± 1.8	7.5 - 18.5	

[a]Half-time. Values are means ± standard error.

[b]Significant at 0.05 level in test of difference between control and test group means.

palmitate might prolong the duration of its depressive action. To test this hypothesis, mice were given a 25 mg dose of the lipid and, 3 days later, a second injection of the same amount. Clearances were done 4, 7 and 10 days after the initial injection. The results are given in Table 12. At days 4 and 7 approximately half the test animals had normal clearance rates and by day 10 all had regained normal function. Thus, this treatment did not extend the duration of RES depression over that produced by a single administration of lipid.

Combined Dose of Ethyl Stearate and Methyl Palmitate

Since ethyl stearate and methyl palmitate are believed to exert their depressive effect on the RES by different mechanisms, it was thought that a combined dose of the two lipids might result in a more severe and/or prolonged depression than that caused by either lipid alone. Animals were divided into 4 test and control groups. The test mice received an injection consisting of 30.0 mg of ethyl stearate and 22.5 mg of methyl palmitate. This combination was not toxic. The results of this experiment (Table 13) show all treated animals to be significantly depressed at days 1 and 4. In fact, at day 1, 2 animals, not shown in the table, were completely depressed ($T/2 - \infty$). By day 7 one-third of the test mice had normal clearance rates. Three of the 12 treated animals were significantly depressed at day 10; the rest had normal or nearly normal half-times. Although these results were not markedly different from those employing only 1 lipid at a time, it did appear that a somewhat higher percentage of the test group remained depressed for a slightly longer period of time. In view of this, the two lipids were used in conjunction as RES depressive agents in the

TABLE 12

EFFECT OF TWO SUCCESSIVE 25 mg DOSES OF METHYL PALMITATE OBTAINED FROM APPLIED SCIENCE LABORATORIES ON RES FUNCTION

Days After Initial Methyl Palmitate Treatment	Animal Group	Number of Animals	Carbon T/2[a] (min.)	Range	F Value
4	Test	11	33.4 ± 2.1	23.7 - 44.9	4.96[b]
4	Control	5	25.4 ± 3.8	18.5 - 39.1	
7	Test	7	25.4 ± 2.7	18.0 - 36.3	14.44[b]
7	Control	6	11.7 ± 1.9	6.4 - 20.1	
10	Test	5	10.7 ± 1.7	7.5 - 17.0	0.20
10	Control	5	9.1 ± 1.4	6.4 - 13.7	

[a]Half-time. Values are means ± standard error.

[b]Significant at 0.05 level in test of difference between control and test group means.

TABLE 13

EFFECT OF A COMBINED DOSE OF ETHYL STEARATE AND METHYL PALMITATE ON RES FUNCTION

Days After Lipid Administration	Animal Group	Number of Animals	Carbon T/2[a] (min.)	Range	F Value
1	Test	12	69.5 ± 13.7	36.3 - 177.1	19.59[b]
1	Control	13	14.1 ± 3.6	5.2 - 15.0	
4	Test	14	43.2 ± 4.4	20.5 - 75.2	8.04[b]
4	Control	14	7.7 ± 1.2	2.4 - 17.4	
7	Test	12	52.1 ± 23.0	12.9 - 301.0	10.99[b]
7	Control	15	10.6 ± 1.1	3.1 - 18.0	
10	Test	12	27.8 ± 3.6	12.2 - 50.2	1.66
10	Control	14	11.6 ± 0.9	5.4 - 17.7	

[a]Half-time. Values are means ± standard error.

[b]Significant at 0.05 level in test of difference between control and test group means.

subsequent tolerance induction studies.

The effect of ethyl stearate-methyl palmitate treatment on the RES activity of C57BL/6 mice was also determined since this strain, as well as BALB/c mice, were employed in the subsequent tolerance induction experiments. The results, presented in Table 14, indicate that C57BL/6 mice are more susceptible than the BALB/c strain to lipid-induced RES depression. All but 1 animal (T/2 - 39.1 minutes) were severely depressed (T/2's ranged from 130.9 to 1,003.3 minutes) and another, not shown in the table was completely depressed (T/2 - ∞). The C57BL/6 mice were also more sensitive to the toxic effects of the lipid, since 5 of 13 did not survive treatment.

Histological Studies

Spleens and livers were removed from mice 1 and 4 days after the following treatments: (1) ethyl stearate, (2) methyl palmitate, (3) the two lipids given together, (4) tween-20 in dextrose water and (5) none. Paraffin sections were stained with either hematoxylin and eosin or with a lipid stain. The spleens and livers of all animals treated with tween-20 in dextrose water were normal as were the livers of all the lipid treated animals. Examination of the spleens of those animals injected with ethyl stearate revealed marked and diffuse necrosis throughout the white pulp with neutrophil infiltration after 24 hours. However, the germinal centers were not affected. At 4 days focal areas of necrosis were still visible in the white pulp but regeneration had begun as evidenced by the marked proliferation of histiocytes and fibroblasts. The methyl palmitate-treated animals displayed almost identical splenic pathology. Regeneration began somewhat earlier in these animals

TABLE 14

EFFECT OF ETHYL STEARATE-METHYL PALMITATE ON RES FUNCTION OF C57BL/6 MICE 24 HOURS AFTER TREATMENT

Group	Number of Animals	Carbon T/2[a] (min.)	Range
Lipid-Treated	5	305.5 ± 176.3	39.1 - 1,003.3
Control	13	7.1 ± 0.7	3.2 - 11.2

[a]Half-time. Values are means ± standard error.

as some histiocyte and fibroblast infiltration was noted 1 day after administration of the methyl palmitate. Those animals treated with the two lipids in combination also had similar splenic changes. Again, at 1 day following treatment, marked necrosis of the white pulp with neutrophil infiltration was evident. By 4 days focal areas of necrosis were observed in both the white and red pulp. Regeneration was also evident at this time.

The lipid stain revealed some fat present in the splenic histiocytes 4 days after treatment with the combination of lipids. In no other instance was fat observed in the liver or spleen sections.

Tolerance Induction

Effect of Centrifugation Speed and Duration on the Tolerogenicity of HGG

The tolerogenic or soluble antigen was prepared by ultracentrifugation of a saline solution of HGG. It was of interest to know what effect, if any, the speed and duration of centrifugation had on the tolerogenic property of this antigen. To determine the effect of speed, antigen preparations were centrifuged at 40,000 x g and 100,000 x g for 4 hours. Mice, previously treated with lipid, were given 5.0 mg doses of the supernatant obtained from the top one-third of the centrifuge tube. From the results presented in Table 15 it can be seen that the percent recovery was greater at the slower speed. However, there was no correlation between centrifugation speed and tolerogenic capacity.

To determine the effect of duration of centrifugation, antigen preparations were centrifuged at 100,000 x g for 2.5 and 4 hours. No correlation existed between duration of centrifugation and tolerogenic

TABLE 15

EFFECT OF SPEED OF CENTRIFUGATION ON PERCENT RECOVERY AND TOLEROGENIC CAPACITY OF 5.0 mg OF HUMAN GAMMA GLOBULIN

	Centrifugation x g		
	100,000	100,000	40,000
Percent Recovery	56.6	64.7	80.0
Tolerogenic Capacity	3.2[a] (7)[b]	0.7 (8)	1.8 (13)
Controls[c]	5.3 (3)	3.0 (5)	5.6 (8)

[a]Mean $\log_2$ titer at two weeks.

[b]Number of animals tested.

[c]Control animals received saline rather than sHGG prior to immunization with aggHGG.

ability of the sHGG (Table 16). Percent recovery was slightly higher, in most cases, with the shorter centrifugation period.

In the first experiment of the tolerance induction series the sHGG was prepared by centrifuging at 40,000 x g for 4 hours with an angle head rotor. The yield was extremely low (36.5%). Also, it was impossible to control the monomeric purity of the sHGG since antigen which sedimented on the walls of the tubes during centrifugation could wash off into the supernatant after cessation of centrifugation. For these reasons a swinging bucket rotor was used for all subsequent preparations of sHGG.

The Effect of Lipid on Tolerance Induction by Several Doses of sHGG

Single injections of various concentrations of sHGG were given to lipid-treated mice to determine whether a dose which was normally antigenic would become tolerogenic when the RES was depressed. The results of these experiments are shown in Figures 3 through 17, which will be discussed later. In each of these figures, EX represents the lipid-treated or experimental group, and TC and IC denote the tolerance control and immunization control groups, respectively. Each dot represents the passive hemagglutination titer of an animal at the specified time. The horizontal line in the middle of each vertical bar designates the mean hemagglutination titer of all animals in that group. The upper and lower horizontal lines indicate the limits of one standard deviation above and below this mean. In certain instances the lower limit of one standard deviation was less than zero in which case the vertical line stops at zero.

TABLE 16

EFFECT OF DURATION OF CENTRIFUGATION ON PERCENT RECOVERY AND TOLEROGENIC CAPACITY OF 5.0 mg OF HUMAN GAMMA GLOBULIN

	Duration of Centrifugation (hours)					
	2.5	2.5	2.5	2.5	4	4
Percent Recovery	59.4	71.7	75.8	81.0	56.6	64.7
Tolerogenic Capacity	1.6[a] (8)[b]	3.5 (13)	4.0 (6)	3.6 (13)	3.2 (7)	0.7 (8)
Controls[c]	7.4 (7)	6.7 (7)	6.6 (5)	4.8 (26)	5.3 (3)	3.0 (5)

[a]Mean $\log_2$ titer at two weeks.

[b]Number of animals tested.

[c]Control animals received saline rather than sHGG prior to immunization with aggHGG.

In evaluating the data represented in Figures 3 through 17, it should be borne in mind that the unsensitized SRBC control titrations often had $\log_2$ titers of one. Therefore, test titers of one may not be significant in all instances. Further, titers determined on different days sometimes varied ± one $\log_2$ unit. Similar variations with this assay have been reported by Unanue and Askonas (1968).

Low doses of sHGG. Two experiments were performed employing a 0.5 mg dose of sHGG. The results of one of these experiments are presented in Figure 3. At 2 and 3 weeks following challenge, approximately half of the lipid-treated animals had somewhat lower titers than those of the control groups and 4 appeared to be tolerant or hyporesponsive. By 4 weeks, however, all lipid-treated animals had titers similar to those of the tolerance controls. No marked difference was noticed in titers of the tolerance and immunization control animals at 2 and 3 weeks post-challenge. At 4 weeks, approximately half of the immunization controls had titers ranging from 8 to 10 $\log_2$ units.

A second experiment employing 0.5 mg sHGG was terminated following the 2 weeks post-challenge titration since there was no difference in titers of the 3 groups. Similar findings were observed when a 0.1 mg dose of sHGG was used.

For comparative purposes, an additional experiment was performed using C57BL/6 mice rather than the BALB/c strain since the former strain is much more susceptible to tolerance induction than is the latter (Golub and Weigle, 1969). Mice were divided into 5 groups. Two groups were treated with lipid. One of these groups was then injected with 0.5 mg of sHGG and the other with an equal quantity of uncentrifuged

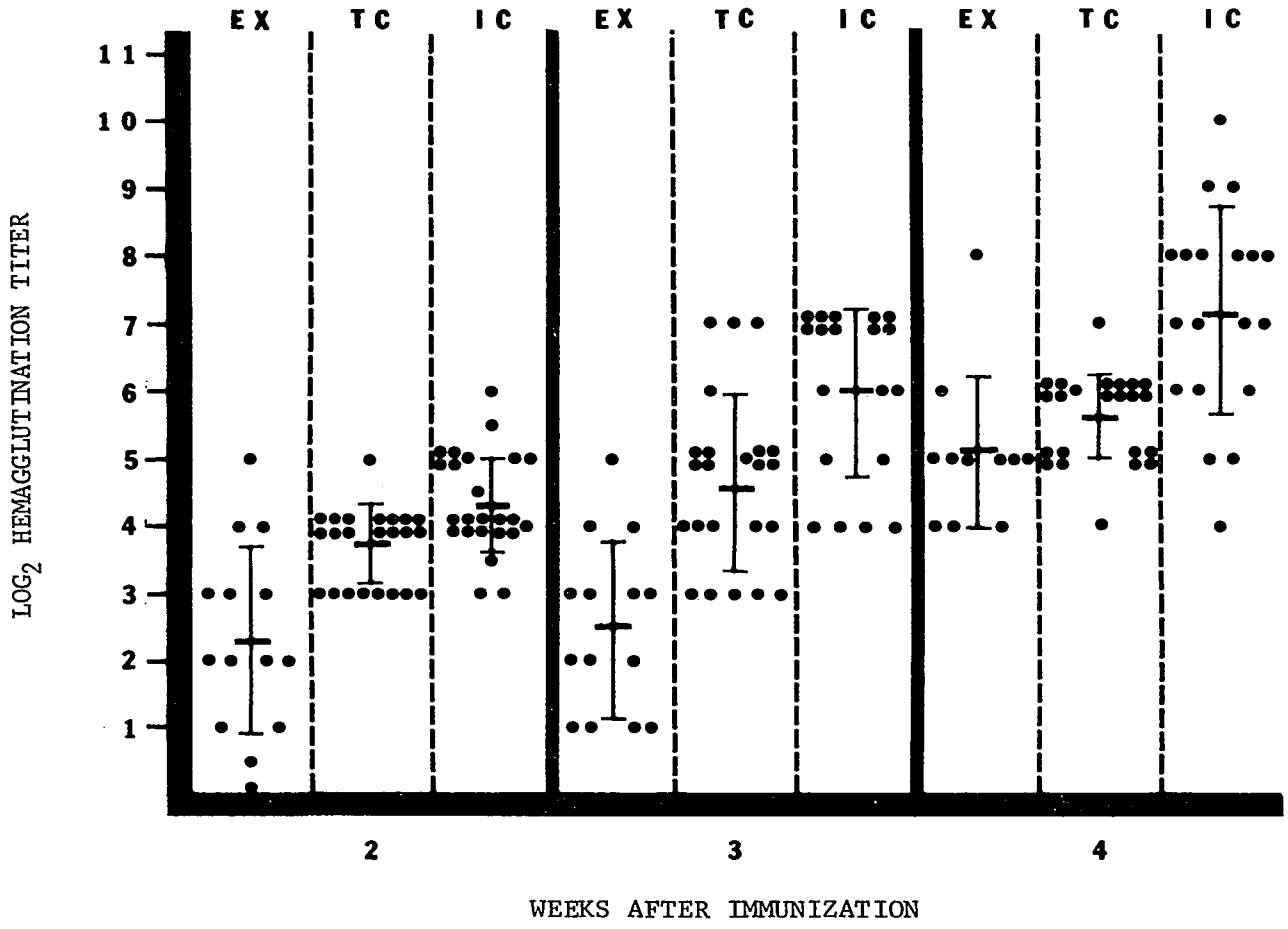

Figure 3. Effect of ethyl stearate-methyl palmitate treatment on tolerance induction in BALB/c mice by 0.5 mg of sHGG. EX - experimental or lipid-treated group; TC - tolerance controls, IC - immunization controls.

HGG. Two groups were used as tolerance controls for the 2 preparations of HGG and the last group served as immunization controls. The response of the animals which received sHGG or uncentrifuged HGG are presented in Figures 4 and 5, respectively. At 2 and 3 weeks post-challenge most of the lipid-treated animals and approximately half of the tolerance controls which had received sHGG appeared to be unresponsive or hyporesponsive (Figure 4). At 4 and 5 weeks after immunization 5 of the 8 lipid-treated animals were still hyporesponsive.

Most of the mice which received uncentrifuged HGG appeared to be hyporesponsive 2 weeks following immunization. The titers of all of these mice increased gradually during the next 3 weeks and by 5 weeks post-challenge none was unresponsive and most had titers of 3 $\log_2$ units or more. The surprising finding was that the titers of the tolerance controls failed to increase at the same rate as those of the immunization controls. At 5 weeks post-challenge 3 of the 6 tolerance control mice had titers from 1 to 2 $\log_2$ units less than those of the lowest immunization control animals.

Intermediate dose of sHGG. A dose of 1.0 mg of sHGG severely depressed the immune response of a majority of both the experimental and tolerance control animals (Figure 6). This depression was evident throughout the test period from 3 to 6 weeks post-challenge. Tolerance, in this case, was apparently due to the dose of sHGG used rather than to any influence of the lipid.

A second experiment employing the same dose of sHGG gave similar results. Titers of the experimental and tolerance control groups were quite comparable, although not as severely depressed as in the previous

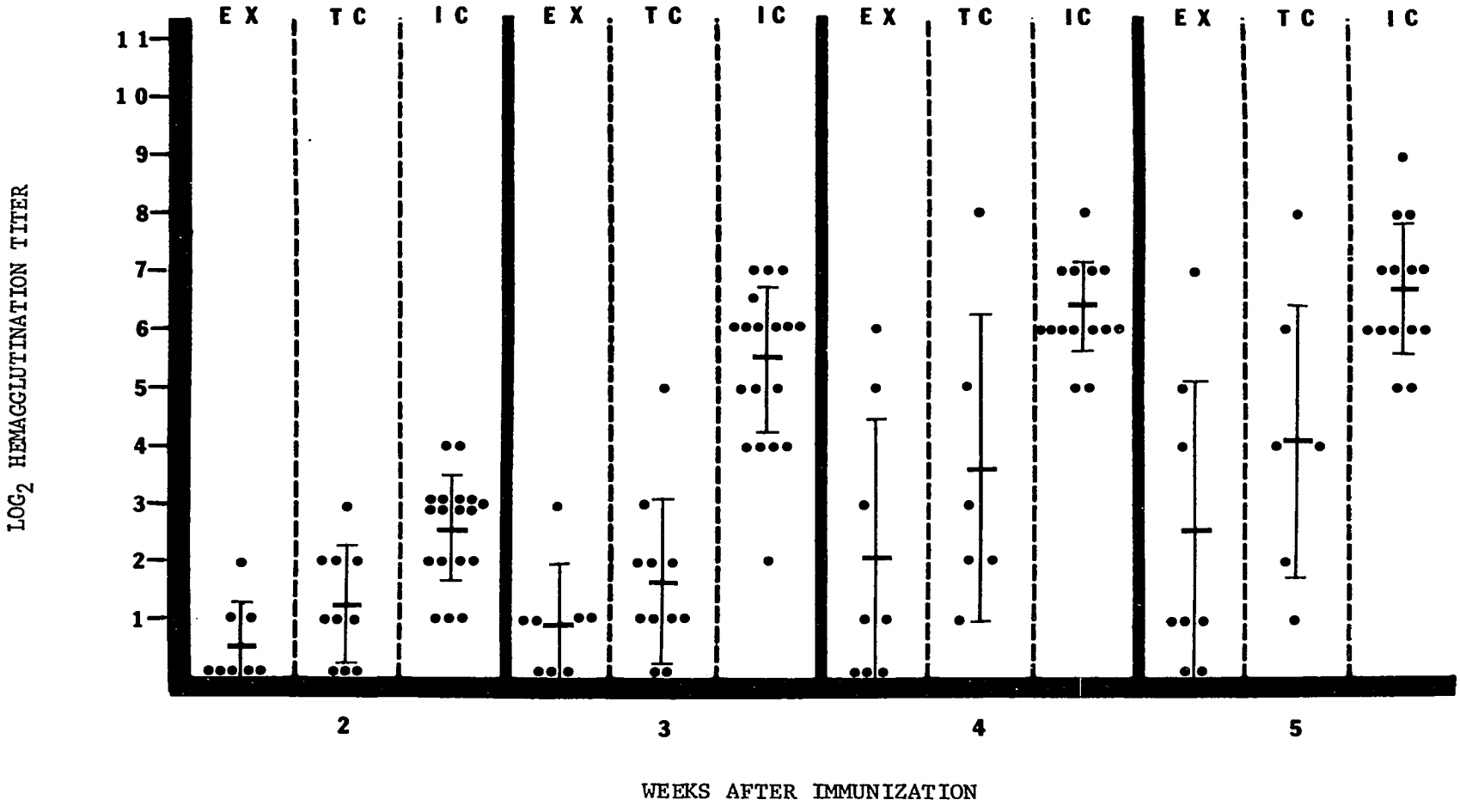

Figure 4. Effect of ethyl stearate-methyl palmitate treatment on tolerance induction in C57BL/6 mice by 0.5 mg of sHGG. EX - experimental or lipid-treated group; TC - tolerance controls; IC - immunization controls.

