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Investigation of the generation of a pseudo-secondary response to LPS induced by macrophage toxins

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AN INVESTIGATION OF THE GENERATION OF A PSEUDO-SECONDARY RESPONSE TO LPS INDUCED BY MACROPHAGE TOXINS

By

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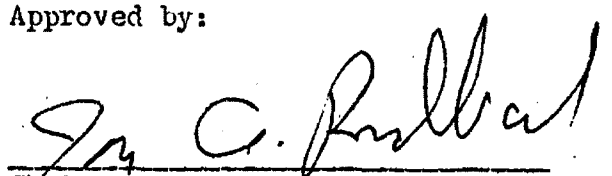

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ABSTRACT

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An Investigation of the Generation of a Pseudo-Secondary Response to LPS Induced by Macrophage Toxins (79 pp.)

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A single dose of lipopolysaccharide (LPS), administered 21 d after mice had been treated with the macrophage toxins, carrageenan (CAR) or microparticulate crystalline silica (MUS), generated a typical secondary-type antibody response. The phenomenon of pseudo-secondary responsiveness was investigated to determine a possible mechanism for its generation. The use of ι-, κ-, and λ-carrageenans and thorotrast (TT), in addition to CAR and MUS, in attempts to generate the pseudo-secondary response, indicated that lysosomal destabilization of macrophages was a necessary requisite for the generation of the response. The extent and duration of modulation of the reticuloendothelial system (RES) by CAR, MUS, and TT was measured by the ability of mice to clear colloidal carbon from the peripheral blood. It was determined that phagocytic activity had recovered by 5 d after treatment with macrophage toxins and that it remained normal or enhanced thereafter. Attempts to generate the pseudo-secondary response in mice which were genetically incapable of responding to the lipid A region of LPS were unsuccessful; thus a requirement for the genetic capability to trigger a pseudo-secondary response by LPS emerged. Administration of LPS at various intervals after a primary injection of LPS or CAR established, first, the kinetics of development of true secondary responsiveness to LPS and then the kinetics of pseudo-secondary responsiveness. The temporal kinetics of true secondary responsiveness and pseudo-secondary responsiveness were identical. The generation of pseudo-secondary responsiveness was attempted in athymic nude mice. Failure to generate the response in these mice indicated the T cell dependency of the response. It was postulated, then, that pseudo-secondary responsiveness was the result of priming of B lymphocytes by the consequences of the macrophage toxins, and secondary responsiveness to LPS was generated in a normal manner.

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ABBREVIATIONS

α	phagocytic index corrected for differences in body and organ weight
BAF	B cell activating factor
B cell	B lymphocyte
BDF	B cell differentiation factor
B1γ	TI-2 responding memory B cells
B2γ	T-dependent responding memory B cells
i-CAR	iota carrageenan
K-CAR	kappa carrageenan
λ-CAR	lambda carrageenan
CAR	Seakem carrageenan
IL-1	interleukin-1
IL-2	interleukin-2
i.p.	intraperitoneal
i.v.	intravenous
K	phagocytic index
KHF	killer cell helper factor
LAF	lymphocyte-activating factor
LPS	lipopolysaccharide
MP	mitogenic protein
MUS	microparticulate crystalline silica (Min-U-Sil)
PBS	phosphate buffered saline
PFC	plaque-forming cell
RES	reticuloendothelial system
RML	Rocky Mountain Laboratories
SCIF	secondary cytotoxic T cell inducing factor

SRBC	sheep red blood cells
T cell	T lymphocyte
TCGF	T cell growth factor
TI	T cell independent
TMF	thymocyte mitogenic factor
TSF	thymocyte stimulating factor
TT	thorotrast
2°	secondary

I. INTRODUCTION

Historical

The production of antibodies in response to a foreign substance is an integral part of an animal's immunological defense. Antibody production is a highly organized and specific system that can eliminate invading microorganisms and requires the cooperation of several cell types.

Antibodies are synthesized and released by a class of lymphocytes, called B lymphocytes, derived from bone marrow cells. At birth, an individual's B lymphocytes bear on their surfaces a repertoire of antigen-specific recognition units (66). During the course of the animal's life, contact with an antigen will cause that antigen-specific B lymphocyte to differentiate and to give rise to a clone of specific immunoglobulin-producing plasma cells. As the stimulus terminates, and antigen is no longer present, antibody synthesis declines. Many of the B lymphocytes become memory cells which, upon subsequent contact with the same antigen, can synthesize antibody rapidly (1).

Although B lymphocytes are responsible for the actual synthesis and release of antibodies, they are by no means the sole participant in the process. Thymus-derived, or T lymphocytes, play a regulatory role in humoral immunity, modulating the activities of B cells positively or negatively. A subpopulation of T lymphocytes, representing approximately one-third of the peripheral T cell population, bears Ly1 surface markers. Ly1 cells interact with antigen and aid in the

antibody response, thereby earning the name helper T cells (13, 14).

Another subpopulation of T lymphocytes, the amplifier T cells, are functionally distinct from helper T cells and act to drive antigen-stimulated B cells to further proliferation (5).

Not all T cells enhance the immune response. An additional subpopulation of T lymphocytes bear Ly2,3 surface antigens and suppress B cell activity. Suppressor T cells, together with helper and amplifier T cells, are important regulators of antibody production (13, 14).

While many antigens require the cooperation of T cells for antibody production, some antigens can stimulate the development of an immune response in the absence of T cells. Thymus-independent antigens are biochemically different from thymus-dependent antigens. Whereas T-dependent antigens are usually foreign proteins and erythrocytes, and are easily degraded, T-independent antigens generally are large polymeric molecules with repetitive identical subunits. T-independent antigens are degraded slowly and tend to persist for a long time in host tissues (6).

T-independent antigens, examples of which are pneumococcal polysaccharides, lipopolysaccharides, and polymerized flagellin, generate the production of antibodies that are predominately of the IgM class. These antigens are often B cell mitogens and may be non-immunological activators of the complement cascade (6).

Recently, it was determined that the thymus-independent (TI) antigens can be divided into two groups, based on their differing ability to stimulate B cells early or late in ontogeny and on their

ability to cause antibody formation in the immune defective CBA/N strain of mice (57, 72). Trinitrophenyl Brucella abortus (TNP-BA), TNP-lipopolysaccharide, and TNP-polyacrylamide beads are characteristic of TI-1 antigens, in that they can stimulate antibody production by early neonatal and CBA/N spleen cells. TI-2 antigens, such as TNP-Ficoll, levan, dextran, and pneumococcal polysaccharide, fail to induce an antibody response in CBA/N cells and cannot stimulate neonatal cells until 7 to 10 days after birth (72).

Tittle and Rittenburg (57) have found that memory B cell subpopulations differ in their susceptibility to stimulation by these two classes of TI antigens. TI-1 antigens can cause both B1δ (TI-2 responding) and B2δ (T-dependent responding) subpopulations of memory B cells to initiate cell division, whereas TI-2 antigens selectively stimulate B1δ subpopulations.

Suppressor T cells may aid in the regulation of thymus independent antigens. It has been shown that Ly2,3 cells block antibody formation by interference with helper T cell activity (which would suppress a response to T-dependent antigens) or by acting on B cells and plasma cells (which would suppress responses to both T-dependent and T-independent antigens) (14).

Macrophages have been known to play a part in the immune response for many years, but their precise role is still not completely understood. In the early years of immunology, macrophages were thought to be responsible for both the uptake of antigen and for the production of antibodies (58). As the science of immunology matured, lymphocytes and plasma cells were shown to be involved in the actual synthesis

of antibodies, and interest in the macrophage as an effector cell of humoral immunity subsided (58).