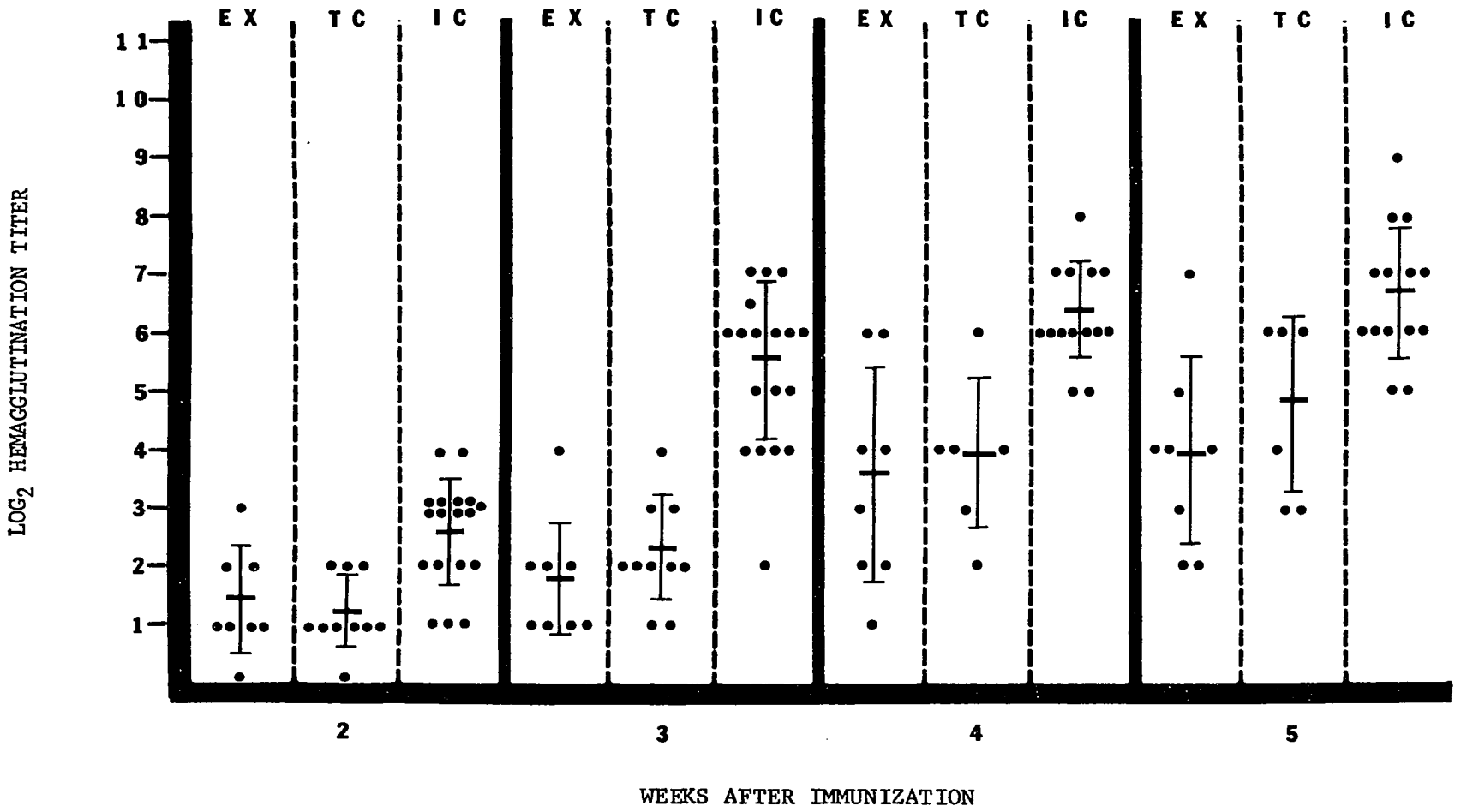

Figure 5. Effect of ethyl stearate-methyl palmitate treatment on tolerance induction in C57BL/6 mice by 0.5 mg of native (uncentrifuged) HGG.. EX - experimental or lipid-treated group; TC - tolerance controls; IC - immunization controls.

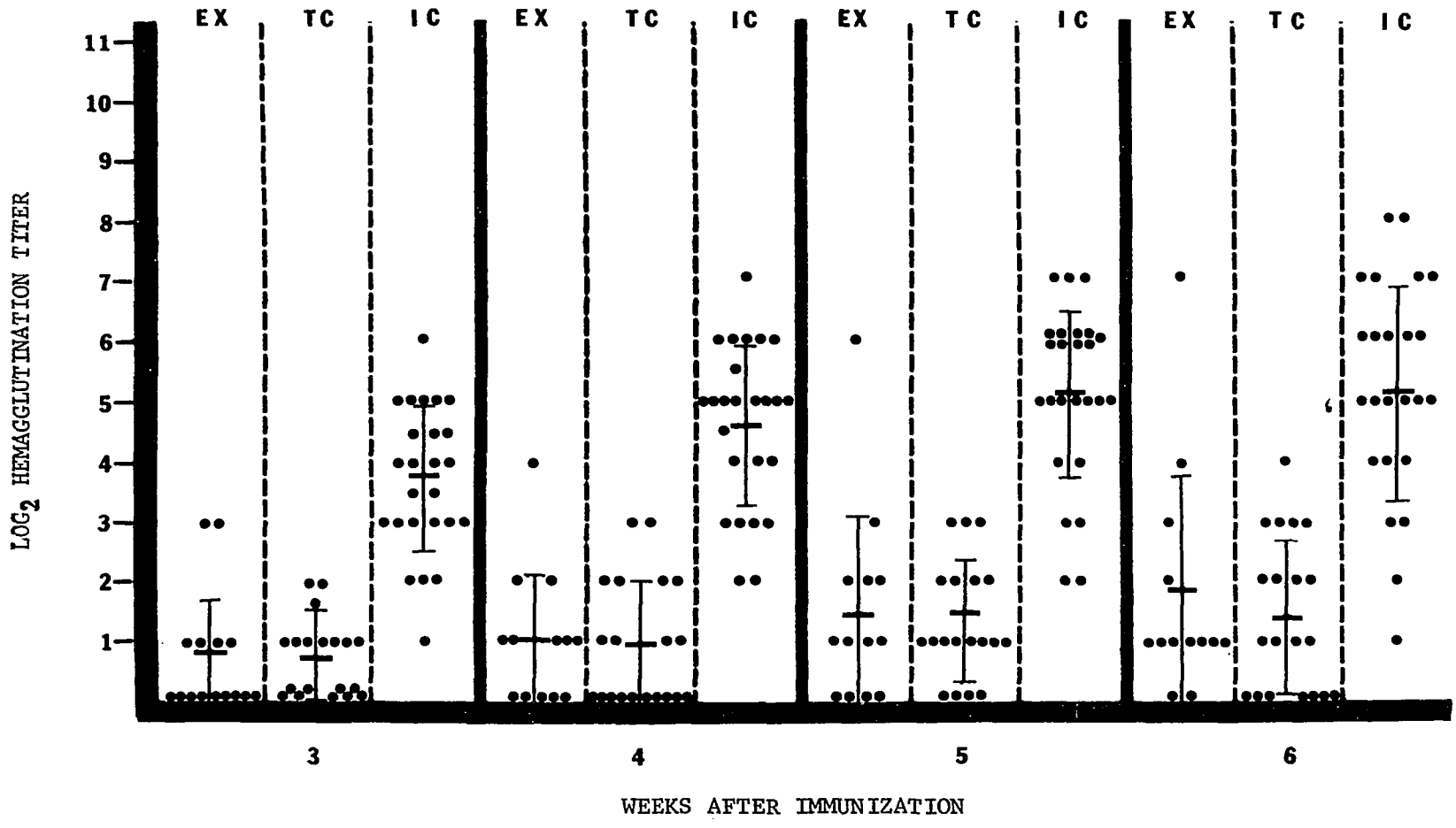

Figure 6. Effect of ethyl stearate-methyl palmitate treatment on tolerance induction in BALB/c mice by 1.0 mg of sHGG. EX - experimental or lipid-treated group; TC - tolerance controls; IC - immunization controls.

experiment.

High doses of sHGG. Six experiments were carried out using 5.0 mg sHGG for tolerance induction. They will be reported in the order in which they were performed. The results of the first experiment (Figure 7) were very similar to those obtained with 1.0 mg sHGG in that the titer means and ranges were remarkably alike for the experimental and tolerance control groups. Further, the mean titers of these 2 groups were not significantly different from those of the immunization controls.

The second experiment employing a 5.0 mg dose of sHGG gave remarkably different results (Figure 8). A significant proportion of the lipid-treated mice were tolerant or hyporesponsive, as compared to both control groups, throughout the experiment. Several of the tolerance control mice had titers somewhat lower than those of the immunization control animals at 2 and 3 weeks post-challenge. By 4 weeks the tolerance control titers approached those of the immunization controls and by 5 weeks the titers of both control groups were identical in range.

In the third experiment, using the same dose of sHGG, the lipid-treated animals again appeared to be immunologically depressed, when compared to the immunization controls, throughout the 5 week post-challenge period (Figure 9). However, the tolerance controls varied greatly in their response with a significant number also displaying hyporesponsiveness and only a few having titers within the range of the immunization control group at the 3, 4 and 5 weeks post-challenge periods.

The results of the fourth experiment (Figure 10) resemble somewhat those of the first experiment (Figure 7) in that no real difference between the lipid-treated and tolerance control groups was discernible.

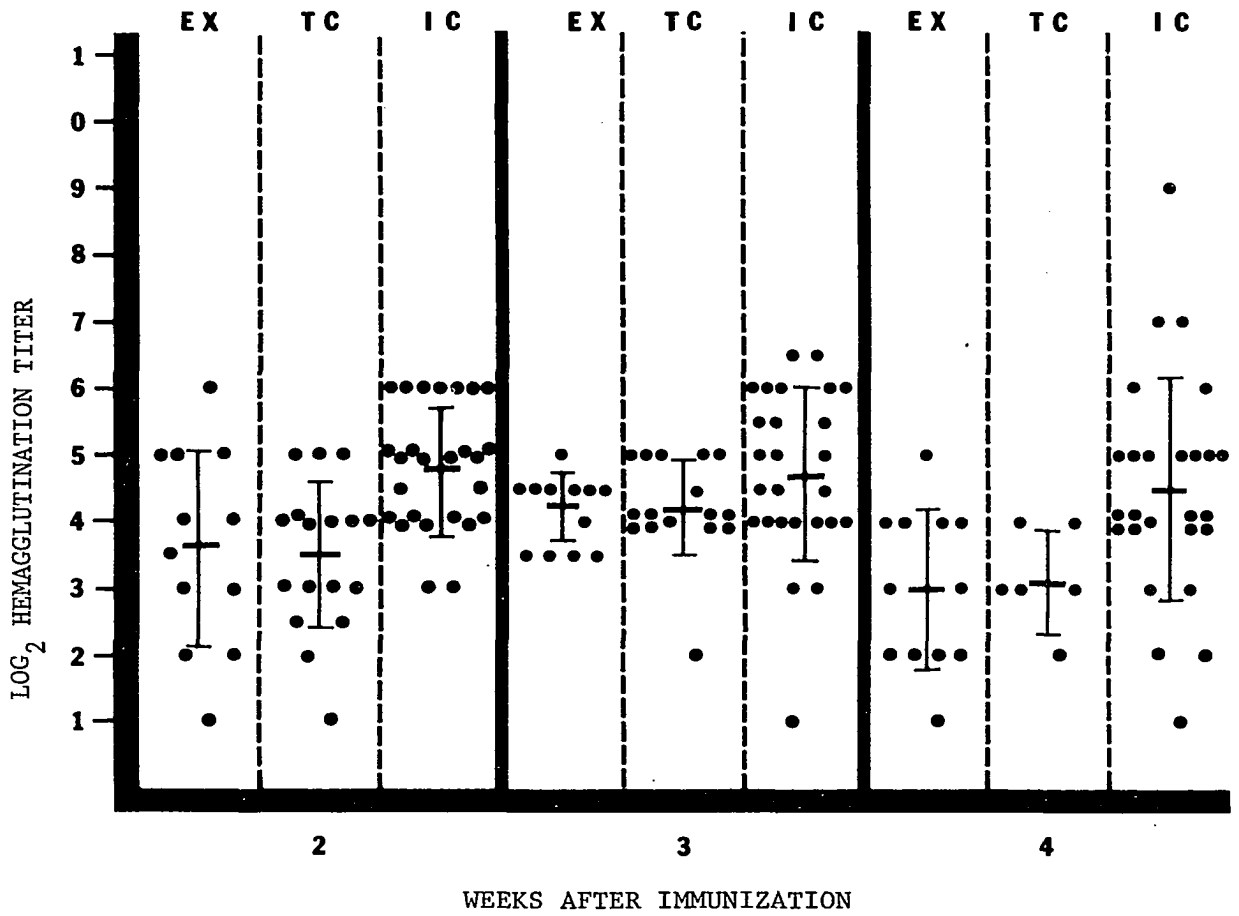

Figure 7. Effect of ethyl stearate-methyl palmitate treatment on tolerance induction in BALB/c mice by 5.0 mg sHGG. EX - experimental or lipid-treated group; TC - tolerance controls; IC - immunization controls.

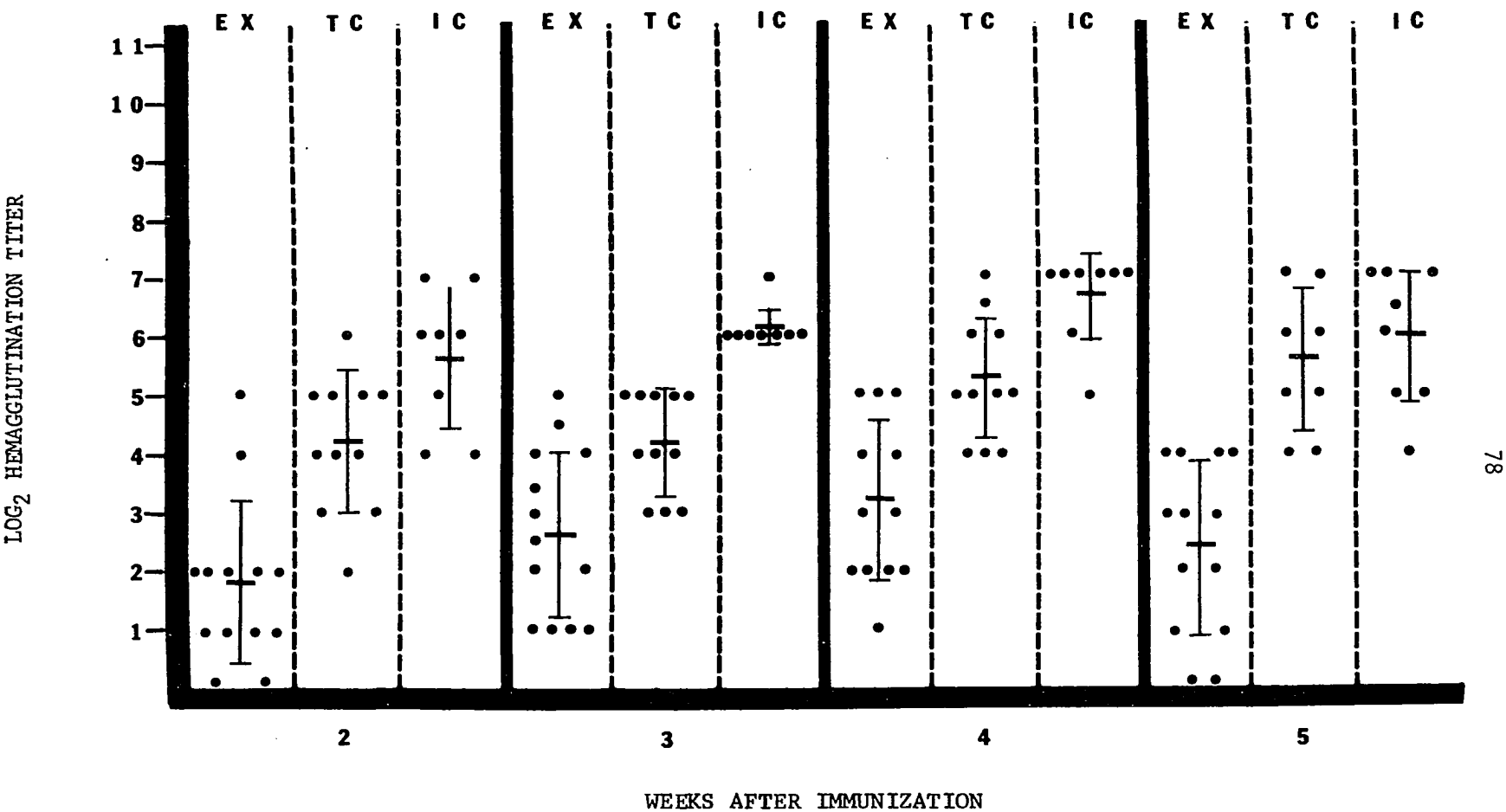

Figure 8. Effect of ethyl stearate-methyl palmitate treatment on tolerance induction in BALB/c mice by 5.0 mg of sHGG. EX - experimental or lipid-treated group; TC - tolerance controls; IC - immunization controls.

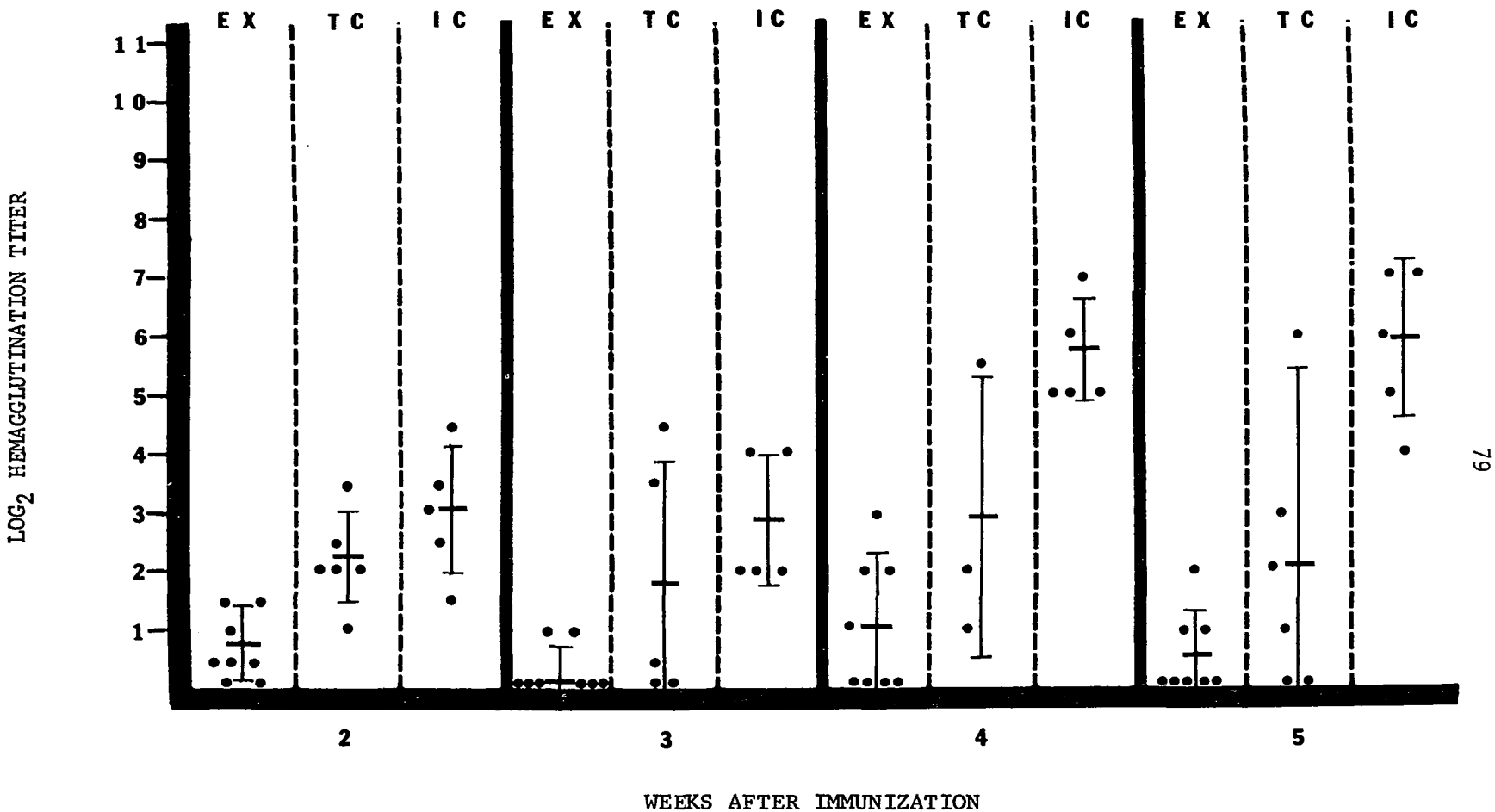

Figure 9. Effect of ethyl stearate-methyl palmitate treatment on tolerance induction in BALB/c mice by 5.0 mg of sHGG. EX - experimental or lipid-treated group; TC - tolerance controls; IC - immunization controls.

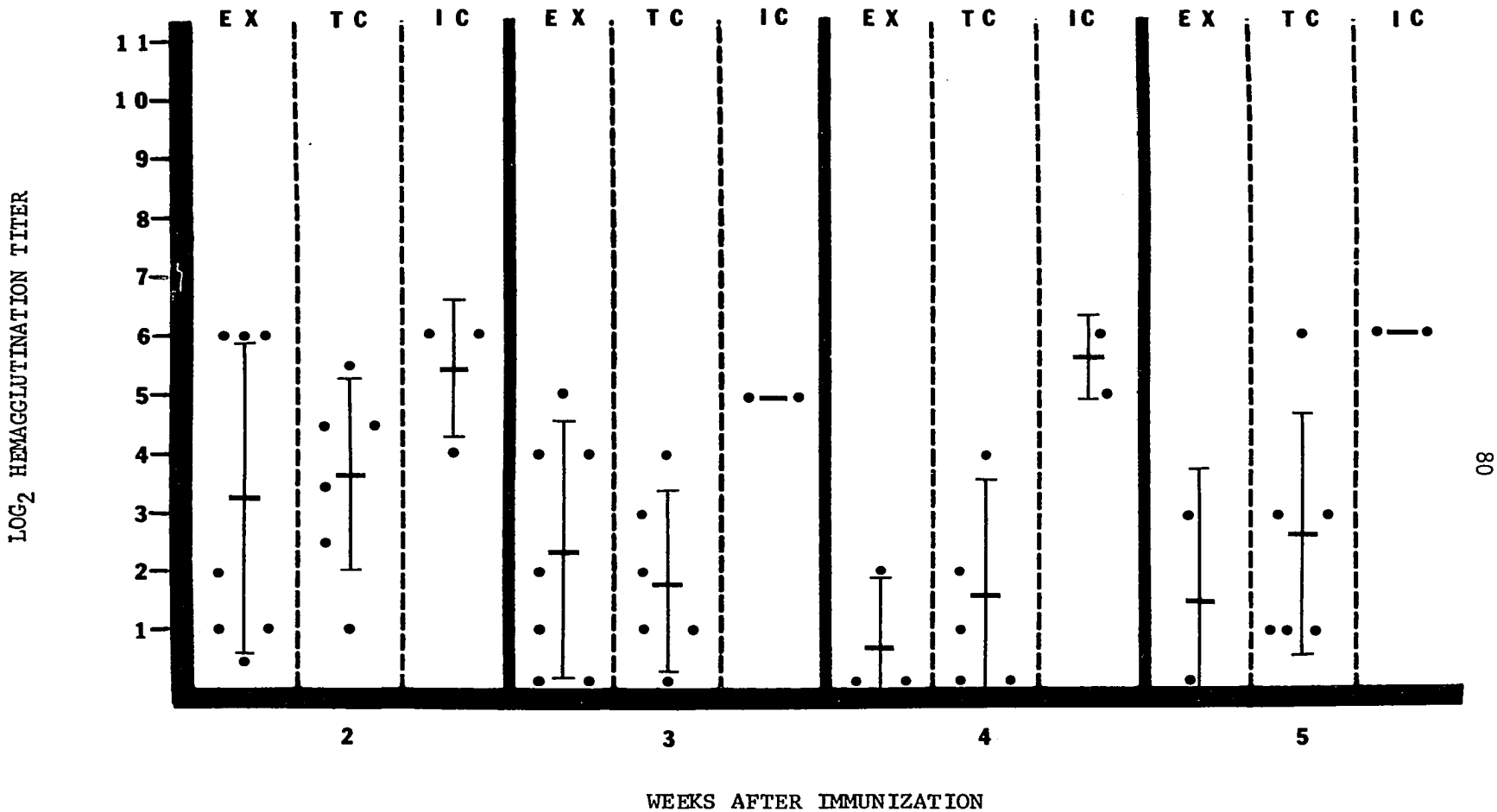

Figure 10. Effect of ethyl stearate-methyl palmitate treatment on tolerance induction in BALB/c mice by 5.0 mg of sHGG. EX - experimental or lipid-treated group; TC - tolerance controls; IC - immunization controls.

Approximately half of the animals in each of these groups were hyporesponsive up to 4 weeks post-challenge. The wide variation in the response observed frequently in tolerance control animals was again evident throughout this experiment. A similar variation in response was seen among the experimental animals at the 2 and 3 week periods. This trend would probably have extended over the 4 and 5 weeks period but this is not evident from an examination of Figure 10 due to the reduced number of experimental animals.

The lipid-treated animals in the fifth experiment again displayed a wide variation in response and only 2 or 3 were hyporesponsive (Figure 11). This hyporesponsiveness did, however, persist throughout the experiment. Some variation in response was also noted in the tolerance control group, although this was not as great as that seen in some of the previous experiments. Two tolerance control mice which appeared to be hyporesponsive at 2 weeks post-challenge did not maintain this state upon subsequent testing. The majority of the tolerance and immunization control animals had titers of the same order of magnitude at 2 and 3 weeks post-challenge. At 4 and 5 weeks post-challenge the mean titers of the immunization controls increased approximately 2 $\log_2$ units, whereas there was no significant change in the titers of most of the tolerance control animals.

The results of the sixth experiment, presented in Figure 12, are quite similar to those obtained in the second experiment (Figure 8) and demonstrate that lipid treatment can, in some cases, enhance the induction of tolerance in BALB/c mice. Examination of Figure 12 reveals that the majority of the lipid-treated mice had titers significantly

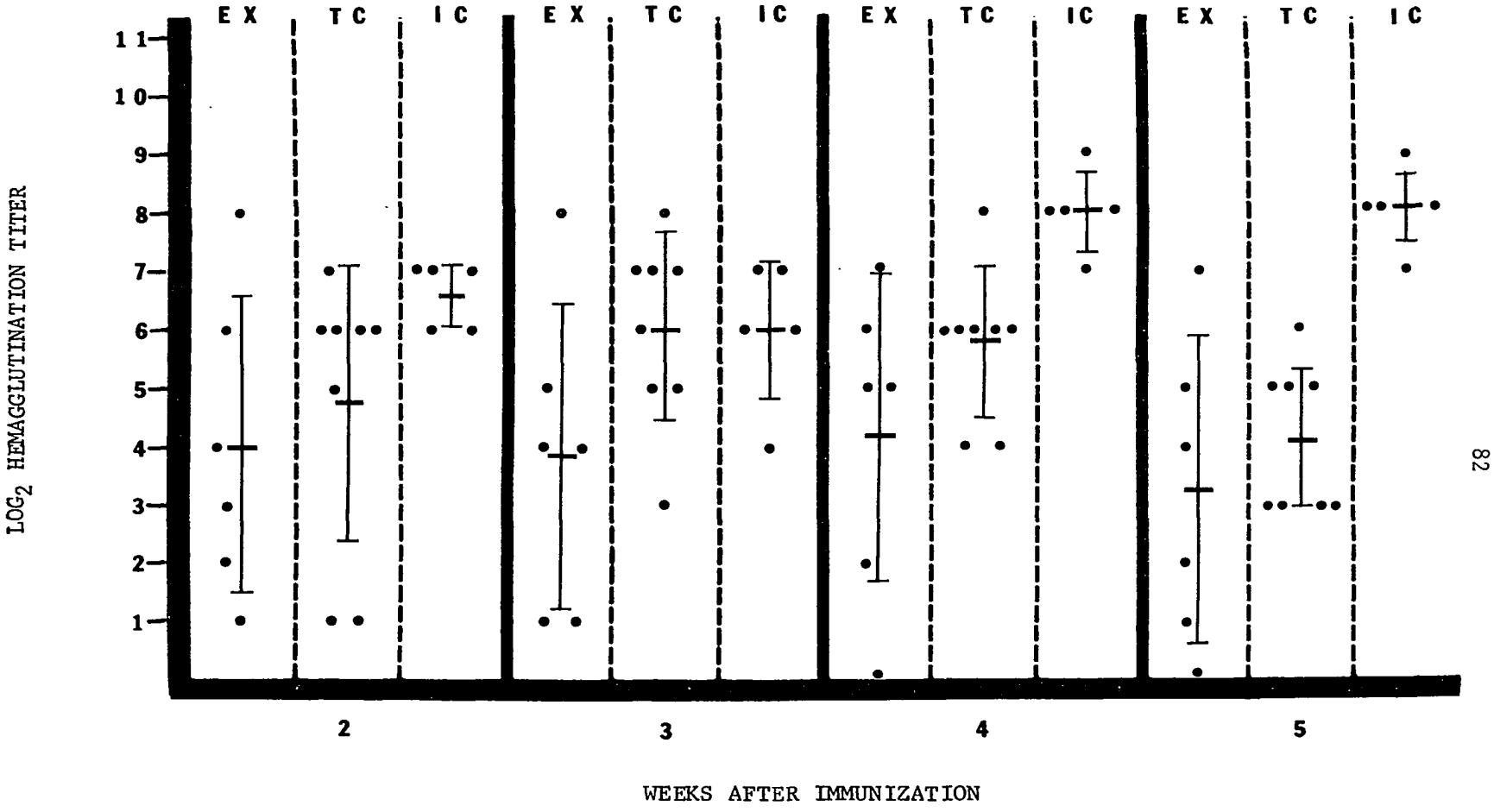

Figure 11. Effect of ethyl stearate-methyl palmitate treatment on tolerance induction in BALB/c mice by 5.0 mg of sHGG. EX - experimental or lipid-treated group; TC - tolerance controls; IC - immunization controls.

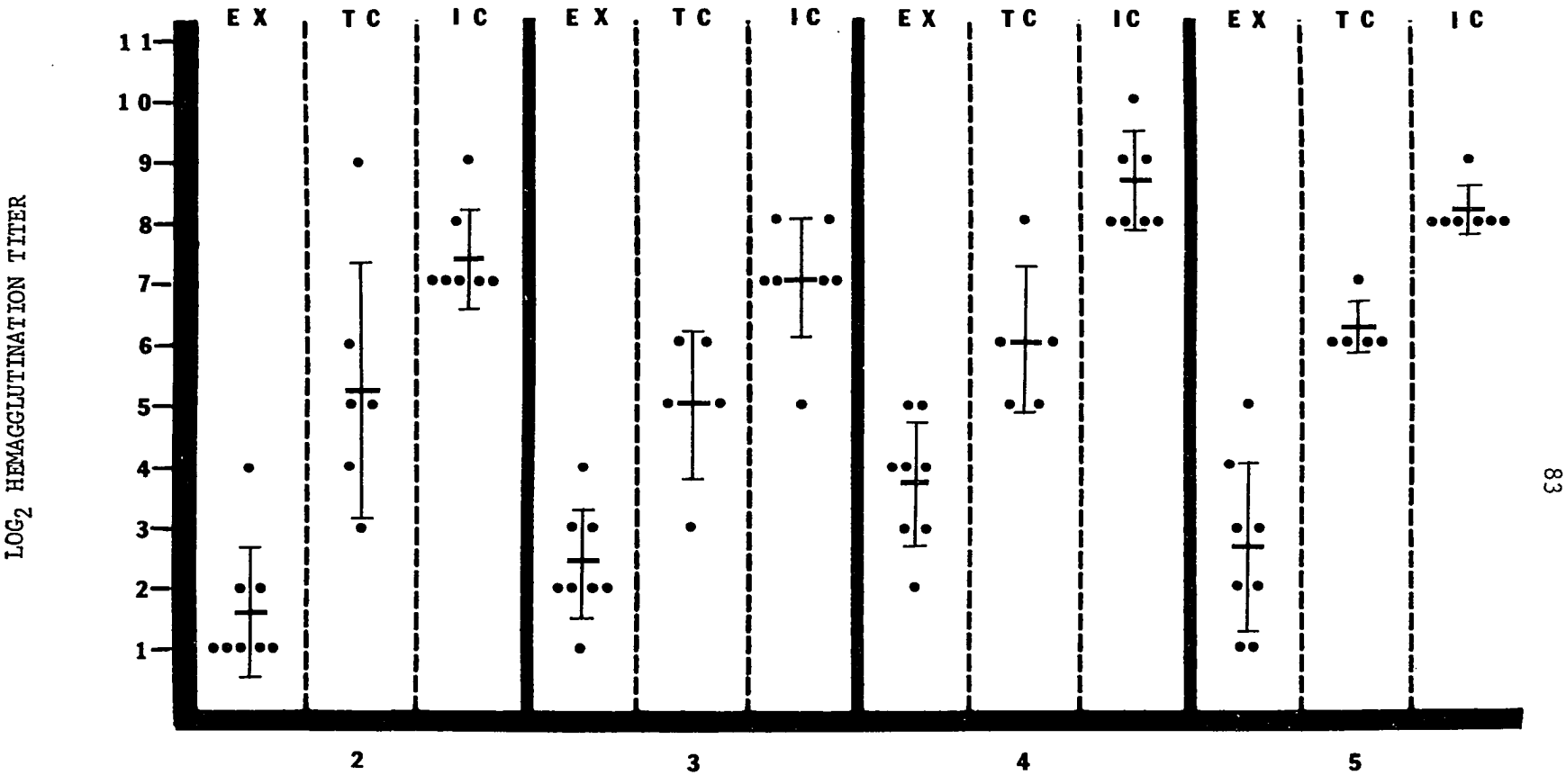

Figure 12. Effect of ethyl stearate-methyl palmitate treatment on tolerance induction in BALB/c mice by 5.0 mg of sHGG. EX - experimental or lipid-treated group; TC - tolerance controls; IC - immunization controls.

lower than those of either control group throughout the experiment. The titers of some of the experimental mice increased slightly over the course of the experiment but they seldom exceeded the lowest titers of the tolerance control animals. In general, the titers of the majority of the tolerance control mice were intermediate between those of the experimental and immunization control animals. The results of this series of experiments, employing 5.0 mg doses of sHGG, have been compiled in Table 17.

The experimental animals in the previous studies were injected with an emulsion containing both ethyl stearate and methyl palmitate. It was deemed of interest to determine if either of these lipids, given separately, would enhance tolerance induction to sHGG to a greater extent than did the two lipids given in combination. For this purpose, mice were divided into 5 groups. One group received ethyl stearate, another received methyl palmitate and a third group received both lipids. The remaining 2 groups were used as tolerance and immunization controls. The experiment was performed as described previously and all experimental and tolerance control animals received 5.0 mg of the same preparation of sHGG. The response of these animals at 2 and 5 weeks after immunization is shown in Figure 13. Once again, a wide variation was seen in the titers of all 3 groups of experimental animals. Although a few animals in each treatment group appeared to be hyporesponsive at 2 weeks, this state rarely persisted. The mean titer of the ethyl stearate-treated mice was somewhat lower than those of the other two experimental groups. At 5 weeks, the methyl palmitate-treated mice had a mean titer intermediate between the ethyl stearate and the ethyl stearate-methyl

TABLE 17

THE EFFECT OF ETHYL STEARATE-METHYL PALMITATE TREATMENT ON THE INDUCTION OF TOLERANCE IN BALB/c MICE BY 5.0 mg OF sHGG: A COMPILATION OF SIX EXPERIMENTS

Weeks After Immunization	Group[a]	Number of Animals	Percentage of Mice in Each Group Having Log_2 Hemagglutination Titers of:				Mean Log_2 Titer
			0-2	3-5	6-8	≥ 9	
2	EX	60	60	27	13		2.5
	TC	70	21	54	23	2	4.2
	IC	61	3	43	52	2	5.4
3	EX	64	41	59			2.8
	TC	67	13	74	13		4.1
	IC	57	7	44	49		5.2
4	EX	60	38	55	7		3.2
	TC	63	10	57	33		4.7
	IC	58	5	41	40	14	6.0
5	EX	48	46	33	21		3.1
	TC	48	17	42	39	2	4.5
	IC	33		15	70	15	7.2

[a]EX - experimental or lipid-treated group; TC - tolerance controls; IC - immunization controls.