In the 1960s, it was observed that some antibody responses required the interaction of lymphocytes with extracts of macrophages that had previously phagocytosed antigen (22, 23). This rekindled interest in the macrophage as an effector cell in the immune response and led to the concept of antigen processing.

It is now understood that an optimal immune response to most antigens requires the cooperation of T lymphocytes, B lymphocytes, and macrophages. Using agents that suppress the reticuloendothelial system or otherwise inactivate macrophages, various investigators obtained evidence that apparently showed an absolute requirement for macrophages in immune responses. Results of these experiments showed that thymus-dependent antigens exhibited a requirement for macrophages in an immune response; however, most thymus independent antigens were also macrophage independent (32, 33, 55, 65, 69, 71). Recently, however, it was shown that some T cell-independent antigens required macrophages; apparently direct B cell-macrophage interaction was required in these immune responses (11, 32, 37).

Whereas T cells and B cells bore antigen recognition units on their surfaces and responded to stimulation by a specific antigen (45, 66), the role of the macrophage in the humoral response was nonspecific. Macrophages, present in lymphoid and nonlymphoid tissues, removed antigen from extracellular fluid nonspecifically. Schmidtke and Unanue (64), found that macrophages did not discriminate between

foreign and autologous proteins. Furthermore, antigen uptake by medullary macrophages was identical in tolerant, normal, and primed animals (31). Any discrimination between antigenic and nonantigenic molecules appeared to be only with antigen-antibody immune complexes; macrophages had surface receptors for the Fc portion of immunoglobulins (31, 54). In fact, Humphrey and Frank (31) have found that the time of localization of antigen in macrophages of the draining lymph nodes corresponded to the appearance of circulating antibody in normal rabbits. Thus, the specificity of the immune response lies entirely upon the lymphocytes precommitted to a specific antigen.

Most of the antigen removed by macrophages was catabolized to a nonimmunogenic form (34, 60). Free, circulating antigen was found to be potentially tolerogenic; therefore an important immunological function of this catabolized antigen was to aid in the circumvention of tolerance (24, 34).

Antigen that escaped catabolism was "presented" by macrophages to lymphocytes; thereby an immunogenic signal was delivered. Antigen presentation is not clearly understood; evidence has led to several models.

Macrophages retain a few molecules of antigen, undegraded or partially degraded, on their surfaces. Macrophage-associated antigen was highly immunogenic. When some antigens were administered in soluble form, a poor immune response was mounted. However, when the same antigen was bound to macrophages and then administered, a good immune response followed (38, 59). Also, it was found that immunogenicity was lost with the removal of antigen from macrophage surfaces (63).

This evidence suggested that the macrophage functions by concentrating antigen and thus promoting its necessary meeting with T and B lymphocytes.

The role of the macrophage in antigen presentation, however, was more than a mere vehicle for antigen. Evidence suggested that the macrophage plays a very active, yet not clearly defined role. For example, Mitchison (38), in macrophage-transfer experiments, found that antigen must be bound to viable macrophages for induction of a good immune response. Furthermore, additional evidence was found that macrophages played an active role in antigen presentation; donor macrophages had to be syngeneic with a recipient, in transfer experiments, for good immunogenicity to be manifested (20, 42, 43, 50).

Not all undegraded antigen was bound to the macrophage surface. Immunogenic antigen molecules were found after surface antigens were removed by proteolytic enzymes or were blocked by specific antibody (19, 62). This suggested another way in which antigen could be presented to lymphocytes by macrophages. Following the "interiorization" of antigen by macrophages, undegraded antigen molecules were released, leading to the stimulation of lymphocytes (62). Such sequestration of antigen by macrophages, followed by slow release, would allow the immune system to be stimulated over a long period of time.

Subsequent to the discovery that processing of antigen by macrophages was necessary for some antibody responses, a highly immunogenic RNA-antigen complex was isolated from extracts of macrophages that had been incubated with antigen (4, 23, 27). Macrophage

processing of antigen was not absolutely necessary to obtain this complex, but yields were significantly greater when antigen was taken up by macrophages (4, 27). Askonas and Rhodes (4) then suggested that macrophages did not form a specific new informational RNA, but processed the antigen in some way to make it more immunogenic. It was postulated by others, however, that the macrophage RNA served as a carrier or an adjuvant for the antigenic determinant and the RNA-antigen complex, although the RNA was nonspecific, could be the means by which information eliciting antibody production was processed (27). Other investigators (47, 48) subsequently found that RNA from various sources (even nonmammalian sources) could complex with antigen to form a very immunogenic "super antigen". This led some to question whether super antigens operated in the in vivo immune response (26). This matter still remains a controversial issue.

The exact role of RNA in immune processes has not yet been defined. It may function in immune induction when an antigen is introduced into the animal or may amplify the immune response by recruiting uncommitted cells receiving antibody mRNA from cells responsive to antigenic stimulation (17).

Although many of the details remain unclear, evidence has shown that the function of macrophages in immune induction is to present antigen to T and B lymphocytes. Macrophages can also influence lymphocytes by the lympho-stimulatory products that they secrete (61). The synthesis and secretion of biologically active products by macrophages has been known for some time. The list of monokines

includes neutral proteinases such as collagenase, elastase, and plasminogen activator, as well as lysozyme and various complement proteins (61).

In recent years, an increasing number of lymphocyte modulating agents have been described. Various immune functions have been ascribed to these factors by the investigators who have reported them, ranging from stimulating the proliferation of thymocytes (21, 25) to substituting for macrophages in the induction of helper T cells in plaque forming cell (PFC) responses (20).

A current concensus is that the numerous catalogue of lymphosimulatory monokines can be reduced to a single factor that has multiple functions (39). This factor, called interleukin 1 (IL-1), is produced exclusively by macrophages and is essential for the production of another factor, interleukin 2 (IL-2), by T cells. IL-2 mediates T cell proliferation. The magnitude of IL-2 production is dependent upon the quantity of IL-1 available; therefore, IL-1 and IL-2 function in a bimodal amplification system which determines the extent of T cell clonal expansion (56).

The many monokines, now determined to be the single factor, interleukin 1, included the lymphocyte-activating factor (LAF) or mitogenic protein (MP), as it is also called (21, 25). LAF stimulated the proliferation of thymocytes and enhanced their responses to lectins (21, 25). Also included in the list of macrophage products that comprised IL-1 are the B cell activating factor (BAF), which encouraged the differentiation of B cells to plasma cells (12, 70), B cell differentiation factor (BDF), and T cell replacing

factors (39).

Rather than a single factor, IL-2 was once thought to be many products of macrophage and T cell interaction. Included in this list were thymocyte stimulating factor (TSF), thymocyte mitogenic factor (TMF), T cell growth factor (TCGF), costimulator, killer cell helper factor (KHF), and secondary cytotoxic T cell inducing factor (SCIF) (39).

Characteristics of each of the interleukins are known. IL-1 is produced exclusively by macrophages, and its activity is neither H-2 nor species restricted. IL-1 is a comparatively small molecule, being 12,000 to 18,000 daltons, and does not possess the ability to maintain in vitro long term cultures of cytotoxic T cells (39).

A larger molecule (30,000 to 35,000 daltons), IL-2 required both macrophages and T cells for its production. The activity of IL-2 is H-2 unrestricted, and it can promote and maintain in vitro long term cultures of cytotoxic T cells (39). Obviously, the interleukins influence the immune response, and their role is becoming increasingly clearer.

Much information of the role of macrophages in humoral immunity has been gained since the time they were thought to synthesize antibody; still their precise function in immunity remains to be defined. Macrophages will continue to be an interesting and controversial topic of research.

Statement of Thesis and Approach to the Problem

Specific macrophage toxins have been used as tools to impair the reticuloendothelial system (RES) for investigating the role of macrophages in generating antibody responses. Recently, in the course of one such study wherein carrageenan (CAR) and microparticulate crystalline silica (MUS) were employed as macrophage toxins, a unique phenomenon was observed. While running controls for a study on the effects of the silica and carrageenan on doses of antigen that were priming mice for a secondary antibody response, Becker and Rudbach (7, 9) observed that one dose of lipopolysaccharide (LPS), given to mice exposed to macrophage toxins 21 days previously, elicited a typical secondary type response. This "pseudo-secondary" response was manifested by an increased production of total antibody and by maintenance of the high antibody titer, the characteristics of a secondary response to a T cell independent antigen.

The purpose of the present study was to explore the mechanism(s) by which the pseudo-secondary response was generated. Aspects of the pseudo-secondary response that were studied included attempts to generate the response with macrophage toxins other than MUS and CAR, examination of RES modulation by the various macrophage toxins, an investigation of the genetic requirements for generating the pseudo-secondary response, an investigation of the temporal kinetics for developing the pseudo-secondary response, and the dependency of the pseudo-secondary response on T cells.

In the investigations of Becker and Rudbach (7, 9), Seakem

carrageenan, a natural mixture of κ- and λ-carrageenans, was used. The present study examined the capability of ι-, κ-, and λ-carrageenans, as well as thorium dioxide (thorotrast) to generate pseudo-secondary responsiveness. First, however, preliminary work was required to establish proper dosage regimens for thorotrast (TT). This was necessary because the carrageenans and MUS exerted their toxic effects by destabilizing lysosomes and causing subsequent autolysis of macrophages; TT was thought to suppress RES function by a physiological blockade of macrophages (68). Once the data had been generated, then an attempt was made to correlate the mechanisms by which RES suppression was achieved with the capability of the various macrophage toxins to generate a pseudo-secondary response.

The extent and duration of RES modulation by MUS, CAR, and TT also were examined, with special consideration given to a study of the state of the systems at the time which the triggering dose of LPS was administered in the pseudo-secondary response.

The use of mice that did not respond to LPS allowed investigation of the genetic requirements of the pseudo-secondary response. The capability of macrophage toxins to "prime" these mice for secondary responsiveness was examined and compared with the genetic inability of the mice to mount a secondary anti-LPS response.

The kinetics of true secondary responsiveness were established and were compared to the kinetic requirements of pseudo-secondary responsiveness.

Finally, an attempt was made to generate the pseudo-secondary response in athymic, nude mice. Preliminary work was necessary to

establish an adequate experimental system; CAR potentiates the endotoxicity of LPS in normal mice and this potentiation was increased in nude mice. Once the system was established, the generation of pseudo-secondary responsiveness was attempted in nude mice. The capability of nude mice to generate pseudo-secondary responsiveness in the absence of T cells was compared with their capability to mount a true secondary response without the aid of T cells.

A strong correlation between true secondary responsiveness and pseudo-secondary responsiveness was observed. It was postulated that the macrophage toxins, MUS and the carrageenans, were responsible for priming mice for a secondary anti-LPS response.

II. MATERIALS AND METHODS

Animals. (i) RML mice. Male Swiss-Webster derived mice were obtained from the Rocky Mountain Laboratories (RML), Hamilton, MT. RML mice are derived from the N.NIH colony and have retained nearly the same amount and type of genetic variation found in natural murine populations (46). They were allowed standard lab chows and water ad libitum. Mice used for experiments were 4 to 8 weeks old.

(ii) Nude mice. Congenitally athymic nude mice were obtained from our colony at the University of Montana. The original stock were aquired from the Rocky Mountain Laboratories, Hamilton, MT. Nude offspring were the product of crossing heterozygous females with nude males. The heterozygous females were obtained by crossing N.NIH Swiss-Webster derived females with nude males.

All mice were housed in cages containing sterilised wood chips as litter. Mice were maintained on autoclaved food (Wayne Lab Blox Sterilizable, Chicago, IL) ad libitum.

Mice received water containing 10 mg oxytetracycline (Roche Laboratories, Nutley, NJ)/liter. This seemed to improve their general health. Nude mice were also given 312 mg metronidazole (Flagyl, Searle and Co., Columbus, OH)/liter of water for 14 days following weaning.

Six to eight week old mice of both sexes were used for investigations.

(iii) C3H/HeJ mice. Male C3H/HeJ mice were purchased from Jackson Laboratories (Bar Harbor, ME) and were maintained on standard lab chow and water ad libitum. Mice were allowed to acclimatize

for one week after their arrival before being used for investigations. Experiments were performed on 6 to 8 week old mice.

Macrophage toxins. (i) Carrageenans. Calcium carrageenan (Seakem 9), K-carrageenan (REGH 5862), λ-carrageenan (REGH 5863), and i-carrageenan (REGH 5864) were obtained from Marine Colloids (Springfield, NJ). The carrageenans were suspended in warm (56C) phosphage buffered saline (PBS) (0.15 M NaCl, 0.0033 M PO_4, pH 7.2) to the desired concentration and were used within 24 h of preparation. The appropriate suspensions were brought to 37C before intraperitoneal injection.

(ii) Microparticulate crystalline silica. Silica particles (Min-U-Sil, Whittaker, Clark and Daniels, Inc., New York, NY) less than 5 um in size were subjected to further fractionation according to the method described by Becker and Rudbach (7, 9).

Briefly, 50 g of silica (Min-U-Sil) were suspended in 500 ml of distilled, deionized water and were subjected to ultrasonic vibrations (Branson, Danbury, CT) at room temperature for 30 s. The suspension was diluted with an equal volume (500 ml) of distilled, deionized water, placed in a 1 l graduated cylinder and allowed to settle at room temperature for 24 h. Fraction III, corresponding to the 500-750 ml portion from the top, was removed and washed twice with distilled, deionized water. It was allowed to dry in a warm (60C) oven for 48 h. Prior to intravenous injection, it was resuspended to the desired concentration in warm (37C) PBS.

(iii) Thorotrast. Stabilized thorium dioxide (Thorotrast, Testagar and Co., Inc., Detroit, MI), containing 24-26% thorium

dioxide by volume (260 mg/ml), 25% aqueous dextrin, and 0.15% methylparasept, was administered intravenously. Appropriate dilutions were made with PBS.

Lipopolysaccharide. Lipopolysaccharide (LPS) was prepared from Escherichia coli 0113 (Braude strain) as a phenol-water extract by the method described by Rudbach, et al. (52). A 1 mg/ml stock solution was made by dissolving the lyophilized lipopolysaccharide in PBS. This solution was dispensed into vials and frozen at -20C until use. Appropriate dilutions of the stock solution were made immediately before use.

Collection of serum for passive hemagglutination assays. Mice were anesthetized with ether (E. R. Squibb and Sons, New York, NY) and exsanguinated by an axillary incision. The whole blood was allowed to clot at room temperature for approximately 1 h and at 4C overnight. The serum was separated from the clot by centrifugation at 1,000 x g for 10 min. It was dispensed into vials, heated at 56C for 30 min and stored at -20C.