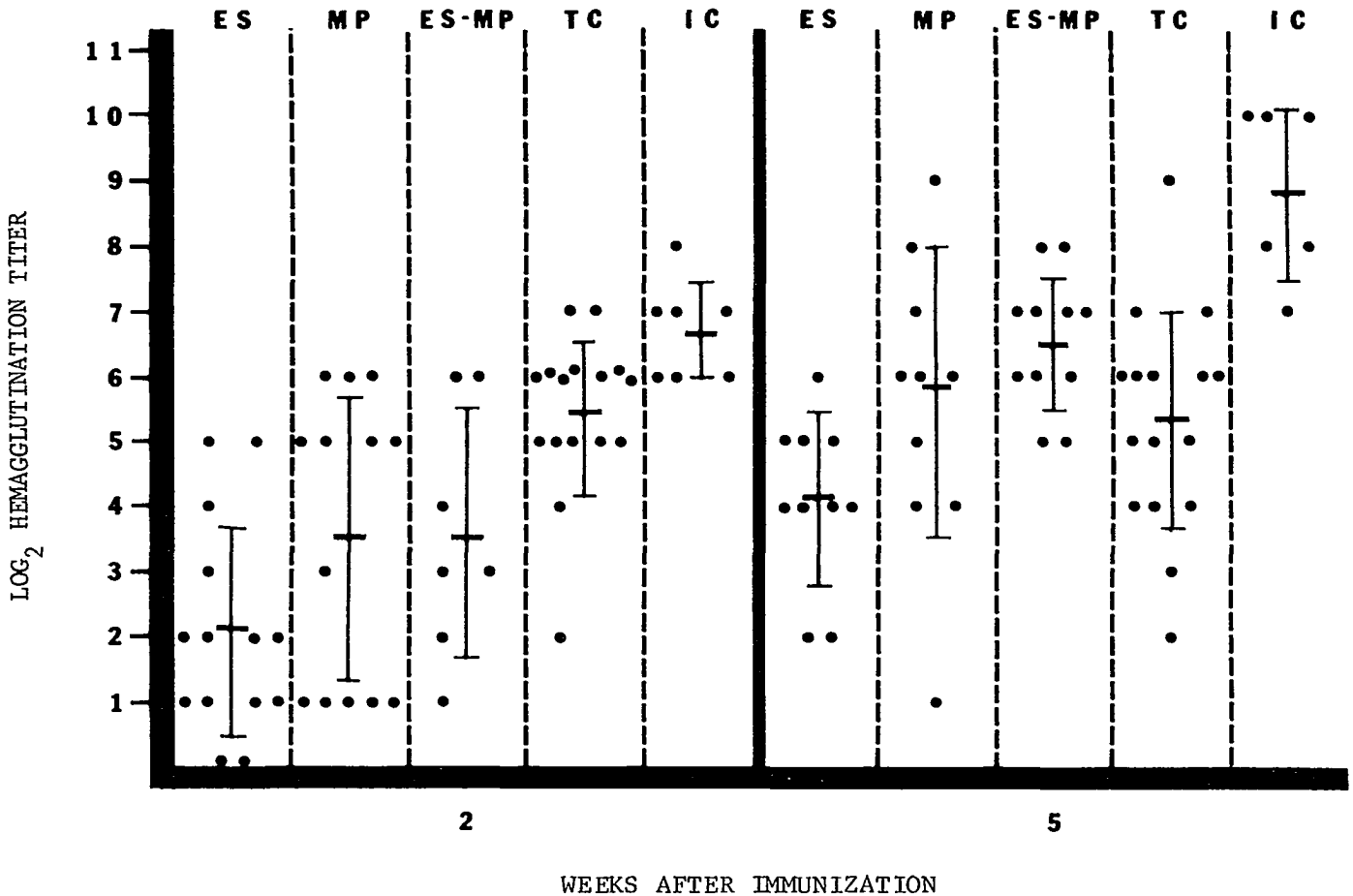

Figure 13. Effect of ethyl stearate and methyl palmitate, given separately and in combination, on tolerance induction in BALB/c mice by 5.0 mg of sHGG. ES - ethyl stearate-treated mice; MP - methyl palmitate treated mice; ES-MP - mice treated with both lipids; TC - tolerance controls; IC - immunization controls.

palmitate-treated groups. The tolerance control animals had generally higher titers at 2 weeks post-challenge than had been seen in previous experiments. Because of the wide range in titers in each of the experimental groups and because the limits of ranges were similar for each group, it is impossible to make a definite statement with regard to the relative effectiveness of the 3 lipid preparations in promoting hyporesponsiveness. The data do suggest, however, that the proportion of antigen in monomeric form in the sHGG preparation used in this experiment was less than that in preparations used in the preceding experiments. This is evidenced by the fact that the titers of mice treated with the combination of lipids were generally higher in this experiment than in the preceding ones, particularly at 5 weeks post-immunization.

The influence of ethyl stearate-methyl palmitate treatment on the induction of tolerance to 10.0 mg of sHGG was investigated in 4 separate experiments. The results of the first experiment of this series are presented in Figure 14. The majority of the lipid-treated animals and the tolerance controls were hyporesponsive throughout the experiment. There was no significant difference in the degree of hyporesponsiveness as evidenced by the similarity in the mean hemagglutination titers of the 2 groups. However, 2 of the tolerance control mice were not hyporesponsive but, rather, responded like the immunization controls. The immunization control animals, in general, had higher titers than the other 2 groups. This was particularly evident at 4 and 5 weeks post-immunization.

The results of the second experiment in this series were surprising and unexpected (Figure 15). At each week following immunization,

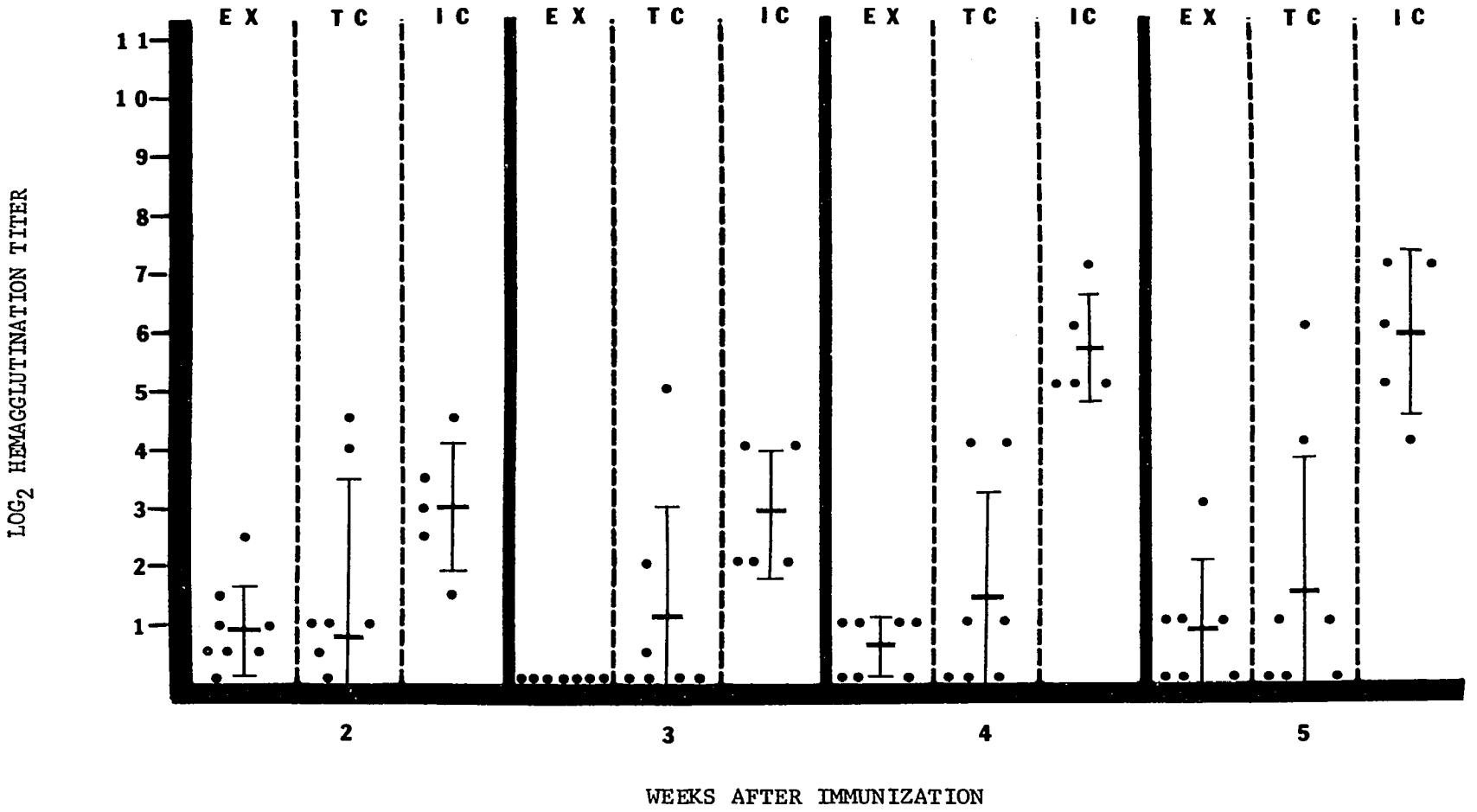

Figure 14. Effect of ethyl stearate-methyl palmitate treatment on tolerance induction in BALB/c mice by 10.0 mg of sHGG. EX - experimental or lipid-treated group; TC - tolerance controls; IC - immunization controls.

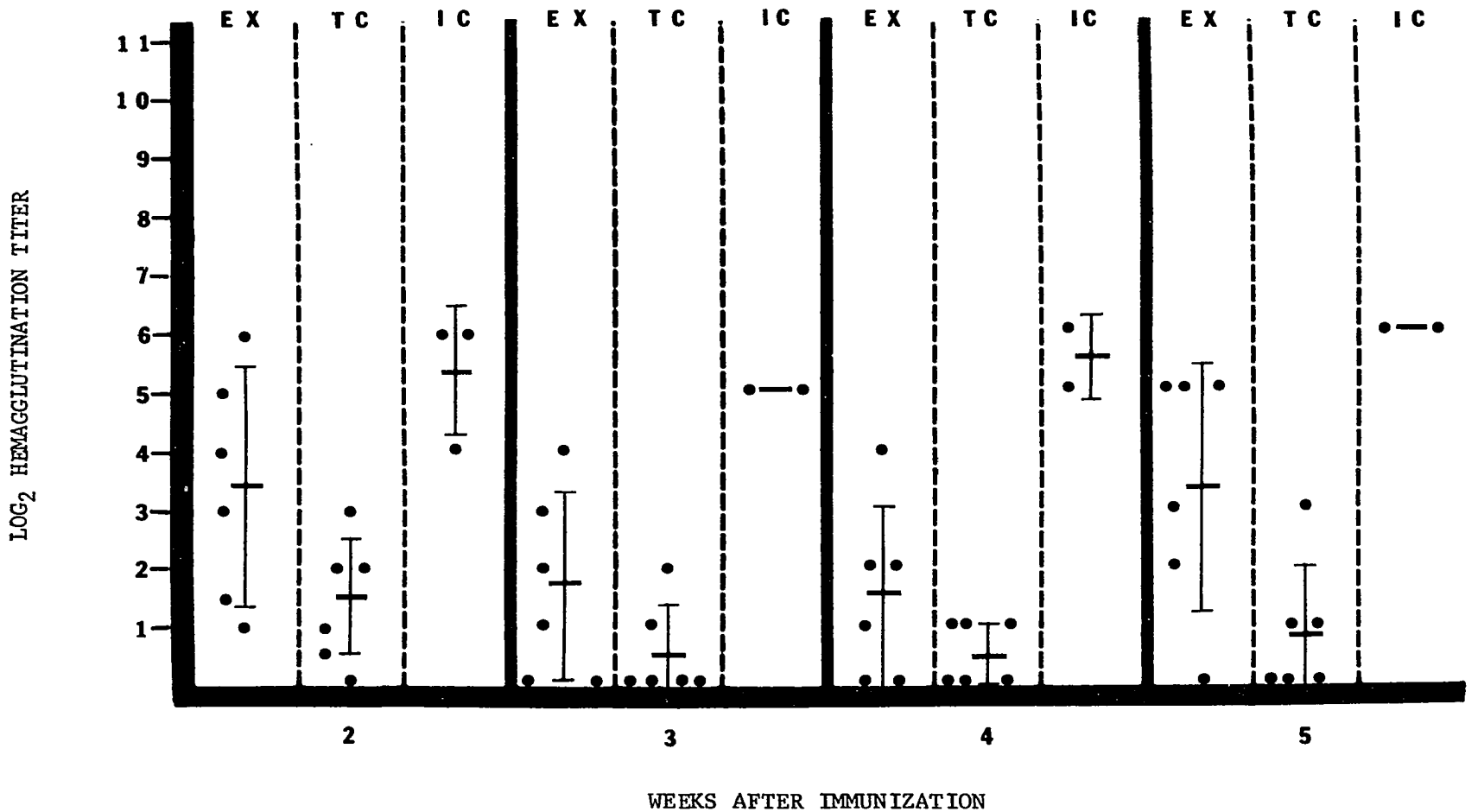

Figure 15. Effect of ethyl stearate-methyl palmitate treatment on tolerance induction in BALB/c mice by 10.0 mg of sHGG. EX - experimental or lipid-treated group; TC - tolerance controls; IC - immunization controls.

the tolerance control group had a larger proportion of hyporesponsive animals than did the lipid-treated group. The possibility that these 2 groups were inadvertently mixed cannot be excluded. The hyporesponders in each group maintained this state throughout the duration of the experiment. The experimental animals, as noted in previous experiments, exhibited a wide range of titers.

A wide range of titers was also seen in the lipid-treated animals in the third experiment in which a 10.0 mg dose of sHGG was employed (Figure 16). Only 1, or possibly 2, of these mice were hyporesponsive. None of the tolerance control animals was hyporesponsive. Titers of the experimental and tolerance control mice tended to decrease somewhat from the second to the fifth week after immunization. This probably is a reflection of the variation in the sensitivity of hemagglutination titrations performed on different days rather than a real decrease in the titers of individual animals. The titrations may have been somewhat more sensitive than usual at 2 weeks and less sensitive at 5 weeks.

The results of the final experiment in this series are shown in Figure 17. All but 1 or 2 of the lipid-treated animals were significantly hyporesponsive throughout the experiment. The titers of the tolerance control group varied over a range of 5 $\log_2$ units, however, the titers of most of these animals were intermediate between the experimental and the immunization control mice. Only one of the tolerance controls appeared to be as hyporesponsive as the lipid-treated mice.

Table 18 contains a summary of the results of all of the experiments utilizing a 10.0 mg dose of sHGG for tolerance induction.

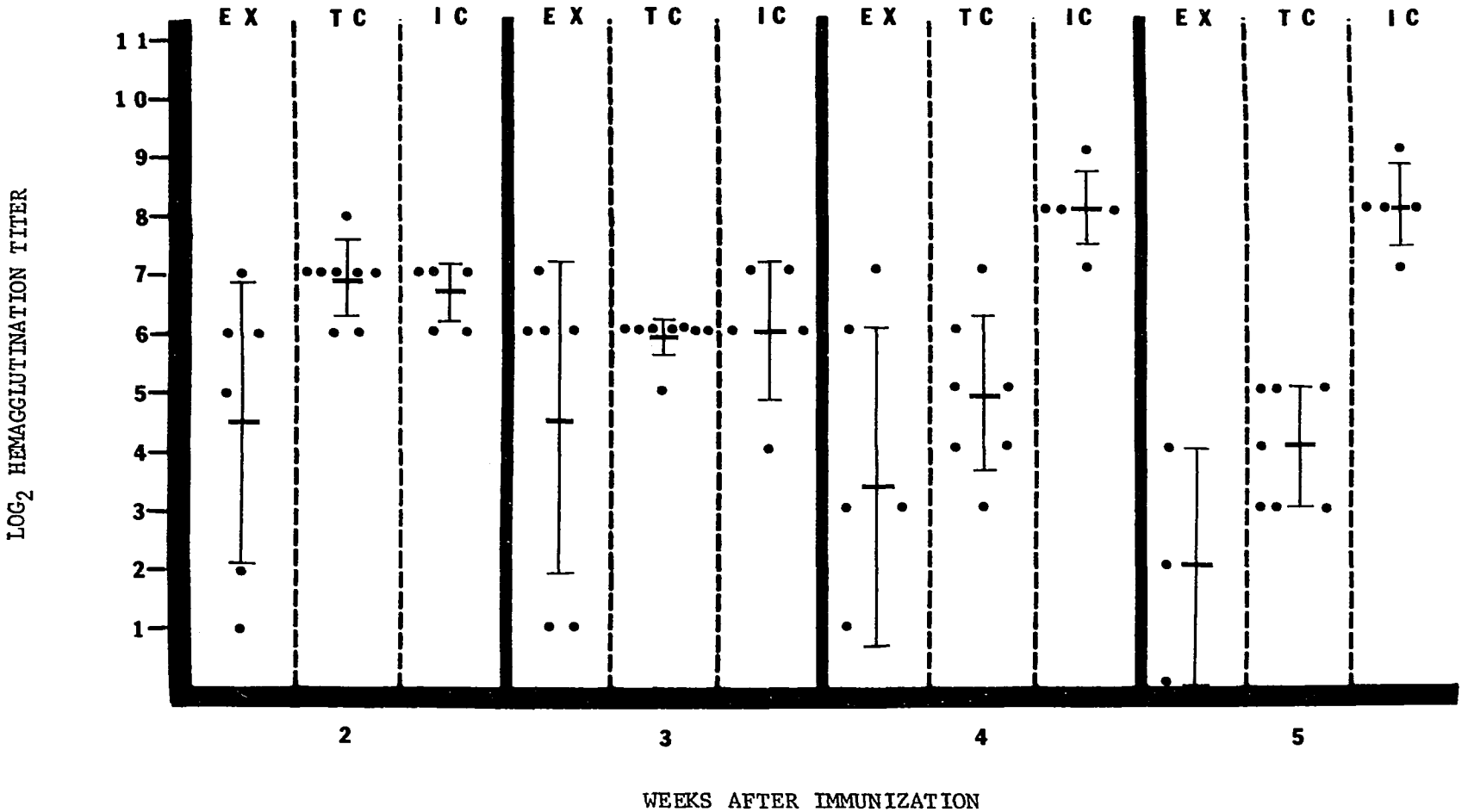

Figure 16. Effect of ethyl stearate-methyl palmitate treatment on tolerance induction in BALB/c mice by 10.0 mg of sHGG. EX - experimental or lipid-treated group; TC - tolerance controls; IC - immunization controls.