Anti-LPS titers. Antibodies specific for LPS were determined by passive hemagglutination assays. Sheep red blood cells (SRBC), coated with LPS from E. coli 0113 were used as indicator cells. For sensitization, 1 mg of LPS was dissolved in 1 ml of 0.1 M IO_4 buffer (pH 7.2) and then placed in a boiling water bath for 2½ h. Sensitized SRBC were prepared by combining 0.25 ml of packed, washed SRBC (Colorado Serum Co., Denver, CO), 9.0 ml of PBS, and 1.0 ml of the boiled LPS. This mixture was incubated at 37C for 30 min with frequent mixing. The cells were then washed thrice in

cold PBS and finally resuspended in microtiter diluent (1% normal rabbit serum (inactivated (56C for 30 min) and adsorbed with SRBC) in PBS) to a concentration of 0.5%. The sensitized SRBC were used immediately after preparation.

Normal SRBC were prepared by resuspending packed, washed SRBC, in microtiter diluent, to a concentration of 0.5%.

To each well of a microtiter plate (Titertek, Linbro Chemical Co., Inc., New Haven, CT), 0.05 ml of diluent was added. Then, 0.05 ml of test serum was added to the first well and serial two-fold dilutions of the serum were made with 0.05 ml dilutors (Cooke Engineering Co., Alexandria, VA). In all cases, duplicate tests of each serum were performed. Next, 0.05 ml of the sensitized SRBC was added to wells 1-11 and 0.05 ml of normal SRBC was added to the 12th well of each row. Each plate was gently agitated, covered, and placed in a humid chamber. Hemagglutination was recorded after incubation for 2 h at room temperature (20-22C) and again after overnight incubation at 4C. A positive hemagglutination titer was read as the reciprocal of that dilution of serum that did not give a button control pattern.

Hemagglutination titers are expressed as values of x, derived from the equation $x = \log_2 (HD/2)$, where HD was the reciprocal of the highest dilution of serum that produced hemagglutination of the sensitized SRBC. Thus, the titer is the tube number of the endpoint when the first well contained a 1/4 dilution of antiserum. Sera that gave no hemagglutination at the lowest dilution were arbitrarily assigned a titer of 0, ie., a dilution of 1/2. Titers

were expressed as the geometric mean of the duplicate tests.

Measurement of phagocytic activity by RES clearance of colloidal carbon. Phagocytic activity was measured by the ability of normal and macrophage toxin-treated mice to clear carbon particles from the peripheral blood. Colloidal carbon (Pelikan C11/1431a, Koh-I-Noor, Bloomsbury, NJ) was stabilized with 1% gelatin (Baker Chemical Co., Phillipsburg, NJ) and was injected intravenously as a volume of 0.1 ml that contained 16.9 mg of carbon. At 5 min and 15 min after the injection of carbon, mice were anesthetized with ether (E. R. Squibb and Sons, New York, NY) and 0.02 ml of blood was removed from the retro-orbital plexus in heparinized capillary tubes (Capillary micro hematocrit 73810, Kimble, Toledo, OH). Blood was lysed in 2.0 ml of 0.1% Na_2CO_3 (J. T. Baker Chemical Co., Phillipsburg, NJ). Optical densities of the lysed blood samples were measured at 650 nm in a spectrophotometer (Beckman DU-2, Beckman Instruments, Inc., Palo Alto, CA). After the 15 min bleeding, the mice were sacrificed and the wet weights of the livers and spleens were determined.

The phagocytic index (K) was determined for each mouse by the following formula:

$$K = \frac{\log_{10} \text{OD at 5 min} - \log_{10} \text{OD at 15 min}}{10 \text{ min}}$$

The phagocytic index corrected for differences in body and organ weight (α) was determined for each mouse by the following formula:

$$\alpha = \sqrt[3]{\frac{K \times (\text{liver weight} + \text{spleen weight})}{\text{total body weight}}}$$

III. RESULTS

Determination of amount of thorotrast to suppress the RES.

Extensive work by other investigators had established the standard dosages for CAR and MUS (7,9). Only limited reports in older literature had described the use of TT to suppress the RES in mice; therefore, preliminary experiments sought to determine effective doses for TT. Alteration of RES activity was determined by comparing, with normal mice, the ability of test mice to clear carbon particles from the peripheral blood 24 h after the final injection of TT. Fig. 1 shows the effect of varying amounts of TT, administered i.p., on the clearance of colloidal carbon. Significant RES suppression occurred with 250 mg doses of TT. However, even multiple i.p. injections of 150 mg TT on consecutive days did not alter RES activity.

The injection of varying amounts of TT i.v. also influenced phagocytic activity, as shown in Fig. 2. Doses of 50 mg and 100 mg TT i.v. were comparable in suppressing RES function. Again, the phagocytic activity of mice that were given multiple injections of 50 mg TT i.v. on successive days was similar to those mice that received PBS. With consideration given to the endotoxicity potentiating effects of thorium dioxide (8), 50 mg i.v. of TT was the chosen dose to be used. This was a compromise between an amount that would suppress effectively the RES activity, but would not potentiate, too greatly, endotoxicity.

Fig. 1. RES activity following injection of varying amounts of TT i.p. RES activity was measured by the ability of test mice to clear carbon particles from the peripheral blood. Mice were tested 24 h after the final injection of TT. Each value represents the arithmetic mean of two mice.

Symbols:

Open bars:	Single injection of TT
Vertical crosshatches:	TT given on two consecutive days
Horizontal crosshatches:	TT given on five consecutive days