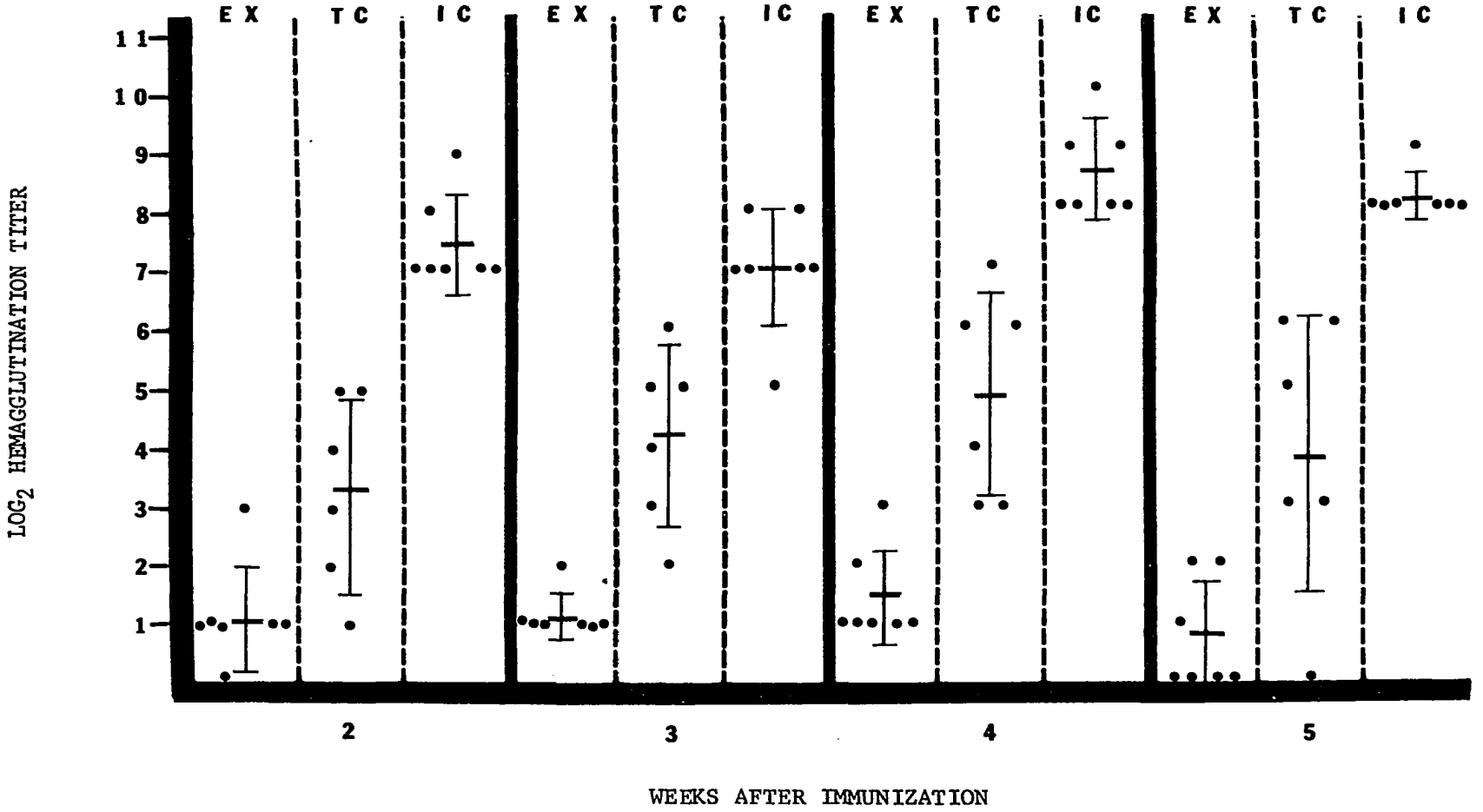

Figure 17. Effect of ethyl stearate-methyl palmitate treatment on tolerance induction in BALB/c mice by 10.0 mg of sHGG. EX - experimental or lipid-treated group; TC - tolerance controls; IC - immunization controls.

TABLE 18

THE EFFECT OF ETHYL STEARATE-METHYL PALMITATE TREATMENT ON THE INDUCTION OF TOLERANCE IN BALB/c MICE BY 10.0 mg OF sHGG: A COMPILATION OF FOUR EXPERIMENTS

Weeks After Immunization	Group[a]	Number of Animals	Percentage of Mice in Each Group Having Log_2 Hemagglutination Titers of:				Mean Log_2 Titer
			0-2	3-5	6-8	≥ 9	
2	EX	27	67	18	15		2.2
	TC	26	50	25	25		3.5
	IC	20	10	20	65	5	3.7
3	EX	26	77	8	15		1.7
	TC	27	45	24	31		3.0
	IC	19	15	32	53		5.4
4	EX	26	77	15	8		1.6
	TC	26	41	33	26		3.0
	IC	19		21	58	21	7.3
5	EX	23	74	26			1.6
	TC	26	42	50	8		2.8
	IC	19		10	80	10	6.7

[a]EX - experimental or lipid-treated group; TC - tolerance controls; IC - immunization controls.

Hemagglutination-Enhancement Effect of BSA

The hemagglutination titers of the immunization control animals in tolerance induction experiments conducted early in this study were considerably lower than had been expected. The titrations were repeated substituting BSA for the PBSG diluent to detect the possible presence of an incomplete antibody. Without exception, the BSA titer of each animal was markedly higher than its PBSG counterpart. Therefore, in all subsequent tolerance induction experiments both the PBSG and BSA hemagglutination titers were determined for each animal. The results of a comparison of mean PBSG and BSA titers for 196 BALB/c mice used in the 5.0 mg dose tolerance induction experiments are presented in Figure 18. It is obvious that the mean BSA titers were significantly higher than the mean PBSG titers in both the experimental and the two control groups at each week tested. The difference in the two titers was greatest in the experimental mice and least in the immunization control animals. Further, it is evident that the mean BSA titers varied no more than 1.5 $\log_2$ units among the 3 test groups, whereas, a significant variation was noted in the corresponding PBSG titers. In fact, the sera of several mice which had high hemagglutination titers in the BSA assay were completely negative when titrated in the PBSG diluent. For lack of a more precise term at present, the substance responsible for the BSA-enhancement effect will subsequently be referred to as "BSA-detectable antibody" (BSA-DAB).

Several attempts were made to characterize BSA-DAB. To study its heat stability, the serum from each animal in the three test groups was pooled. The serum pools were divided in half and one-half was heated

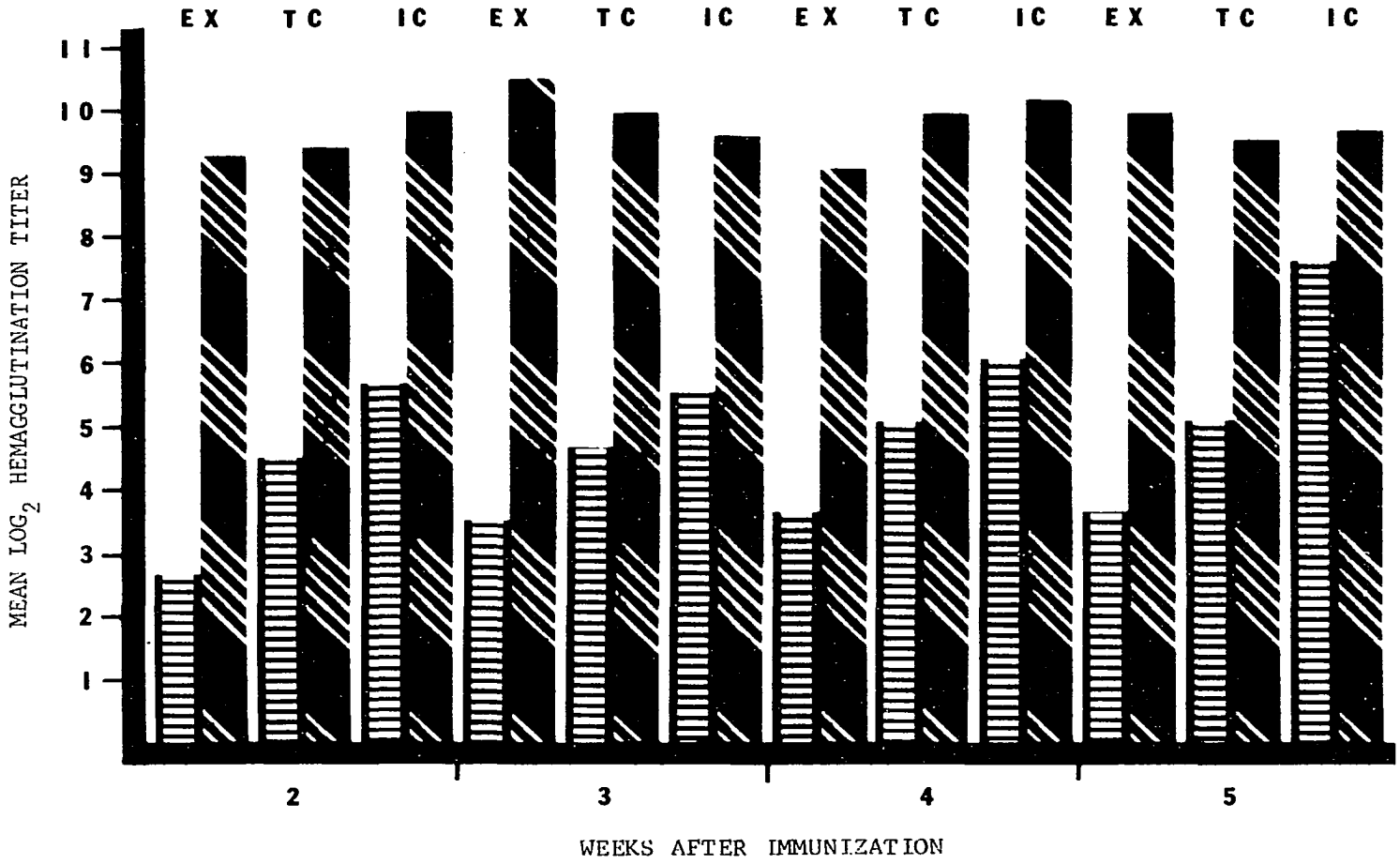

Figure 18. Effect of BSA on mean hemagglutination titers of 196 BALB/c mice. Horizontal bars - mean titer in PBSG; slanted bars - mean titer in BSA. EX - experimental or lipid-treated group; TC - tolerance controls; IC - immunization controls.

in a water bath at 56C for 30 minutes while the other half of each pool was refrigerated (4C). The BSA and PBSG titers of each serum pool, heated and unheated, were determined. The results of three such experiments are presented in Table 19. Heating did not significantly alter the PBSG titer. BSA titers, however, were drastically reduced in the heated serum pools, the titer loss ranging from 2 to 8 $\log_2$ units. In all cases, the BSA titer of the heated pooled sera equaled or approximated the PBSG titer of the corresponding unheated serum pool.

To test the affect of prior absorption of mouse serum with HGG on hemagglutination titers, individual sera from the 3 test groups were pooled and a 0.2 ml aliquot from each serum pool was added to equal volumes of both HGG (0.05 mg/0.2 ml) and saline. The absorption mixtures were incubated at room temperature for 2 hours and then refrigerated overnight. The tubes were then centrifuged at 1,500 rpm for 5 minutes. A precipitate was visible in those tubes containing HGG. The supernatant was removed from each tube and titrated in both PBSG and BSA. From the results presented in Table 20 it can be seen that both BSA-DAB and the PBSG anti-HGG can be completely removed from mouse sera by absorption with 0.05 mg of HGG.

Similar observations were made when hemagglutination assays were performed in the presence of excess HGG. A 20 mg/ml solution of HGG was diluted 1:10 and 1:100 in PBSG or in BSA to give final concentrations of 2 mg/ml (0.05 mg/well) and 0.2 mg/ml (0.005 mg/well), respectively. Both the PBSG and BSA titers of all 3 test groups were completely inhibited by a 2 mg/ml concentration of HGG (Table 21). The BSA and PBSG titers of the experimental group were also abolished by the

TABLE 19

EFFECT OF TEMPERATURE ON HEMAGGLUTINATION TITER IN PBSG OR BSA DILUENTS

Experiment Number	Serum Pool[a]	Log_2 Hemagglutination Titers					
		Mean Titer of Individuals Within Pool		Pooled Sera			
				PBSG		BSA	
		PBSG	BSA	Unheated	Heated[b]	Unheated	Heated
1	EX	2.9	10.6	3.0	2.0	9.0	3.0
	TC	5.3	10.2	4.0	4.0	9.0	5.0
	IC	6.6	10.2	5.0	5.0	10.0	6.0
2	EX	3.1	10.4	3.0	2.0	9.0	3.0
	TC	5.3	10.0	4.0	3.0	9.0	5.0
	IC	8.3	12.3	6.5	6.0	10.5	8.0
3	EX	2.1	8.3	4.0	2.0	11.0	3.0
	TC	4.4	8.5	6.0	4.0	11.0	6.0
	IC	8.1	10.5	8.0	8.0	12.0	10.0

[a]EX - experimental group; TC - tolerance control group; IC - immunization control group.

[b]Heated at 56C for 30 minutes.

TABLE 20

EFFECT OF ABSORPTION WITH HGG ON HEMAGGLUTINATION TITER IN PBSG OR BSA DILUENTS

Serum Pool[a]	Absorption Material	Log_2 Hemagglutination Titer	
		PBSG	BSA
EX	Saline	1	5
EX	HGG	0	0
TC	Saline	2	6
TC	HGG	0	0
IC	Saline	4	7
IC	HGG	0	0
Control[b]	Saline	4	4
Control	HGG	0	0

[a]EX - experimental group; TC - tolerance control group; IC - immunization control group.

[b]Commercial rabbit anti-HGG.

TABLE 21

HEMAGGLUTINATION INHIBITION EFFECT OF EXCESS HGG ON TITRATIONS IN PBSG OR BSA DILUENTS

Diluent	HGG Concentration (mg/ml)	Log_2 Hemagglutination Titer			
		EX[a]	TC[b]	IC[c]	Control[d]
PBSG	0	2	3	5	5
	0.2	1	2	2	0
	2.0	1	1	1	0
BSA	0	5	7	8	5
	0.2	1	2	2	0
	2.0	1	1	1	0

[a]Pooled serum of experimental animals.

[b]Pooled serum of tolerance control animals.

[c]Pooled serum of immunization control animals.

[d]Commercial rabbit anti-HGG.

0.2 mg/ml concentration of HGG and both titers of the tolerance and immunization control groups were severely reduced. Titers of one $\log_2$ unit were not considered significant, since the unsensitized cell controls had similar titers.

It was of interest to know if BSA-DAB was specific for HGG. For this purpose, mouse gamma globulin (MGG) was conjugated to SRBC employing the same procedure as that with HGG. A concentration of 2 mg MGG/ml was used since this has been found to give the highest hemagglutination titers (Kramer, 1970). Titrations were performed as described previously, using both PBSG and BSA as diluents. The results, presented in Table 22, demonstrated that BSA-DAB reacts with both HGG and MGG. In fact, higher titers were observed with MGG than with HGG. The difference in titers ranged from 1-3 $\log_2$ units. The anti-HGG antibody detected with the PBSG diluent did not cross react with MGG. The $\log_2$ anti-MGG titers of one for the experimental and tolerance control groups were not considered to be significant as the corresponding unsensitized cell controls also had titers of one, indicative of a non-specific reaction with the SRBC. Further evidence for the cross reactive nature of BSA-DAB was the observation that the reaction with MGG could be inhibited when the titration was carried out in the presence of HGG (Table 22).

During the course of these investigations it was noticed that the BSA titer of many sera decreased as the duration of storage increased and also decreased after multiple freeze-thawing. Upon completion of the final tolerance induction experiment the remaining individual sera were frozen and stored for varying periods of time. In addition, these sera

TABLE 22

CROSS REACTIVITY BETWEEN MGG AND ANTI-HGG PRODUCED IN BALB/c MICE

Diluent	Log_2 Hemagglutination Titer Specific for:							
	MGG				HGG			
	EX[a]	TC[b]	IC[c]	Control[d]	EX	TC	IC	Control[e]
PBSG	1	1	-	9	2	3	5	5
BSA	10	11	9	10	5	7	8	5
BSA + HGG (2 mg/ml)	0	1	-	8	-	-	-	-

[a]Experimental group pooled sera.

[b]Tolerance control group pooled sera.

[c]Immunization control group pooled sera.

[d]Rabbit anti-MGG serum.

[e]Rabbit anti-HGG serum.

were subjected to a varying number of freeze-thaw cycles. Titrations were performed in PBSG and BSA after each of these test procedures. Examination of Table 23 reveals that PBSG titers were not affected by prolonged storage or multiple freeze-thawing of serum. The BSA titers of the immunization control animals were similarly unaffected by these procedures. However, BSA titers of many of the experimental and tolerance control animals were markedly reduced. Multiple freeze-thawing appeared to have a more profound effect on the tolerance control mice than did prolonged storage since after 40 days of storage none of the 4 sera tested had decreased in titer by 3 or more $\log_2$ hemagglutination units. Both prolonged storage and multiple freeze-thawing appeared to account for the decreased BSA titers in the experimental mice. These results are merely suggestive. Before any definite statement can be made a much larger number of sera would have to be tested.

TABLE 23

EFFECT OF MULTIPLE FREEZE-THAWING AND DURATION OF STORAGE AT -10C ON HEMAGGLUTINATION TITERS IN PBSG OR BSA DILUENTS

Number of Freeze-thaws	Number of Days in Storage	Diluent					
		PBSG			BSA		
		EX[a]	TC[b]	IC[c]	EX	TC	IC
1	7	0/2[d]	0/4	0/2	0/2	0/4	0/2
2	11	0/4	0/4	0/2	4/4	2/4	0/2
3	18	0/3	0/4	0/2	2/3	3/4	0/2
4	23	2/4	0/4	0/2	4/4	3/4	0/2
1	40	0/4	0/4	0/1	3/4	0/4	0/1

[a]Experimental or lipid-treated animals.

[b]Tolerance controls.

[c]Immunization controls.

[d]Number of mice with a titer decrease of 3 or more $\log_2$ hemagglutination units/total tested.

CHAPTER IV

DISCUSSION

The primary aim of this investigation was to determine whether RES depression would enhance the induction of tolerance to sHGG in BALB/c mice. Two lipids, ethyl stearate and methyl palmitate, were employed as RES depressants. The extent and duration of RES depression was determined by measuring the intravascular clearance rate of colloidal carbon. In these studies the mice were routinely anesthetized with sodium pentabarbital for ease in handling. It was found that anesthesia did not alter the clearance rates of normal animals (Table 2).

Ethyl stearate, obtained from two sources, was evaluated as to the dose required for maximum RES depression, the duration of the induced depression and the toxicity for mice. The two lipid preparations from both sources were nearly identical in their depressant activity. Doses ranging from 20 - 80 mg were equally effective in producing a significant depression of RES function. Higher doses caused an even more profound depression but were extremely toxic. Percent survival for 60 and 80 mg doses were 100 and 70 percent, respectively. Maximum RES depression occurred 24 hours following a 60 mg dose of ethyl stearate. Thereafter, the extent of the depression gradually lessened and normal RES function was regained within 4 to 7 days. These findings are in accord with those of Stuart, et al. (1960) and Stuart and Cooper (1962). Marked and

diffuse necrosis of splenic white pulp was observed within 24 hours after lipid administration. Regeneration was evident within 4 days. This supports the belief of Stuart and Cooper (1962) that ethyl stearate exerts its RES depressive effect through a cytotoxic mechanism. These investigators reported that the Kupffer cells were the primary target of ethyl stearate toxicity. However, in the present study no liver pathology was detected. This discrepancy could be due to the use of different doses and source of lipid by these workers. Probably of more importance, a different strain of mice was used in their investigation. Prosnitz, et al. (1969) have shown that animal species and even strains differ in their response to certain lipids.

Methyl palmitate preparations obtained from two suppliers were evaluated in the same manner as that used with ethyl stearate. The preparation obtained from Nutritional Biochemicals Corp., while effective in depressing RES activity, was toxic at doses as low as 20 mg. The methyl palmitate supplied by Applied Science Laboratories was much less toxic. Doses of this lipid ranging from 15 - 45 mg induced RES depression. The 15 mg dose only mildly impaired RES function, whereas the 25 mg and 35 mg doses caused marked depression. The most profound impairment was produced by a 45 mg dose. Mice treated with 45 mg of methyl palmitate were maximally depressed 24 hours later, as evidenced by their mean carbon half-time of 97.1 minutes which was in marked contrast to the 17.4 minute mean half-time of the controls. Normal RES function was regained within 4 to 10 days following lipid treatment. Two successive 25 mg doses of methyl palmitate, given 4 days apart, did not increase the duration of RES depression. These results do not agree

entirely with those obtained by other investigators using methyl palmitate from the same supplier (DiLuzio and Wooles, 1964; DiLuzio and Blickens, 1966). These investigators obtained maximum RES depression in C57BL/6 mice with a 35 mg dose of methyl palmitate. They observed marked impairment over a period of 5 hours to 7 days following lipid treatment with the most profound depression occurring at 48 hours. Normal function was regained in 12 to 17 days. It appears, therefore, that C57BL/6 mice respond to methyl palmitate treatment in a different manner than do BALB/c mice. This is further substantiated by the fact that these and other investigators (Ohbuchi, 1968) found no histological evidence of hepatic or splenic pathology associated with methyl palmitate treatment, whereas in the present study all mice examined exhibited marked splenic necrosis. In fact, the splenic pathology of the methyl palmitate-treated mice was almost identical to that seen in mice treated with ethyl stearate, the major difference being that regeneration began somewhat earlier following methyl palmitate treatment. This might explain, in part, the shorter duration of RES depression in BALB/c mice. There are other factors, however, which influence the response of mice to lipid treatment. For example, the particle size of the emulsified lipid determines its organ distribution. DiLuzio and Wooles (1964) prepared emulsions of methyl palmitate by mechanical agitation. Particle size ranged from 3 - 15 μ. This preparation was highly toxic since the larger particles were trapped in the lung resulting in the formation of pulmonary emboli, edema and finally death. When emulsions were prepared by sonication, the particle size ranged from 1 - 6 μ (DiLuzio and Blickens, 1966). This preparation was much less toxic and did not cause any increase in

lung weight. The methyl palmitate emulsion used in the present study was also prepared by sonication. Most particles were within the range of 3 - 8 μ although a small proportion were somewhat larger. This difference in particle size of the lipid emulsions could provide one explanation for the different responses observed in C57BL/6 and BALB/c mice.

To summarize, ethyl stearate and methyl palmitate are quite similar in their action on the RES. Both depress RES function maximally at 24 hours, although methyl palmitate exerts a more profound impairment at this time, and the duration of their effect is approximately the same. The unusually long mean carbon half-time seen at 7 days after ethyl stearate treatment (Table 6) resulted from the inclusion of one animal which was severely depressed (T/2 - 231.5 minutes). Half-times of the other mice ranged from 13.5 to 91.2 minutes. It should be noted that in the summarizing procedures required in statistical analyses, interest becomes focused upon population characteristics, such as mean values, rather than upon the behavior of individual animals. The peculiar response of specific animals, such as those with extremely long half-times (T/2 - ∞) which were excluded from the mean comparisons, may be of particular interest since it may indicate a subpopulation which is extremely sensitive to lipid-induced RES depression. Occasionally an animal was observed to have a half-time less than infinity but still markedly prolonged as compared with those of other mice in the same group. These animals were included in the statistical analysis since their number was not sufficient to allow rejection of the normality assumption. Such animals may also be members of the distinctive subpopulation rather than the chance observing of an extreme from a normal distribution. They,

together with the excluded animals, provide a basis for speculation regarding the possible existance of a subpopulation of extremely lipid-sensitive animals.

It was thought that ethyl stearate and methyl palmitate, administered together, might produce a more severe and/or prolonged depression of the RES than that caused by either lipid given singly, since it is generally believed that these two lipids exert their influence on the RES by different mechanisms. Mice treated with 30 mg of ethyl stearate plus 22.5 mg of methyl palmitate exhibited RES depression very similar in extent and duration to that of mice given the lipids separately. Further, the splenic pathology of these mice was similar to those treated with methyl palmitate. Fat was observed in the splenic histiocytes in one of these animals 4 days following treatment. Fat was not evident at either 1 or 4 days post-lipid injection in the spleens or livers of any of the other animals treated with either lipid alone or in combination. The absence of fat in these organs at these times is probably a reflection of the efficiency of the RES in metabolizing and degrading this material. Prosnitz, et al. (1969) found the livers of mice treated with ethyl palmitate to be extensively infiltrated with fat one hour after injection. However, the lipid had virtually disappeared within the next hour. The livers and spleens of methyl palmitate-treated mice did not increase in weight at any time from 1 hour to 17 days after lipid administration (DiLuzio and Blickens, 1966) indicating the absence of a large or prolonged deposition of fat in these organs.

Treatment of C57BL/6 mice with the ethyl stearate-methyl palmitate emulsion indicated that this strain is considerably more susceptible

to the RES depressive influence of these lipids than are BALB/c mice. The mean half-times, 24 hours after lipid administration, for C57BL/6 and BALB/c mice were 305.5 minutes and 69.5 minutes, respectively. It also appeared that normal, untreated C57BL/6 mice are more efficient in removing colloidal carbon from the circulation than are BALB/c mice. The mean half-time for the C57BL/6 controls was 7.1 minutes, whereas that for the BALB/c controls was 14.1 minutes. A comparison of RES depression in C57BL/6 mice produced by methyl palmitate (DiLuzio and Blickens, 1966) and by the ethyl stearate-methyl palmitate emulsion (this study) suggests that the combination of lipids is far more effective than methyl palmitate alone. Mean half-times 24 hours after injection were 305.5 minutes for ethyl stearate-methyl palmitate-treated mice and 54.4 minutes for mice treated with only methyl palmitate. Mean half-times for control mice were similar in both studies (7.1 and 9.6 minutes). Although caution should be exercised when comparing results from different laboratories, if this difference is, indeed, a real and significant one, it can be concluded that either the two lipids in combination or ethyl stearate in relatively low dosage (30 mg) are more effective RES inhibitors for C57BL/6 mice than is methyl palmitate alone.

Several attempts were made to induce RES depression in rabbits with either ethyl stearate or methyl palmitate. All were unsuccessful and it was later learned that both rabbits and dogs are refractory to lipid-induced RES depression (DiLuzio, 1969).

Throughout the RES depression studies, a wide variation in carbon half-times was observed in both lipid-treated animals and, to a lesser extent, in control mice. Inherent differences in the

physiological function of individual animals would undoubtedly account for part of this variation. In the case of lipid-treated animals, variation would also result if an animal failed to receive a full dose of lipid. Within a given control group, variation in clearance rates of individual animals was not as great as that in the corresponding test mice although an occasional animal with an exceptionally short or long half-time was encountered. The mean half-time for 245 control animals used in this study was 15.3 minutes. Individual half-times ranged from 2.4 - 60.2 minutes. However, only 22.8 percent of the control mice had half-times longer than 20 minutes and 7.7 percent were over 30 minutes. Animals suffering from a subclinical or chronic infection tend to have hyperactive RE systems and would, therefore, have shorter carbon half-times. Obviously unhealthy animals were excluded from the study. Prolonged half-times could result from metabolic disorders affecting hepatic function. More perplexing was the occasional occurrence of significantly depressed RES function in nearly all animals in a given control group. This could be explained by alterations in environmental conditions such as temperature and humidity changes, or by fluctuations in food intake, or cyclic variations in physiological function. Whatever the causes of this variation, its existence emphasizes the necessity for concomitant controls for experiments performed at different times.

Soluble HGG in low doses (0.1 mg and 0.5 mg) failed to induce a persistent tolerant or hyporesponsive state in BALB/c mice pre-treated with the ethyl stearate-methyl palmitate emulsion. However, both lipid treatment and sHGG influenced the immune response of these animals. In Figure 3 it can be seen that the hemagglutination titers of a majority

of the tolerance control animals ranged from 3 - 6 $\log_2$ units throughout the course of the experiment. This level was somewhat less than the titers of most of the immunization control mice 4 weeks post-challenge. Therefore, it can be concluded that the prior injection of sHGG limited the capacity of these animals to respond maximally to challenge with aggHGG, a highly immunogenic material. The fact that the lipid-treated animals had even lower titers initially than did the tolerance control mice strongly suggests that the lipids, as well as the sHGG, acted to impair the immune response of these animals and, further, that the inhibitory effect of the two materials was additive. A possible explanation could be based upon the fact that soluble materials are not efficiently phagocytized by the RES. Therefore, only a portion of the injected dose of sHGG was phagocytized and processed by the tolerance control mice. The lipid-treated mice, with depressed RES function, would require a longer time to engulf and/or process an amount of sHGG equivalent to that handled by the tolerance control mice. The problem with this hypothesis is that one would expect that even the small amount of sHGG processed would prime the animals for a secondary response upon challenge with aggHGG. It has been demonstrated with other protein antigens that the injection of antigen in quantities too small to elicit a primary response can prime an animal for an anamnestic response. Perhaps, in the case of sHGG, a significant proportion of the immunocompetent cells are rendered tolerant resulting in the development of fewer memory cells. Upon challenge, then, the remaining immunocompetent cells would respond normally but the total amount of antibody synthesized would be less than normal due to the reduced size of this cell population.

A 0.5 mg dose of sHGG induced tolerance or hyporesponsiveness in approximately half of the lipid-treated C57BL/6 mice (Figure 4). This substantiates prior observations that the C57BL/6 strain is less refractory to tolerance induction than are BALB/c mice (Golub and Weigle, 1969). Four of the 6 tolerance control animals failed to respond as rapidly or to the same degree to challenge with aggHGG as did the immunization control mice. The observation that lipid-induced RES depression was more profound in C57BL/6 mice than in BALB/c mice could explain why a significant number of the former strain was rendered tolerant to 0.5 mg sHGG, whereas, the latter was not. The percentage of tolerant C57BL/6 mice was less than expected. Golub and Weigle (1969) reported 100 percent tolerance in normal C57BL/6 mice to doses of sHGG ranging from 0.1 - 10.0 mg. Moreover, these investigators prepared sHGG by centrifugation at 20,000 x g for 30 minutes, conditions much less stringent than those employed in the present study. It would, therefore, be expected that their preparation would contain less monomeric antigen and, thus, be more immunogenic. However, certain differences in experimental procedure might account for the apparent quantitative differences in these two investigations. Golub and Weigle (1969) evaluated tolerance by immune elimination of labeled HGG rather than by passive hemagglutination. Although these two methods of measuring an immune response generally agree (Frei, et al., 1968), positive hemagglutination titers have been found in neonatal rabbits tolerant to HGG as determined by immune elimination (Weigle and Fudenberg, 1966). Another variable was the time at which tolerance was determined. Golub and Weigle (1969) tested their animals for immune elimination only once, at 1 week

post-challenge. A longer interval between challenge and assay of the immune response might have revealed some responsive animals.

A 0.5 mg dose of uncentrifuged, native HGG failed to induce tolerance in lipid-treated C57BL/6 mice (Figure 5). At 2 weeks post-challenge both the experimental and tolerance control animals appeared to be hyporesponsive. However, both groups of animals displayed titers which increased gradually with time. Throughout the experiment both groups had remarkably similar titers and most were significantly lower than the titers of the corresponding immunization control animals. It might be expected that the lipid-treated mice would exhibit a delayed or limited response but why this also occurred in the tolerance control animals is perplexing and no ready explanation is available. From the data presented in Figure 5 it appears that the native HGG interfered with the response of these animals to challenge with aggHGG. This is contrary to what would be expected, namely, that the HGG would prime the animals for a secondary response upon contact with the aggregated antigen. By virtue of their different physical conformations, native and aggregated HGG have determinant groups exposed which differ in quantity, specificity, and immunogenicity. It could be hypothesized that the experimental and tolerance control animals responded to determinant groups which were more prevalent on the native HGG molecules and which were only weakly immunogenic compared to the available determinant groups on the aggHGG. If this were so, at the time of challenge with aggHGG, the bulk of the available immunocompetent cells of these animals would have been previously committed to synthesizing antibody specific for the determinants of the native HGG molecule. The slight increase in the

titer of these animals with time would be due to the presence, on the aggHGG, of a few of the determinant groups of the native HGG.

A 1.0 mg does of sHGG produced a significant degree of hyporesponsiveness in both lipid-treated and untreated, tolerance control BALB/c mice. This demonstrates, as noted above, the affect of the physical form of the antigen on its tolerogenicity. At this dose the sHGG was apparently just as influential as the lipid-mediated RES depression in inducing tolerance.

A similar response was observed to 10.0 mg doses of sHGG. The results of the 4 experiments employing this dose were compiled in Table 18. Over 70 percent of the lipid-treated animals had titers of 2 or less $\log_2$ units and were considered to be tolerant or hyporesponsive. From 41 to 50 percent of the tolerance controls also had titers in this range. It appears, therefore, that both RES depression and the solubility of the antigen influenced the response of these animals but that the latter factor exerted the stronger influence.

The ability of lipid-induced RES depression to alter the immune response to sHGG was most evident when a 5.0 mg dose was employed. In Table 17 it can be seen that at least 38 percent of the lipid-treated animals were hyporesponsive, whereas only 10 percent of the tolerance controls were depressed to the same extent ($\log_2$ titers of 2 or less). Approximately half of the tolerance controls had titers ranging from 3 to 5 $\log_2$ units and the rest had titers of 6 or more, similar to those of the immunization control animals.

Perusal of Figures 3 through 17 reveals a high degree of variation between (1) individual animals within a given experimental or

control group, (2) the 3 groups assayed at a specific time after immunization and, (3) the results of individual experiments employing identical dosages of sHGG. Some of this variation is undoubtedly the result of innate physiological differences commonly observed among individuals of a given strain or species. That individual animals vary considerably in their ability to respond to antigenic stimulation is evident from an examination of the hemagglutination titers within the various immunization control groups employed in these experiments. For example, in Figure 3, the 4 week titers of the immunization control animals ranged from 4 - 10 $\log_2$ units. Differences in the overall response of the various immunization control groups were also noted, i.e., at 2 weeks post-immunization the mean $\log_2$ titers of these control groups ranged from 3.0 - 7.4, a difference of 4.4 $\log_2$ units. At 3, 4 and 5 weeks after immunization, these groups exhibited differences in mean titers of 4.8, 4.1 and 3.0 $\log_2$ units, respectively. The mean titers of some immunization control groups increased gradually throughout the experiment (Figure 6) while other such groups maintained a relatively constant mean titer during the course of the investigation (Figures 7, 8, 10, 12, 15 and 17). The mean titers of still other immunization control groups were somewhat low (3.0 $\log_2$ units) at 2 and 3 weeks post-immunization but increased by 2.5 - 3.0 $\log_2$ units by the fourth week as exemplified in Figures 4, 5, 9 and 14. A few groups which had relatively high mean titers 2 weeks post-challenge also manifested an increased mean titer by 4 weeks (Figures 11 and 16) but this increase was generally not as great as in the case of those groups having low titers initially. Other than inherent individual animal variation, the major factors responsible for

the variation in the immune response of the different immunization control groups were the preparation and administration of the antigen. Although the same preparation of aggHGG was employed in all experiments, it was emulsified in incomplete Freund's adjuvant just prior to injection and slight variations in the emulsification procedure from experiment to experiment undoubtedly occurred. The antigen in adjuvant was injected subcutaneously. Undoubtedly some animals received slightly more or less than the specified dose of antigen. It is equally likely that some animals were inadvertently injected intraperitoneally rather than subcutaneously and in some cases, antigen may have entered by both routes. The effect of the route of antigen administration on antibody production has been reviewed by Hyde (1967).

The variation in the immune response of the immunization control animals was minimal compared to that observed in tolerance control and lipid-treated animals. In several experiments the titers of the tolerance control animals generally ranged between those of the experimental and immunization control groups (Figures 11, 12 and 17), suggesting that sHGG limited the response to a subsequent injection of aggHGG. However, in other cases, these control animals had low titers initially but by 4 or 5 weeks most were similar to the immunization control titers (Figure 3). Evidently, sHGG can delay the achievement of a normal immune response. In some instances a significant proportion of the tolerance control animals were tolerant or hyporesponsive (Figures 14 and 15). Thus, no consistent pattern of response related to dose, or to any other factor, was evident for these control groups. Responsibility for the variation in these animals must, therefore, be attributed to sHGG

preparations. The preparations used in these experiments were no doubt quite heterogeneous with regard to their relative proportions of aggregated and monomeric components and, thus, would be expected to differ in tolerogenicity. The more these preparations are handled following ultracentrifugation the more opportunity there is for aggregation of the monomers. Such variables could not be identically controlled for each experiment. In addition to the factors already mentioned, the variation in the immune response of lipid-treated mice probably reflects the possibility that some mice did not receive a full dose of the lipid emulsion. Further, it was evident from the carbon clearance half-times of lipid-treated mice that these animals vary considerably in the extent and duration of the lipid-induced RES depression. It has been shown that, for tolerance to be induced in C57BL/6 mice, an interval of 4 days must intervene between administration of the tolerizing antigen and subsequent challenge (Golub and Weigle, 1967b). It has not been established whether a similar interval is required for tolerance induction in BALB/c mice although it is reasonable to assume that such an interval would not vary greatly among different mouse strains. It may be that the RES of a significant proportion of the lipid-treated mice was not sufficiently depressed throughout this 4 day period to allow the establishment of a completely tolerant state. It is also possible that BALB/c mice require a longer interval for tolerance induction. If so, this may be one reason why this strain is so difficult to render tolerant.