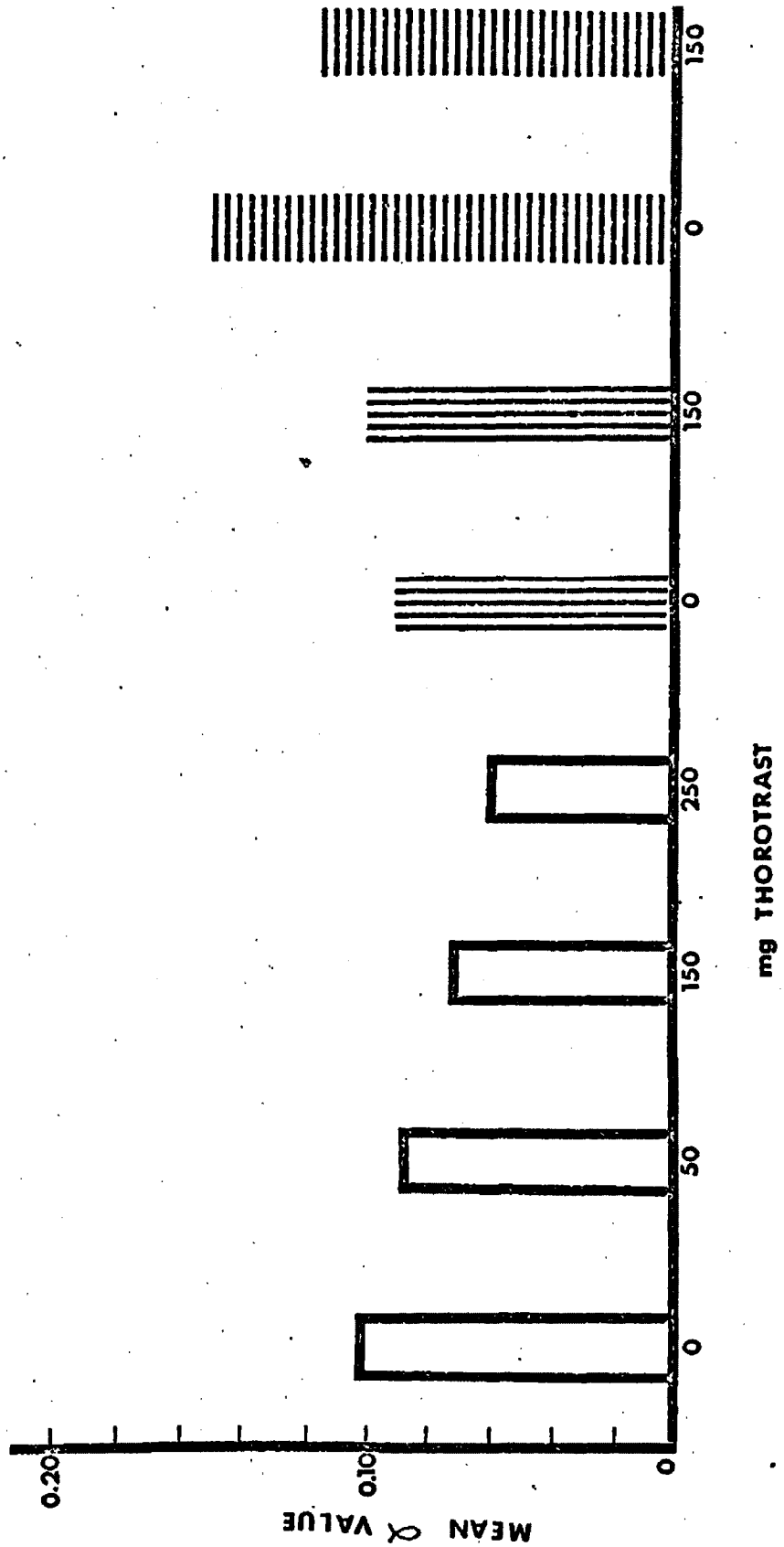
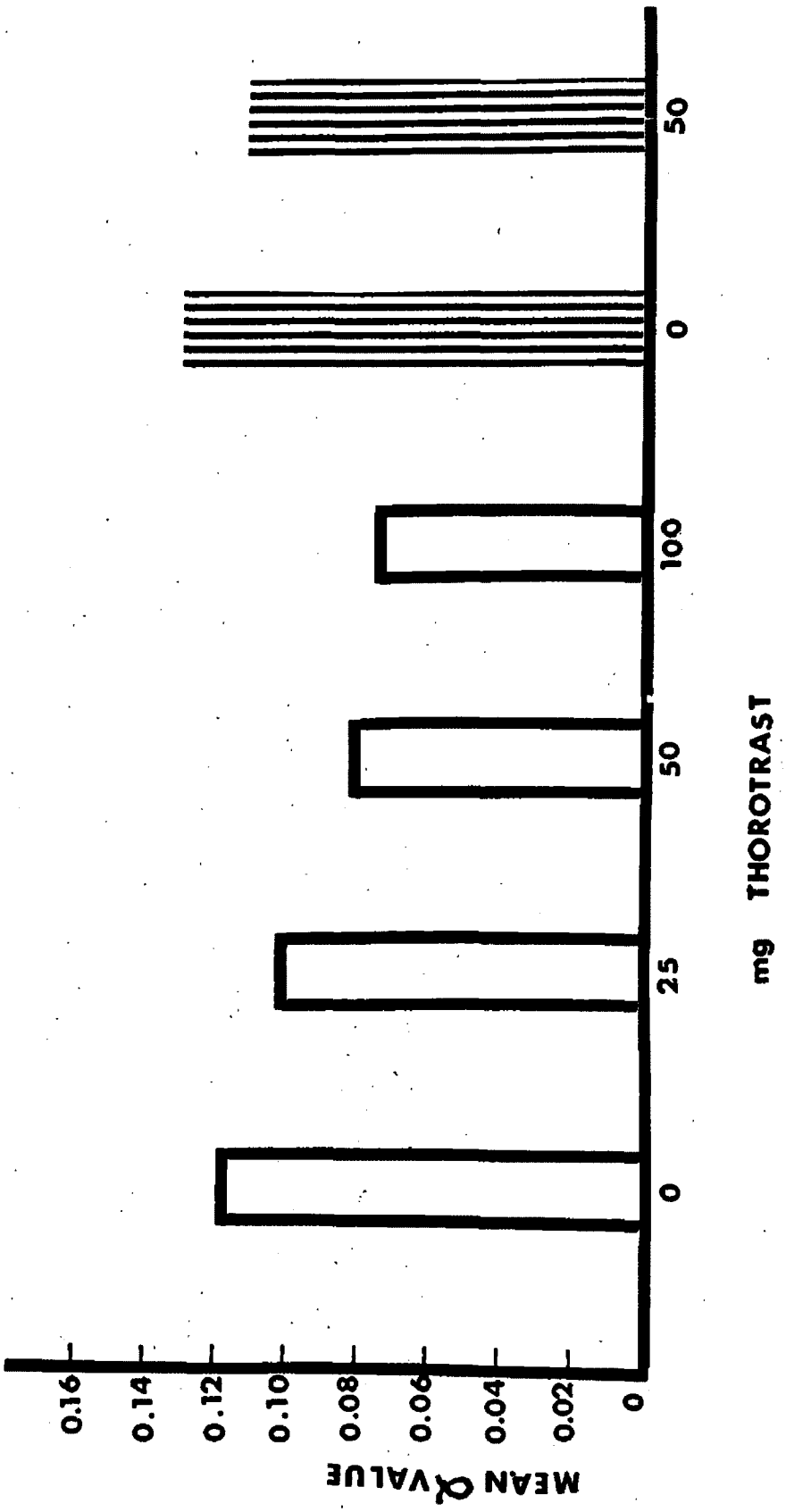


Fig. 2. RES activity following injection of varying amounts of TT i.v. RES activity was measured by the ability of test mice to clear carbon particles from the peripheral blood. Mice were tested 24 h after the final injection of TT. Each value represents the arithmetic mean of two mice.

Symbols:

Open bars: Single injection of TT

Vertical crosshatches: TT given on two consecutive days



Attempts to generate a pseudo-secondary response with various macrophage toxins. It had been shown previously by Becker and Rudbach (7, 9) that a single dose of LPS, given to mice exposed to MUS or CAR 21 days earlier, elicited a typical secondary type response. This "pseudo-secondary" response could not be generated in the absence of the macrophage toxins (ie. when PBS was administered in place of MUS or CAR); therefore, it became of interest to determine the role the macrophage toxins played in the generation of the pseudo-secondary response. The use of macrophage toxins that would suppress the RES by a mechanism other than that employed by MUS and CAR was felt to be an important step in the investigation of the role of the macrophage toxin in the pseudo-secondary response.

In an attempt to re-establish the system, groups of 5 mice were treated with MUS or CAR on day 0 and then were given one or two injections of LPS. Single doses of LPS were given either at 6 h after treatment with macrophage toxin or on day 21. Mice receiving two injections of LPS were injected 6 h after treatment with macrophage toxins and again on day 21. Control mice were given PBS on day 0 and LPS at the times designated. Sera were collected on day 25.

Tables 1 and 2 show that mice given MUS or CAR on day 0 and a single dose of LPS 21 days later do indeed generate a pseudo-secondary antibody response (groups A). The magnitude of this response was comparable to a true secondary response (ie. a response to primary and secondary injections of LPS) (groups E and F). The pseudo-secondary response of groups A contrast sharply with the

Table 1

Generation of a pseudo-secondary response with silica (MUS) in white mice.

GROUP	TREATMENT[a]	1° [b]	2° [c]	AP TITER[d]
	DAY 0	DAY 0	DAY 21	DAY 25
A	MUS	NONE	LPS	10
B	PBS	NONE	LPS	5.5
C	MUS	LPS	NONE	5
D	PBS	LPS	NONE	6
E	MUS	LPS	LPS	10.5
F	PBS	LPS	LPS	11

[a] 10 mg MUS i.v.
[b] 0.1 ug LPS i.v. 6 h after MUS or PBS
[c] 1.0 ug LPS i.p.
[d] Antibody titers were obtained by passive hemagglutination tests. Each value represents the antibody titer of sera pooled from five mice. Titers are expressed as values of x, derived from the equation, $x = \log_2 (HD/2)$, where HD was the reciprocal of the highest dilution of sera that produced hemagglutination.

Table 2

Generation of a pseudo-secondary response with Seakem carrageenan (CAR) in white mice.

GROUP	TREATMENT[a]	1° [b]	2° [c]	AB TITER[d]
	DAY 0	DAY 0	DAY 21	DAY 25
A	CAR	NONE	LPS	10.5
B	PBS	NONE	LPS	7.5
C	CAR	LPS	NONE	8.5
D	PBS	LPS	NONE	5
E	CAR	LPS	LPS	11
F	PBS	LPS	LPS	10.5
G	CAR	NONE	NONE	2.5
H	PBS	NONE	NONE	0

[a] 5.0 mg CAR i.p.
[b] 0.1 ug LPS i.v. 6 h after CAR or PBS
[c] 1.0 ug LPS i.p.
[d] Antibody titers were obtained by passive hemagglutination tests. Each value represents the antibody titer of sera pooled from 5 mice. Titers are expressed as values of x, derived from the equation, $x = \log_2 (HD/2)$, where HD was the reciprocal of the highest dilution of sera that produced hemagglutination.

response of control mice that received PBS on day 0 and the single injection of LPS on day 21 (groups B).

Once the system was re-established, the use of macrophage toxins other than MUS or CAR were employed in attempts to generate pseudo-secondary responses. Various isomers of carrageenan exist which suppress the RES in a manner similar to CAR (ie. by lysosomal destabilization). i-, κ-, and λ-carrageenans were given to groups of 5 mice on day 0. LPS was administered on day 21. It can be seen in Tables 3, 4, and 5 that, like CAR, the i, κ, and λ isomers of carrageenan would also elicit pseudo-secondary responses (groups A). These responses also mirrored true secondary responses in magnitude (groups E and F). Contol mice, receiving PBS on day 0 and LPS on day 21 (groups B) responded in a primary fashion, thus indicating that the secondary responsiveness of groups A was generated by the i-, κ-, and λ-carrageenans.

The carrageenans and MUS suppressed the RES by lysosomal destabilization and concomitant cytotoxicity (3, 15). To determine if the mechanism of RES suppression by macrophage toxins played a part in the generation of the pseudo-secondary response, TT was employed. TT is a macrophage toxin that suppressed phagocytic activity by RES blockade; no cytotoxicity was observed (68). Table 6 demonstrated that mice given TT on day 0 and LPS on day 21 do not generate a pseudo-secondary response (group A). The anti-LPS response of these mice was essentially the same as the mice that received PBS on day 0 and LPS on day 21 (group B). Mice that were given PBS or TT and two injections of LPS (groups E and F) generated

Table 3

Generation of a pseudo-secondary response with i-carrageenan (i-CAR) in white mice.

GROUP	TREATMENT[a]	1° [b]	2° [c]	AB TITER[d]
	DAY 0	DAY 0	DAY 21	DAY 25
A	i-CAR	NONE	LPS	9
B	PBS	NONE	LPS	7.5
C	i-CAR	LPS	NONE	4
D	PBS	LPS	NONE	5
E	i-CAR	LPS	LPS	11
F	PBS	LPS	LPS	10.5
G	i-CAR	NONE	NONE	1.5
H	PBS	NONE	NONE	0

[a] 5.0 mg i-CAR i.p.
[b] 0.1 ug LPS i.v. 6 h after i-CAR or PBS
[c] 1.0 ug LPS i.p.
[d] Antibody titers were obtained by passive hemagglutination tests. Each value represents the antibody titer of sera pooled from five mice. Titers are expressed as vlaues of x, derived from the equation, $x = \log_2 (HD/2)$, where HD was the reciprocal of the highest dilution of sera that produced hemagglutination.

Table 4

Generation of a pseudo-secondary response with K-carrageenan (K-CAR) in white mice.

GROUP	TREATMENT[a]	1° [b]	2° [c]	AB TITER[d]
	DAY 0	DAY 0	DAY 21	DAY 25
A	K-CAR	NONE	LPS	11
B	PBS	NONE	LPS	5.5
C	K-CAR	LPS	NONE	6
D	PBS	LPS	NONE	6
E	K-CAR	LPS	LPS	10.5
F	PBS	LPS	LPS	11

[a] 5.0 mg K-CAR i.p.
[b] 0.1 ug LPS i.v. 6 h after K-CAR or PBS
[c] 1.0 ug LPS i.p.
[d] Antibody titers were obtained by passive hemagglutination tests. Each value represents the antibody titer of sera pooled from 5 mice. Titers are expressed as values of x, derived from the equation, $x = \log_2 (HD/2)$, where HD was the reciprocal of the highest dilution of sera that produced hemagglutination.

Table 5

Generation of a pseudo-secondary response with λ-carrageenan (λ-CAR) in white mice.

GROUP	TREATMENT[a]	1° [b]	2° [c]	AB TITER[d]
	DAY 0	DAY 0	DAY 21	DAY 25
A	λ-CAR	NONE	LPS	8
B	PBS	NONE	LPS	5.5
C	λ-CAR	LPS	NONE	7
D	PBS	LPS	NONE	6
E	PBS	LPS	LPS	11

[a] 5.0 mg λ-CAR i.p.
[b] 0.1 ug LPS i.v. 6 h after λ-CAR or PBS
[c] 1.0 ug LPS i.p.
[d] Antibody titers were obtained by passive hemagglutination tests. Each value represents the antibody titer of sera pooled from 5 mice. Titers are expressed as values of x, derived from the equation, $x = \log_2 (HD/2)$, where HD was the reciprocal of the highest dilution of sera that produced hemagglutination.

Table 6

Attempt to generate a pseudo-secondary response with thorotrast (TT) in white mice.

GROUP	TREATMENT[a]	1° [b]	2° [c]	AB TITER[d]
	DAY 0	DAY 0	DAY 21	DAY 25
A	TT	NONE	LPS	5
B	PBS	NONE	LPS	6.5
C	TT	LPS	NONE	5
D	PBS	LPS	NONE	4.5
E	TT	LPS	LPS	10
F	PBS	LPS	LPS	9.5
G	TT	NONE	NONE	0
H	PBS	NONE	NONE	2

[a] 50 mg TT i.v.
[b] 0.1 ug LPS i.v. 6 h after TT or PBS
[c] 1.0 ug LPS i.p.
[d] Antibody titers were obtained by passive hemagglutination tests. Each value represents the antibody titer of sera pooled from 3 mice. Titers are expressed as values of x, derived from the equation, $x = \log_2 (HD/2)$, where HD was the reciprocal of the highest dilution of sera that produced hemagglutination.

true secondary responses. Thus, it appeared that the lysosomal destabilization of macrophages was essential for the generation of a pseudo-secondary response.