The results of this investigation, while not as definitive as had been hoped, are far from equivocal. It has been amply demonstrated that a significant proportion of mice treated with the RES depressants,

ethyl stearate and methyl palmitate, are severely limited in their ability to respond to an antigenic stimulus. The fact that this was demonstrated in BALB/c mice, a strain well known for its resistance to tolerance induction, is particularly significant. Certainly these findings support the hypothesis that at least one mechanism of tolerance induction involves the absence of appropriate antigen-processing by cells of the RES.

When the sera of the BALB/c mice employed in the tolerance induction experiments were titrated using BSA as a diluent, all animals exhibited a marked increase in hemagglutination titer. This high BSA titer was evident at 2 weeks post-immunization and remained remarkably constant throughout the course of these experiments (Figure 18). This BSA-DAB was not induced as a result of either lipid treatment or the administration of sHGG since both the tolerance controls, which did not receive lipid, and the immunization controls, which did not receive sHGG, had similar hemagglutination titers in the BSA diluent. It is noteworthy that C57BL/6 mice did not have significantly increased BSA titers. In fact, the PBGS and BSA titers of these animals were nearly identical.

BSA-detectable antibody (BSA-DAB) was not detected in the serum of normal BALB/c mice. It appears to have reached near maximum levels by 2 weeks after immunization in all groups of animals. The relative increase in hemagglutinin activity in the presence of BSA is obviously greatest in mice which have the most profound immune depression as detected by titration in PBSG. In fact, animals which would be considered completely tolerant by the PBSG titration criterion had BSA-DAB levels

similar to those seen in immunization control mice. Hence, absolute tolerance was never achieved in this study. Similar observations have been documented in which animals deemed tolerant by one assay were found to have specific antibody titers when evaluated by a different procedure (Linscott and Weigle, 1965; Weigle and Fudenberg, 1966; Henney and Ishizaka, 1967). Another point worth mentioning is the fact that the passive hemagglutination assay, which was used in the present study, is a more sensitive detector of IgM than IgG antibody (Borel, et al., 1968). There is, however, no evidence to suggest that employing BSA as a diluent should further enhance its sensitivity for IgM or any other class of complete antibody. One would not expect IgM to be the predominating antibody throughout the experiments reported here. However, it is known that considerable variation exists among mouse strains with regard to the relative concentration and duration of immunoglobulin classes comprising an antibody response to antigenic stimulation. Warner, et al. (1968) immunized 5 strains of mice with dinitrophenylated hemocyanin. Two strains synthesized relatively less IgG than the others but had significant amounts of 2-mercaptoethanol-sensitive antibody present as long as 80 days post-immunization. The difference in PBSG and BSA hemagglutination titers of an individual animal is reminiscent of an immunological phenomenon known as immune deviation or split tolerance. Immune deviation refers to the antigen-induced abolishment or diminution of certain types of immune responses, while other types are unaffected. The classic example of immune deviation is the suppression of delayed hypersensitivity without affecting the humoral antibody response. Asherson and Stone (1965) demonstrated suppression of delayed hypersensitivity to

BGG or HSA in guinea pigs by treating them with alum-precipitated BGG or HSA prior to immunization with the respective antigen in complete Freund's adjuvant. Total antibody production was not altered. In some instances depression of γ_2-antibody synthesis was observed concomitantly with suppression of delayed hypersensitivity, while production of γ_1-antibody remained unchanged. In fact, by manipulating the conditions required for the induction of immune deviation, such as the dosage and time of administration of the alum-precipitated antigen, delayed hypersensitivity, delayed hypersensitivity and γ_1-antibody production or delayed hypersensitivity, γ_2- and γ_1-antibody production could be suppressed (Asherson, 1967; Dvorak and Flax, 1966; Dvorak, et al., 1965). Immune deviation has also been produced by administering the antigen in incomplete Freund's adjuvant prior to sensitization (Dvorak, et al., 1966; Loewi, et al., 1966) or with soluble antigen (Dvorak and Flax, 1966). Immune deviation is probably a fairly common occurrence. It has been observed frequently in guinea pigs (Asherson and Stone, 1965; Dvorak, et al., 1965, 1966) and in mice (Crowle and Hu, 1966) employing numerous antigens including HSA, BGG, BSA, ribonuclease (Dvorak, et al., 1965, 1966), blood-group substance (Loewi, et al., 1966) and egg albumin (Loewi, et al., 1966; Crowle and Hu, 1966). Other observations which might be classed as examples of immune deviation, had the appropriate humoral antibody quantitations been made, include suppression of delayed hypersensitivity in guinea pigs to tuberculin, which normally follows immunization with BCG, by the prior injection of purified protein derivative (PPD) (Boyden, 1957) and the prolonged survival of skin homografts in mice previously immunized with lyophilized spleen tissue, emulsified

in incomplete Freund's adjuvant, from donor mice (Chutná, 1968). More germane to the present study are examples of possible split tolerance which pertain only to variations in humoral antibody formation. Borel, et al. (1968) noted that IgM antibody production was inhibited more readily and to a greater extent than was IgG antibody following the administration of deaggregated (ultracentrifuged) BGG to rabbits. Chickens injected with very small amounts of HSA (500 γ) synthesized only IgM antibody, whereas massive quantities of HSA (2500 mg) induced a form of split tolerance in that the only serum antibody detectable was IgG. Subsequent challenge with 10 mg of HSA, which normally stimulates IgM production, failed to do so in this case (Černý and Iványi, 1967).

Characterization of the BSA-DAB revealed it to be heat labile at 56C for 30 minutes. Titration of heat-inactivated sera using fresh, normal mouse serum as a diluent failed to reconstitute activity. Therefore, the decreased BSA titers of heated sera were not due to inactivation of complement. Further, the BSA-DAB titers ranged from 9 to 10.5 $\log_2$ units and it is doubtful that a 1:1,024 dilution of serum contains a sufficient amount of complement to effectively participate in an antigen-antibody reaction. Further observations suggested that the BSA-DAB in the serum of lipid-treated and tolerance control animals may be sensitive to multiple freeze-thawings and prolonged storage in a frozen state.

The specificity of the antibodies produced in the BALB/c mice was investigated by means of absorption and in vitro inhibition assays. Both PBSG and BSA titers were abolished by prior absorption of mouse sera with a 2 mg/ml concentration of HGG. Further, titration of mouse sera in the presence of excess HGG also abolished or drastically decreased

both the PBSG and BSA titers.

The BSA-DAB differed from the antibody detected by titration in the PBSG diluent in that the former cross-reacted with MGG, whereas the latter did not. In fact, higher hemagglutination titers were obtained when SRBC were sensitized with MGG than with HGG. This would be expected if less MGG was conjugated to SRBC than was HGG which would result in a more sensitive assay. On the other hand, BSA-DAB may be an antibody which cross reacts with a determinant group present on HGG and Swiss-Webster MGG (the commercially obtained MGG was prepared from Swiss-Webster mice), but not on BALB/c gamma globulin, and is more specific for Swiss-Webster MGG than for HGG.

Several similarities were noted in the characteristics of BSA-DAB and rheumatoid factor (RF). Both react with isoantigens and heteroantigens and both react with IgG (RF reacts with IgG exclusively). Titration in a protein diluent potentiates the titer of both materials. When titrating RF by the SRBC agglutination test, substituting sheep serum for saline as a diluent increases the RF titer 4 to 500-fold (Epstein, 1957). BSA-DAB and RF appear to differ in their heat stability. Rheumatoid factor is stable when heated up to 78C for 30 minutes (Ziff, 1957), whereas BSA-DAB was inactivated at 56C for 30 minutes. However, preliminary investigations have shown that heated, normal BALB/c serum, present in a 1:10 dilution in BSA, inhibits BSA-DAB produced in BALB/c mice (Hyde and Rice, 1970). They suggest that the apparent heat lability of BSA-DAB may be an artifact resulting from the "sponging up" of antibody by gamma globulin which was denatured or aggregated during serum inactivation. If this is true, it further supports the thesis that

BSA-DAB and RF are the same since RF is currently thought to react primarily with gamma globulin determinant groups which are not exposed unless the molecule is immunologically denatured, as in formation of an antigen-antibody complex (Christian, 1967). It is quite probable that some HGG is denatured in the process of becoming coupled to SRBC with BDB. Perhaps, then, BSA-DAB reacts with HGG determinant groups exposed during this procedure. One problem with this hypothesis is the fact that reaction between RF and sensitized particles is not inhibited by native IgG, whereas, as demonstrated in this study, BSA-DAB is inhibited. Thus, additional characterization will be required to identify the elusive BSA-DAB.

CHAPTER V

SUMMARY

Ethyl stearate and methyl palmitate, administered separately or in combination, were equally effective as RES depressants in mice, as determined by the intravascular clearance of colloidal carbon. Maximum depression of RES function occurred 24 hours after lipid administration. Normal function was regained in 4 to 10 days. A combination of these lipids produced a decidedly more profound impairment of RES activity in C57BL/6 mice than in BALB/c mice. However, normal C57BL/6 mice cleared carbon from the circulation more rapidly than did mice of the BALB/c strain. Considerable variation existed in the susceptibility of individual animals to lipid-induced RES depression. Animals also varied in normal RES activity as evaluated by the carbon clearance technique.

Lipid-induced RES depression enhanced the induction of a tolerant or hyporesponsive state to sHGG in a significant proportion of BALB/c mice. This was most evident when a 5.0 mg dose of sHGG was employed. Soluble HGG, alone, rendered some normal animals hyporesponsive or tolerant and appeared to delay or limit the normal immune response of many animals to a subsequent injection of aggHGG. These findings support the hypothesis that tolerance may be induced to an antigen when it has not been appropriately processed by macrophages.

Titration of BALB/c sera, but not C57BL/6 sera, in the presence

of BSA resulted in markedly increased hemagglutination titers in all animals. These titers had reached maximum levels by 2 weeks post-immunization and remained relatively constant throughout the experiment. The similarity between these observations and those seen in immune deviation were noted.

Characterization of BSA-DAB revealed it to be heat-labile. Inactivation of complement was not thought to be responsible for the heat-lability. The BSA-DAB cross reacted with HGG and MGG from the Swiss-Webster strain. Some evidence was obtained which suggested that BSA-DAB may lose activity when subjected to multiple freeze-thawing or prolonged storage in a frozen state. Similarities in the characteristics of BSA-DAB and rheumatoid factor exist and it seems possible that these materials might be a single entity.

BIBLIOGRAPHY

Allison, A. C., Harington, J. S. and Birbeck, M. 1966. An examination of the cytotoxic effects of silica on macrophages. J. Exp. Med., 124: 141-153.

Argyris, B. F. 1968. Role of macrophages in immunological maturation. J. Exp. Med., 128: 459-467.

Argyris, B. F. and Askonas, B. A. 1968. Mouse peritoneal cells: their ability to elicit or produce antibody after exposure to antigen. Immunol., 14: 379-392.

Argyris, B. F. and Plotkin, D. H. 1969. Effect of antimacrophage serum on antibody production and phagocytosis in mice. J. Immunol., 103: 372-373.

Arteta, J. L. 1956. The microglia and the reticuloendothelial system. Research on the histiocytic significance of the microglia. RES Bull., 2: 9-13.

Aschoff, L. 1924. Lectures on Pathology. Paul B. Hoeber, Inc., New York.

Asherson, G. L. 1967. Antigen-mediated depression of delayed hypersensitivity. Brit. Med. Bull., 23: 24-29.

Asherson, G. L. and Stone, S. H. 1965. Selective and specific inhibition of 24 hour skin reactions in the guinea pig. I. Immune deviation: description of the phenomenon and the effect of splenectomy. Immunol., 9: 205-217.

Askonas, B. A. and Rhodes, J. M. 1965. Immunogenicity of antigen-containing ribonucleic acid preparations from macrophages. Nature 205: 470-474.

Baillif, R. N. 1956. Delineation of the RES. RES Bull., 2: 3-4.

Balner, H., Old, L. J. and Clarke, D. A. 1962. Accelerated rejection of male skin isografts by female C57BL mice infected with Bacillus Calmette-Guérin (B.C.G.). Proc. Soc. Exp. Biol. Med., 109: 58-62.

Benacerraf, B., Biozzi, G., Halpern, B. N. and Stiffel, C. 1957. Physiology of phagocytosis of particles by the RES, in Physiopathology of the Reticuloendothelial System, Edited by B. N. Halpern, B. Benacerraf and J. F. Delafresnaye, p. 52-77, Charles C. Thomas, Springfield.

Benacerraf, B., Halpern, B. N., Biozzi, G. and Benos, S. A. 1954. Quantitative study of the granulopectic activity of the reticuloendothelial system. III. The effect of cortisone and nitrogen mustard on the regenerative capacity of the RES after saturation with carbon. Brit. J. Exp. Path., 35: 97-106.

Berglund, K. 1956. Studies on factors which condition the effect of cortisone on antibody production. I. The significance of time of hormone administration in primary hemolysin response. Acta Pathol. Microbiol. Scand., 38: 311-328.

Biozzi, G., Benacerraf, B., and Halpern, B. N. 1953. Quantitative study of the granulopectic activity of the reticulo-endothelial system. II. A study of the kinetics of the granulopectic activity of the RES in relation to the dose of carbon injected. Relationship between the weight of the organs and their activity. Brit. J. Exp. Path., 34 441-457.

Biro, C. E. and Garcia, G. 1965. The antigenicity of aggregated and aggregate-free human gamma-globulin for rabbits. Immunol., 8: 411-419.

Blickens, D. A. and DiLuzio, N. R. 1965. Metabolism of methyl palmitate, a phagocytic and immunologic depressant, and its influence on tissue lipids. RES, J. Reticuloendothelial Soc., 2: 60-74.

Borel, Y., Franklin, E. C. and Miescher, P. A. 1968. The effect of unaggregated bovine γ-globulin on γM and γG antibody formation. Immunol., 14: 899-909.

Box, G. E. P. 1954. Some theorems on quadratic forms applied in the study of analysis of variance problems. Ann. Math. Statist., 25: 290-302 and 484-498. Cited in Statistical Principles in Experimental Design, Winer, B. J. (McGraw-Hill, New York, 1962).

Boyden, S. V. 1957. The effect of previous injections of tuberculoprotein on the development of tuberculin sensitivity following BCG vaccination in guinea pigs. Brit. J. Exp. Path., 38: 611-617.

Buchanan, K. D. and MacGregor, R. F. S. 1964. Prolongation of the survival of human red cells in mice by chemical splenectomy. Brit. J. Exp. Path., 45: 248-251.

Černý, J. and Iványi, J. 1967. Variation in tolerance dose requirements as related to heterogeneity of the immune response. Nature 213: 1017-1018.

Christian, C. L. 1967. Rheumatoid factors. A. Nature and significance of rheumatoid factors, in Laboratory Diagnostic Procedures in the Rheumatic Diseases, Edited by A. S. Cohen, p. 84-95, Little, Brown, and Co., Boston.

Chutná, J. 1968. Enhancement of skin homografts and dissociation of immune responses in mice immunized with lyophilized tissues in Freund's adjuvant. Nature 217: 175-177.

Claman, H. N. 1963. Tolerance to a protein antigen in adult mice and the effect of nonspecific factors. J. Immunol., 91: 833-839.

Cline, M. J. and Swett, V. C. 1968. The interaction of human monocytes and lymphocytes. J. Exp. Med., 128: 1309-1325.

Cohen, E. P. 1967. Conversion of non-immune cells into antibody-forming cells by RNA. Nature 213: 462-465.

Cohen, E. P. 1968. Analysis by molecular hybridization of RNA from immunocompetent cells exposed to antigen. RES, J. Reticuloendothelial Soc., 5: abstract number 29, Fifth Annual National Meeting.

Cohen, E. P., Newcomb, R. W. and Crosby, L. K. 1965. Conversion of non-immune spleen cells to antibody-forming cells by RNA: Strain specificity of the response. J. Immunol., 95: 583-590.

Cohen, E. P. and Parks, J. J. 1964. Antibody production by nonimmune spleen cells incubated with RNA from immunized mice. Science 144 : 1012-1013.

Conning, D. M. and Heppleston, A. G. 1966. Reticuloendothelial activity and local particle disposal. A comparison of the influence of modifying agents. Brit. J. Exp. Path., 47: 388-400.

Cooper, G. N. and Stuart, A. E. 1961. Sensitivity of mice to bacterial lipopolysaccharide following alteration of activity of the reticulo-endothelial system. Nature 191: 294-295.

Cooper, G. N. and Stuart, A. E. 1962. Susceptibility of mice to pneumococcal infection after modification of the reticulo-endothelial system with simple lipids. J. Path. Bact., 83: 227-243.

Crowle, A. J. and Hu, C. C. 1966. Split tolerance affecting delayed hypersensitivity and induced in mice by pre-immunization with protein antigens in solution. Clin. Exp. Immunol., 1: 323-335.

Cruchaud, A. 1968. The effect of reticuloendothelial blockade on antibody formation and immunologic tolerance. Lab. Invest., 19: 15-24.

Cutler, J. L. 1960. The enhancement of hemolysin production in the rat by zymosan. J. Immunol., 84: 416-419.

Despont, J. P. and Cruchaud, A. 1969. In vivo and in vitro effects of anti-macrophage serum. Nature 223: 838-839.

Dietrich, F. M. and Weigle, W. O. 1964. Immunologic unresponsiveness to heterologous serum proteins induced in adult mice and transfer of the unresponsive state. J. Immunol., 92: 167-172.

Diller, I. C., Fisher, M. E. and Gable, D. 1964. Effect of glucan on mouse sarcoma 37. Proc. Soc. Exp. Biol. Med., 117: 107-110.

DiLuzio, N. R. 1969. Personal communication.

DiLuzio, N. R. and Blickens, D. A. 1966. Influence of intravenously administered lipids on reticuloendothelial function. RES, J. Reticuloendothelial Soc., 3: 250-270.

DiLuzio, N. R. and Riggi, S. J. 1964. The development of a lipid emulsion for the measurement of reticuloendothelial function. RES, J. Reticuloendothelial Soc., 1: 136-149.

DiLuzio, N. R. and Wooles, W. R. 1964. Depression of phagocytic activity and immune response by methyl palmitate. Amer. J. Physiol., 206: 939-943.

Dresser, D. W. 1961. Effectiveness of lipid and lipidophilic substances as adjuvants. Nature 191: 1169-1171.

Dresser, D. W. 1962. Specific inhibition of antibody production. II. Paralysis induced in adult mice by small quantities of protein antigen. Immunol., 5: 378-388.

Dresser, D. W. 1963. Specific inhibition of antibody production. III. Apparent changes in the half-life of bovine gamma globulin in paralysed mice. Immunol., 6: 345-355.

Dvorak, H. F., Billote, J. B., McCarthy, J. S. and Flax, M. H. 1965. Immunologic unresponsiveness in the adult guinea pig. I. Suppression of delayed hypersensitivity and antibody formation to protein antigens. J. Immunol., 94: 966-975.