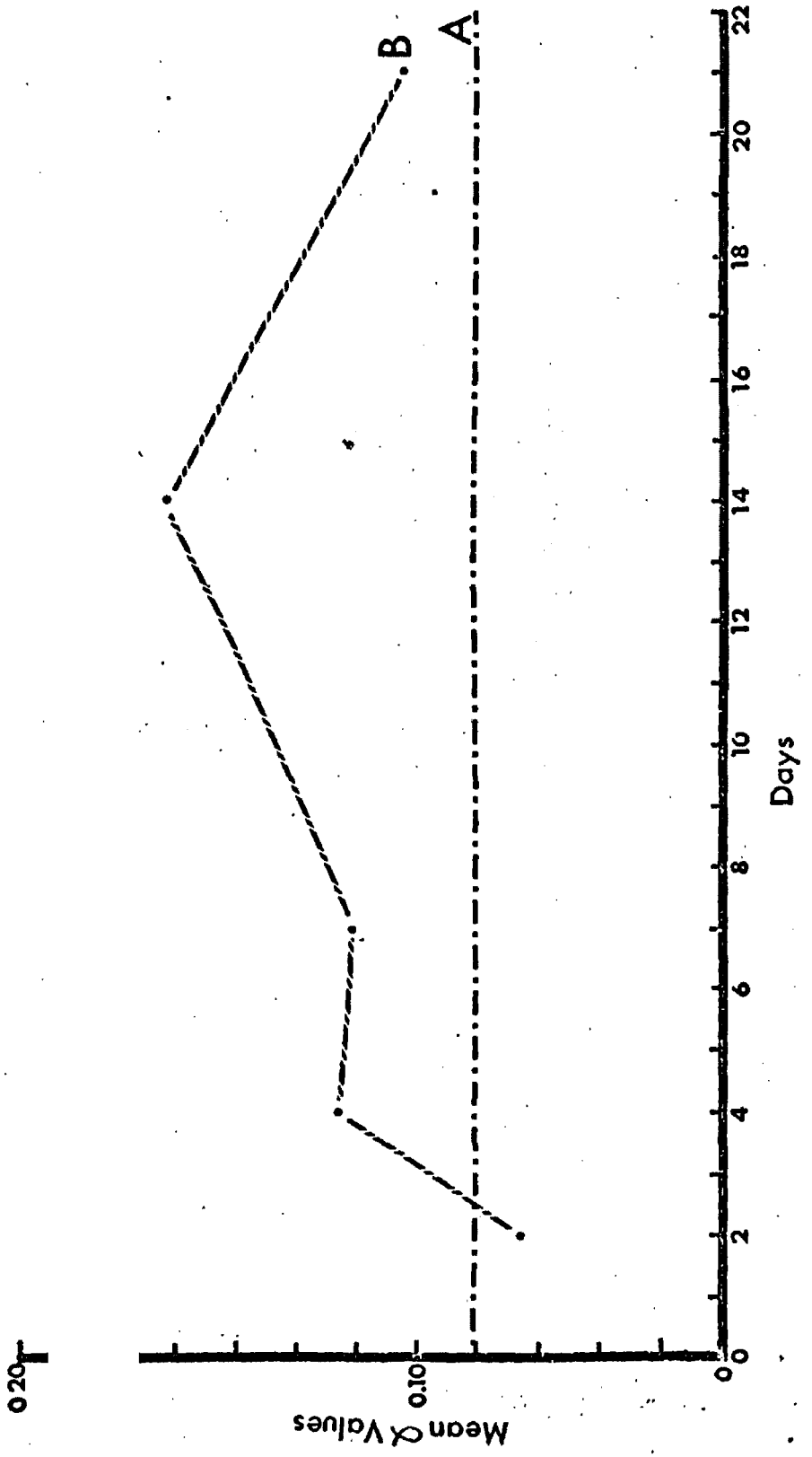
Duration of modulation of RES activity by macrophage toxins. The use of various macrophage toxins in attempts to generate pseudo-secondary responses had indicated that lysosomal destabilization and the concomitant cytotoxicity of macrophages was a requirement for the generation of a pseudo-secondary response. Earlier work by Becker and Rudbach (7, 9), however, had shown that by 4 or 5 days after treatment with MUS or CAR, RES function had returned to normal. Therefore, RES activity was followed for 21 days after treatment with macrophage toxins in order to determine if RES function was still normal at the time the eliciting dose of LPS was given, or if phagocytic activity had again become suppressed, thereby allowing a bolus of antigen to stimulate the antibody producing cells. The macrophage toxins, MUS, CAR, and TT were used to contrast the functional state of the RES at various times after macrophage toxin administration with the means by which RES modulation was achieved (cell cytotoxicity vs. RES blockade). It was assumed that the Seakem carrageenan would produce results similar to the isomers of carrageenan; therefore, the latter were omitted in this study. Groups of three mice were given macrophage toxins on day 0. Phagocytic activity was assessed by the ability of mice to clear carbon particles from the peripheral blood. Control mice received PBS on day 0. Fig. 3 shows the RES activity of mice after receiving MUS. By day 3, phagocytic function had returned to normal, and by day 4 and thereafter,

Fig. 3. Duration of modulation of RES activity following i.v. injection of 10 mg of KUS. RES activity was measured by the ability of test mice to clear carbon particles from the peripheral blood. Mice were given silica at time 0 and were tested on the days indicated. Control mice received PBS at time 0. Each value represents the arithmetic mean of three mice.

Symbols:

Line A: Control mice

Line B: Test mice



phagocytic function was even enhanced. Thus, on day 21, the day the triggering dose of LPS for the pseudo-secondary response was given, phagocytic activity was greater than normal. Similarly, in Fig. 4, it is seen that on day 4, following treatment with CAR, RES function had returned to normal. Thereafter an enhancement of phagocytic activity was observed.

Treatment of mice with TT suppressed RES function for 50 h (Fig. 5). Fig. 6 shows that after day 2 phagocytic function had recovered and was even enhanced. Thus, the state of modulation of the RES was similar for the 21 days following the administration of CAR, MUS or TT, although the toxins affected modulation of the RES by different mechanisms.

The genetic requirements for generating pseudo-secondary responses: attempt to generate the response in LPS nonresponder mice. C3H/HeJ mice can mount a primary response to LPS, but lack the genetic capability to produce a secondary response (53). Experiments were designed to test whether C3H/HeJ mice had the genetic capability to generate a pseudo-secondary response.

Groups of 3 mice were given MUS or CAR on day 0 and LPS on day 21. Control mice received PBS on day 0 and LPS at the appropriate times. Sera was collected on day 25. Table 7 shows the antibody responses of mice that received MUS on day 0. Mice that were given MUS on day 0 and LPS on day 21 (group A) did not generate a pseudo-secondary response. The titer of that group was similar to the primary responses in control groups (groups B, D, and E). Likewise, the data in Table 8 demonstrate that mice that were given

Fig. 4. Duration of modulation of RES activity following i.p. injections of 5.0 mg CAR. RES activity was measured by the ability of test mice to clear carbon particles from the peripheral blood. Mice were given CAR at time 0 and were tested on the days indicated. Control mice received PBS at time 0. Each value represents the arithmetic mean of 3 mice.