Dvorak, H. F., Billote, J. B., McCarthy, J. S. and Flax, M. H. 1966. Immunologic unresponsiveness in the adult guinea pig. III. Variation of the antigen and vehicle of suppression. Induction of unresponsiveness in the adult rat. J. Immunol., 97: 106-111.

Dvorak, H. F. and Flax, M. H. 1966. Immunologic unresponsiveness in the adult guinea pig. II. The kinetics of unresponsiveness. J. Immunol., 96: 546-553.

Epstein, W. 1957. Serological Reactions of Rheumatoid Arthritis, Edited by R. W. Lamong-Havers, p. 17, Issued by The Arthritis and Rheumatism Foundation.

Feldman, M. and Gallily, R. 1967. Cell interactions in the induction of antibody formation. Cold Springs Harbor Symp. Quant. Biol., 32: 415-421.

Fenn, W. O. 1921a. The phagocytosis of solid particles. II. Carbon. J. Gen. Physiol., 3: 465-482.

Fenn, W. O. 1921b. The phagocytosis of solid particles. III. Carbon and quartz. J. Gen. Physiol., 3: 575-593.

Fishman, M. 1959. Antibody formation in tissue culture. Nature 183: 1200-1201.

Fishman, M. 1961. Antibody formation in vitro. J. Exp. Med., 114: 837-856.

Fishman, M. and Adler, F. L. 1963. Antibody formation initiated in vitro. II. Antibody synthesis in X-irradiated recipients of diffusion chambers containing nucleic acid derived from macrophages incubated with antigen. J. Exp. Med., 117: 595-602.

Fishman, M. and Adler, F. L. 1967. The role of macrophage-RNA in the immune response. Cold Springs Harbor Symp. Quant. Biol., 32: 343-348.

Fishman, M., Hammerstrom, R. A. and Bond, V. P. 1963. In vitro transfer of macrophage RNA to lymph node cells. Nature 198: 549-551.

Fong, J., Chin, D. and Elberg, S. S. 1963. Studies of tubercle bacillus-histiocyte relationships. VI. Induction of cellular resistance by ribosomes and ribosomal RNA. J. Exp. Med., 118: 371-386.

Forbes, I. J. 1969. Analysis in vitro of tolerance. Immunol., 16: 699-706.

Frei, P. C., Benacerraf, B. and Thorbecke, G. J. 1965. Phagocytosis of the antigen, a crucial step in the induction of the primary response. Proc. Nat. Acad. Sci. USA, 53: 20-23.

Frei, P. C., Cruchaud, S., Arnaudo, L. and Vannotti, A. 1968. Immunologic tolerance induced in the adult rabbit with modified BSA. J. Immunol., 101: 605-610.

Friedman, H. P., Stavitsky, A. B. and Solomon, J. M. 1965. Induction in vitro of antibodies to phage T2: antigens in the RNA extract employed. Science 149: 1106-1107.

Frisch, A. W. and Wilson, B. J. 1969. Cell transfer studies in immunosuppressed mice: the role of the macrophage. Proc. Soc. Exp. Biol. Med., 132: 42-45.

Gamble, C. N. 1966. The role of soluble aggregates in the primary immune response of mice to human gamma globulin. Int. Arch. Allergy 30: 446-455.

Garvey, J. S. and Campbell, D. H. 1957. The retention of S^{35}-labelled bovine serum albumin in normal and immunized liver tissue. J. Exp. Med., 105: 361-372.

Gershon, H. and Felman, M. 1968. Studies on the immune reconstitution of sublethally irradiated mice by peritoneal macrophages. Immunol., 15: 827-835.

Golub, E. S. and Weigle, W. O. 1967a. Studies on the induction of immunologic unresponsiveness. I. Effects of endotoxin and phytohemagglutinin. J. Immunol., 98: 1241-1247.

Golub, E. S. and Weigle, W. O. 1967b. Studies on the induction of immunologic unresponsiveness. II. Kinetics. J. Immunol., 99: 624-628.

Golub, E. S. and Weigle, W. O. 1969. Studies on the induction of immunologic unresponsiveness. III. Antigen form and mouse strain variation. J. Immunol., 102: 389-396.

Gordon, J. 1968. Role of monocytes in the mixed leukocyte culture reaction. Proc. Soc. Exp. Biol. Med., 127: 30-33.

Halpern, B. N., Benacerraf, B. and Biozzi, G. 1953. Quantitative study of the granulopectic activity of the reticulo-endothelial system. I. The effect of the ingredients present in India ink and of substances affecting blood clotting in vivo on the fate of carbon particles administered intravenously in rats, mice and rabbits. Brit. J. Exp. Path., 34: 426-440.

Halpern, B. N., Biozzi, G., Mené, G., and Benacerraf, B. 1951. Étude quantitative de l'activite granulopexique du systeme reticulo-endothélial par l'injection intravenineuse dencre de chine chez les diverses espéces animales. Ann. Inst. Pasteur 80: 582-604.

Halpern, B. N., Biozzi, G., Stiffel, C. and Mouton, D. 1958. Corrélation entre l'activité phagocytaire du systeme reticulo-endothélial et la production d'anticorps antibactériens. C. R. Soc. Biol., 152: 758-761.

Hanan, R. and Oyama, J. 1954. Inhibition of antibody formation in mature rabbits by contact with the antigen at an early age. J. Immunol., 73: 49-53.

Heller, J. H. 1955. Cortisone and phagocytosis. Endocrinol., 56: 80-85.

Henney, C. S. and Ishizaka, K. 1967. Immunological unresponsiveness to specific antigenic determinants in human γG globulin. Proc. Soc. Exp. Biol. Med., 125: 335-341.

Hersh, E. M. and Harris, J. E. 1968. Macrophage-lymphocyte interaction in the antigen-induced blastogenic response of human peripheral blood leucocytes. J. Immunol., 100: 1184-1194.

Hirsch, M. S., Gary, G. W., Jr., and Murphy, F. A. 1969. In vitro and in vivo properties of antimacrophage sera. J. Immunol., 102: 656-661.

Howard, R. J., Mergenhagen, S. E., Notkins, A. L. and Dougherty, S. F. 1969. Inhibition of cellular immunity and enhancement of humoral antibody formation in mice infected with lactic dehydrogenase virus. Transplant. Proc., 1: 586-588.

Hyde, R. M. 1967. Antiserum production in experimental animals. Advances Appl. Microbiol., 9: 39-67.

Hyde, R. M., Garb, S. and Bennett, A. J. 1967. Demonstration by immunoelectrophoresis of antigen in human myelogenous leukemia. J. Nat. Cancer Inst. 38: 909-919.

Hyde, R. M. and Rice, J. E. 1970. Unpublished data.

Jennings, J. F. and Oates, C. M. 1967. Studies on the nonspecific depression of the immune response. J. Exp. Med., 126: 557-564.

Kalckar, H. M. 1947. Differential spectrophotometry of purine compounds by means of specific enzymes. III. Studies of the enzymes of purine metabolism. J. Biol. Chem., 167: 461-475.

Karthigasu, K., Reade, P. C. and Jenkin, C. R. 1965. The functional development of the reticulo-endothelial system. III. The bactericidal capacity of fixed macrophages of foetal and neonatal chicks and rats. Immunol., 9: 67-73.

Kessel, R. W. I., Monaco, L. and Marchisio, M. A. 1963. The specificity of the cytotoxic action of silica - a study in vitro. Brit. J. Exp. Path., 44: 351-364.

Kong, Y. M. and Cheng, J. K. 1970. Immunologic tolerance to a protein antigen in adult mice: challenge with an aggregated fraction. Fed. Proc., 29: abstract number 352.

Kramer, T. R. 1970. Personal communication.

Linscott, W. D. and Weigle, W. O. 1965. Induction of tolerance to bovine serum albumin by means of whole-body X-radiation. J. Immunol., 94: 430-436.

Loewi, G., Holborow, E. J. and Temple, A. 1966. Inhibition of delayed hypersensitivity by pre-immunization without complete adjuvant. Immunol., 10: 339-347.

Loewi, G., Temple, A., Nind, A. P. and Axelrad, M. 1969. A study of the effects of anti-macrophage sera. Immunol., 16: 99-106.

Mannick, J. A. and Egdahl, R. H. 1962. Ribonucleic acid in "transformation" of lymphoid cells. Science 137: 976-977.

Martin, W. J. 1966. The cellular basis of immunological tolerance in newborn animals. Aust. J. Exp. Biol. Med. Sci., 44: 605-608.

Medawar, P. B. 1963. Concluding remarks in, Acquired or natural tolerance toward defined protein antigens, Edited by E. S. Bussard, C. N. R. S. Symposium, Academic Press, New York and London.

Melnick, H. D. and Friedman, H. 1969. The immunosuppressive effect of RES "blockade" with ethyl stearate emulsion on cellular formation of antibody to sheep erythrocytes. Proc. Soc. Exp. Biol. Med., 133: 423-427.

Metchnikoff, E. 1907. Immunity in Infective Diseases. Binnie, F. G. (trans.), University Press, Cambridge.

Mergenhagen, S. E., Notkins, A. L. and Dougherty, S. F. 1967. Adjuvanticity of lactic dehydrogenase virus: influence of virus infection on the establishment of immunologic tolerance to a protein antigen in adult mice. J. Immunol., 99: 576-581.

Mitchell, G. F. and Miller, J. F. A. P. 1968. Immunological activity of thymus and thoracic-duct lymphocytes. Proc. Nat. Acad. Sci. USA, 59: 296-303.

Mitchell, J. and Nossal, G. J. V. 1966. Mechanism of induction of immunological tolerance. I. Localization of tolerance-inducing antigen. Aust. J. Exp. Biol. Med. Sci., 44: 211-224.

Mitchison, N. A. 1969. The immunogenic capacity of antigen taken up by peritoneal exudate cells. Immunol., 16: 1-14.

Morrow, S. H. and DiLuzio, N. R. 1965. The fate of foreign red cells in mice with altered reticuloendothelial function. Proc. Soc. Exp. Biol. Med., 119: 647-652.

Mosier, D. E. 1967. A requirement for two cell types for antibody formation in vitro. Science 158: 1573-1575.

Mountain, I. M. 1955. Antibody production by spleen in vitro. I. Influence of cortisone and other chemicals. J. Immunol., 74: 270-277.

Neukomm, S., Lerch, P. and Jallut, O. 1957. Physicochemical factors governing the phagocytic function of the RES, in Physiopathology of the Reticulo-endothelial System, Edited by B. N. Halpern, B. Benacerraf and J. F. Delafresnaye, p. 115-127, Charles C. Thomas, Springfield.

Nicol. T. and Bilbey, D. L. J. 1958. Substances depressing the phagocytic activity of the reticulo-endothelial system. Nature 182: 606.

Ohbuchi, S. 1968. Suppression of antibody formation by the RES-blockade. III. Effects of the RES-blockade with methyl palmitate. Acta. Med. Okayama 22: 137-146.

Old, L. J., Clarke, D. A., Benacerraf, B. and Goldsmith, M. 1960. The reticuloendothelial system and the neoplastic process. Ann. N. Y. Acad. Sci., 88: 264-280.

Oppenheim, J. J., Leventhal, B. G. and Hersh, E. M. 1968. The transformation of column-purified lymphocytes with nonspecific and specific antigenic stimuli. J. Immunol., 101: 262-270.

Panijel, J. and Cayeux, P. 1968. Immunosuppressive effects of macrophage antiserum. Immunol., 14: 769-780.

Pearsall, N. N. and Weiser, R. S. 1968. The macrophage in allograft immunity. I. Effects of silica as a specific macrophage toxin. RES, J. Reticuloendothelial Soc., 5: 107-120.

Pierce, C. W. 1969. Immune responses in vitro. I. Cellular requirements for the immune response by nonprimed and primed spleen cells in vitro. J. Exp. Med., 130: 345-364.

Pierce, C. W. and Benacerraf, B. 1969. Immune response in vitro: independence of "activated" lymphoid cells. Science 166: 1002-1003.

Pinchuck, P., Fishman, M., Adler, F. L. and Maurer, P. H. 1968. Antibody formation: initiation in "nonresponder" mice by macrophage synthetic polypeptide RNA. Science 160: 194-195.

Pinckard, R. N., Weir, D. M. and McBride, W. H. 1967. Factors influencing the immune response. I. Effects of the physical state of the antigen and of lymphoreticular cell proliferation on the response to intravenous injection of bovine serum albumin in rabbits. Clin. Exp. Immunol., 2: 331-341.

Pinckard, R. N., Weir, D. M. and McBride, W. H. 1968. Factors influencing the immune response. III. The blocking effect of Corynebacterium parvum upon the induction of acquired immunological unresponsiveness to bovine serum albumin in the adult rabbit. Clin. Exp. Immunol., 3: 413-421.

Pribnow, J. F. and Silverman, M. S. 1967. Studies on the radiosensitive phase of the primary antibody response in rabbits. I. The role of the macrophage. J. Immunol., 98: 225-229.

Prosnitz, L, Kawasaki, S., Cohen, G. S., Dineen, J. L., Perille, P. E. and Finch, S. C. 1969. Ethyl palmitate-induced splenic destruction. RES, J. Reticuloendothelial Soc., 6: 487-497.

Reade, P. C. and Jenkin, C. R. 1965. The functional development of the reticulo-endothelial system. I. The uptake of intravenously injected particles by foetal rats. Immunol., 9: 53-60.

Riggi, S. J. and DiLuzio, N. R. 1961. Identification of a reticuloendothelial stimulating agent in zymosan. Amer. J. Physiol., 200: 297-300.

Roelants, G. E. and Goodman, J. W. 1968. Immunochemical studies on the poly-γ-D- glutamyl capsule of Bacillus anthracis. IV. The association with peritoneal exudate cell ribonucleic acid of the polypeptide in immunogenic and nonimmunogenic forms. Biochem., 7: 1432-1440.

Roelants, G. E. and Goodman, J. W. 1969. The chemical nature of macrophage RNA-antigen complexes and their relevance to immune induction. J. Exp. Med., 130: 557-574.

Roseman, J. 1969. X-ray resistant cell required for the induction of in vitro antibody formation. Science 165: 1125-1127.

Roser, B. 1965. The distribution of intravenously injected peritoneal macrophages in the mouse. Aust. J. Biol. Med. Sci., 43: 553-562.

Roser, B. 1968. The distribution of intravenously injected Kupffer cells in the mouse. RES, J. Reticuloendothelial Soc., 5: 455-471.

Ryder, J. W. and Schwartz, R. S. 1969. Immunosuppression by antibody: localization of site of action. J. Immunol., 103: 970-978.

Saba, T. M. and DiLuzio, N. R. 1968. Evaluation of humoral and cellular mechanisms of methyl palmitate induced reticuloendothelial depression. Life Sciences 7: 337-347.

Sabet, T. and Friedman, H. 1969. Immunologic "amnesia" of antibody-forming cells after RES blockade. Proc. Soc. Exp. Biol. Med., 131: 1317-1322.

Sabet, T., Newlin, C. and Friedman, H. 1968. The effect of RES blockade on cellular antibody formation to sheep erythrocytes. Proc. Soc. Exp. Biol. Med., 128: 274-278.

Sabet, T., Newlin, C. and Friedman, H. 1969. Effects of RES "blockade" on antibody-formation. I. Suppressed cellular and humoral haemolysin responses in mice injected with carbon particles. Immunol., 16: 433-446.

Shivas, A. A. and Fraser, G. P. 1959. Lymphoid necrosis induced by lipid emulsions. Nature 184: 1813.

Smith, R. T. 1961. Immunological tolerance of nonliving antigens. Advances Immunol., 1: 67-129.

Stavitsky, A. B. and Arquilla, E. R. 1955. Micromethods for the study of proteins and antibodies. III. Procedure and applications of hemagglutination and hemagglutination-inhibition reactions with bis-diazotized benzidine and protein-conjugated red blood cells. J. Immunol., 74: 306-312.

Stuart, A. E. 1960. Chemical splenectomy. Lancet 2: 896-897.

Stuart, A. E. 1962. Effect of cholesterol oleate on the phagocytic function of the reticulo-endothelial system. Nature 196: 78-79.

Stuart, A. E., Biozzi, G., Stiffel, C., Halpern, B. N. and Mouton, D. 1960. The stimulation and depression of reticulo-endothelial phagocytic function by simple lipids. Brit. J. Exp. Path., 41: 599-604.

Stuart, A. E. and Cooper, G. N. 1962. Susceptibility of mice to bacterial endotoxin after modification of reticulo-endothelial function by simple lipids. J. Path. Bact., 88: 245-254.

Stuart, A. E. and Davidson, A. E. 1963. The effect of intravenous cholesterol oleate on the function of the reticuloendothelial system. Brit. J. Exp. Path., 44: 24-30.

Sulitzeanu, D. 1968. Affinity of antigen for white cells and its relation to the induction of antibody formation. Bacteriol. Rev., 32: 404-424.

Taber, R. and Irwin, S. 1969. Anesthesia in the mouse. Fed. Proc., 28: 1528-1532.

Unanue, E. R. 1968. Properties and some uses of anti-macrophage antibodies. Nature 218: 36-38.

Unanue, E. R. and Askonas, B. A. 1968. The immune response of mice to antigen in macrophages. Immunol., 15: 287-296.

Unanue, E. R. and Cerottini, J. C. 1970. The function of macrophages in the immune response. Seminars Hematol., 7: 225-248.

Warner, N. L., Vaz, N. M. and Ovary, Z. 1968. Immunoglobulin classes in antibody responses in mice. I. Analysis by biological properties. Immunol., 14: 725-734.

Weigle, W. O. and Fudenberg, H. H. 1966. Specificity of immunological tolerance induced in the rabbit to genetic determinants of human γG globulin. Int. Arch. Allergy 29: 28-35.

Weigle, W. O. and Golub, E. S. 1967. Kinetics of the establishment of immunological unresponsiveness to serum protein antigens. Cold Springs Harbor Symp. Quant. Biol., 32: 555-558.

Weiner, J., Cottrell, J. S., Margaretten, W. and Spiro, D. 1967. An electron microscopic study of steroid induced reticulo-endothelial blockade. Amer. J. Path., 50: 187-201.

Winer, B. J. 1962. Statistical Principles in Experimental Design. McGraw-Hill, New York. pp. 241-244.

Wooles, W. R. and DiLuzio, N. R. 1962. Influence of reticuloendothelial hyperfunction on bone marrow transplantation. Amer. J. Physiol., 203: 404-408.

Wooles, W. R. and DiLuzio, N. R. 1963. Reticuloendothelial function and the immune response. Science 142: 1078-1080.

Wooles, W. R. and DiLuzio, N. R. 1964. Inhibition of homograft acceptance and homo- and hetero-graft rejection in chimeras by reticuloendothelial system stimulation. Proc. Soc. Exp. Biol. Med., 115: 756-759.

Wu, W. G. and Trice, K. L. 1967. Influence of cellular factors on immune unresponsiveness induced by Klebsiella penumoniae capsular antigen. J. Bact., 94: 896-901.

Ziff, M. 1957. Serological Reactions of Rheumatoid Arthritis, Edited by R. W. Lamont-Havers, p. 31, Issued by The Arthritis and Rheumatism Foundation.