Symbols:

Line A: Control mice

Line B; Test mice

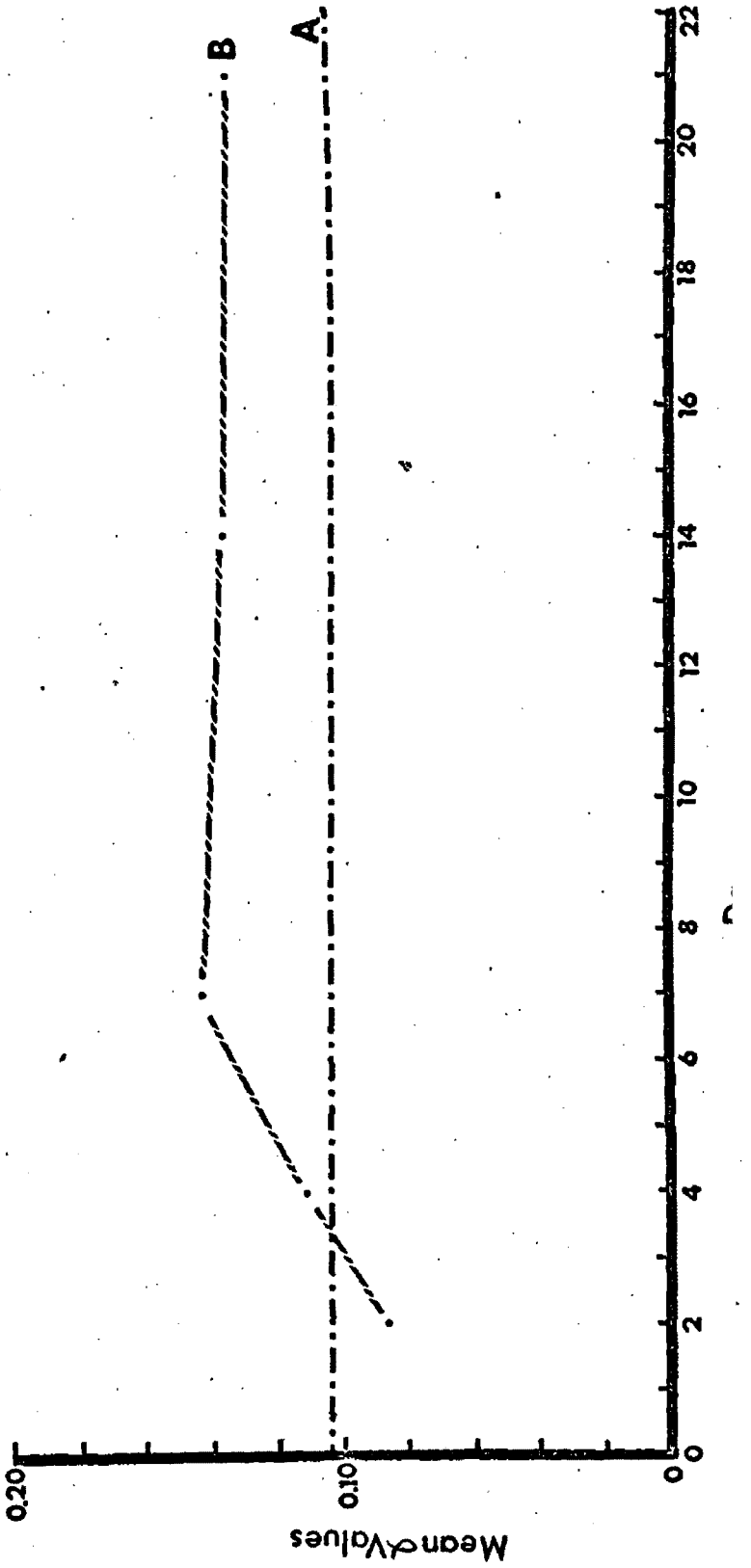


Fig. 5. Duration of modulation of RES activity following i.v. injection of 50 mg TT. RES activity was measured by the ability of test mice to clear carbon particles from the peripheral blood. Mice were given TT at time 0 and were tested at the times indicated. Control mice received PBS at time 0. Each value represents the arithmetic mean of 2 or 3 mice.

Symbols:

Line A: Control mice

Line B: Test mice

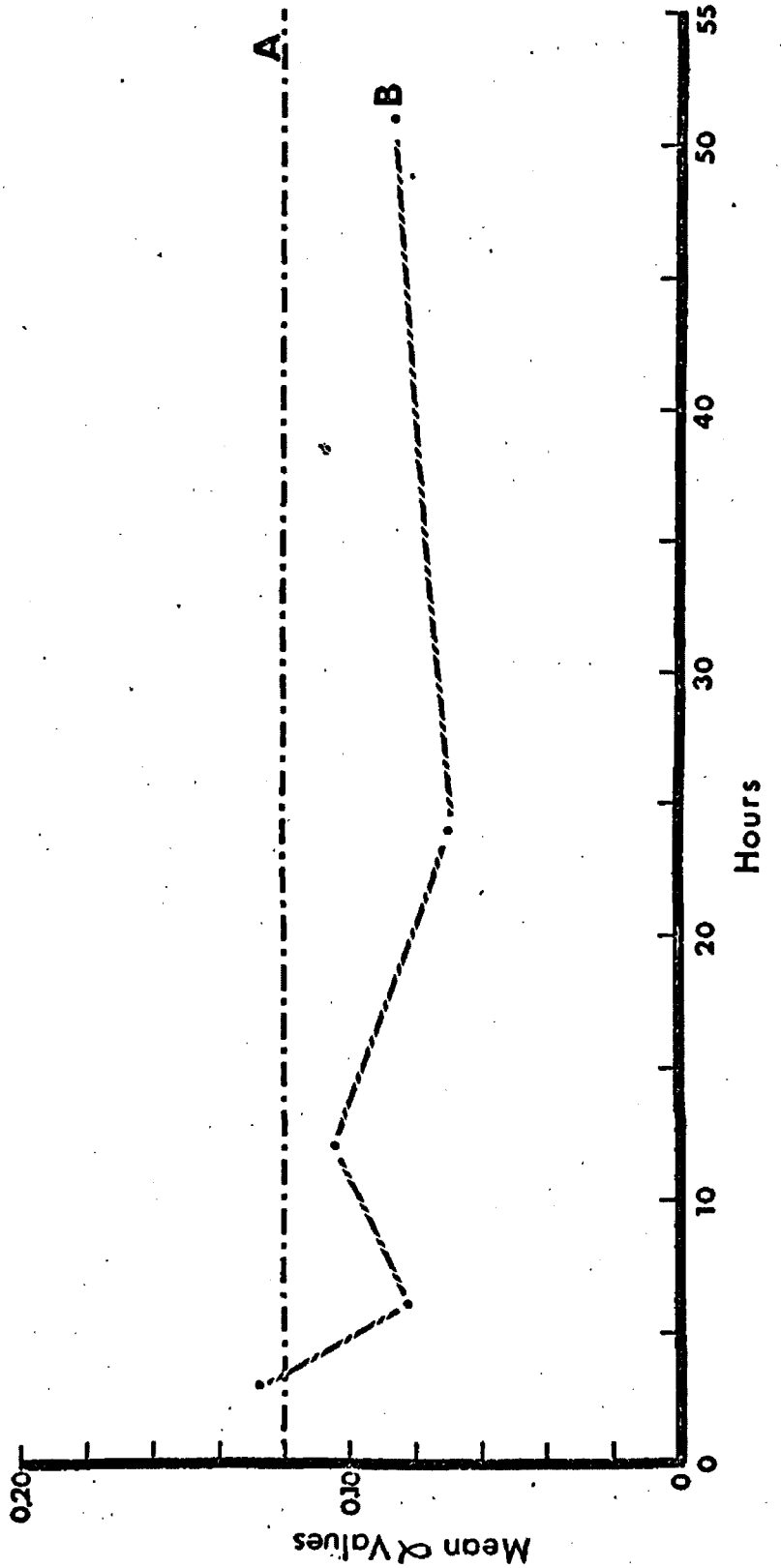


Fig. 6. Duration of modulation of RES activity following i.v. injection of 50 mg TT. RES activity was measured by the ability of test mice to clear carbon particles from the peripheral blood. Mice were given TT at time 0 and were tested at the times indicated. Control mice received PBS at time 0. Each value represents the arithmetic mean of 2 or 3 mice.

Symbols:

Line A: Control mice

Line B: Test mice

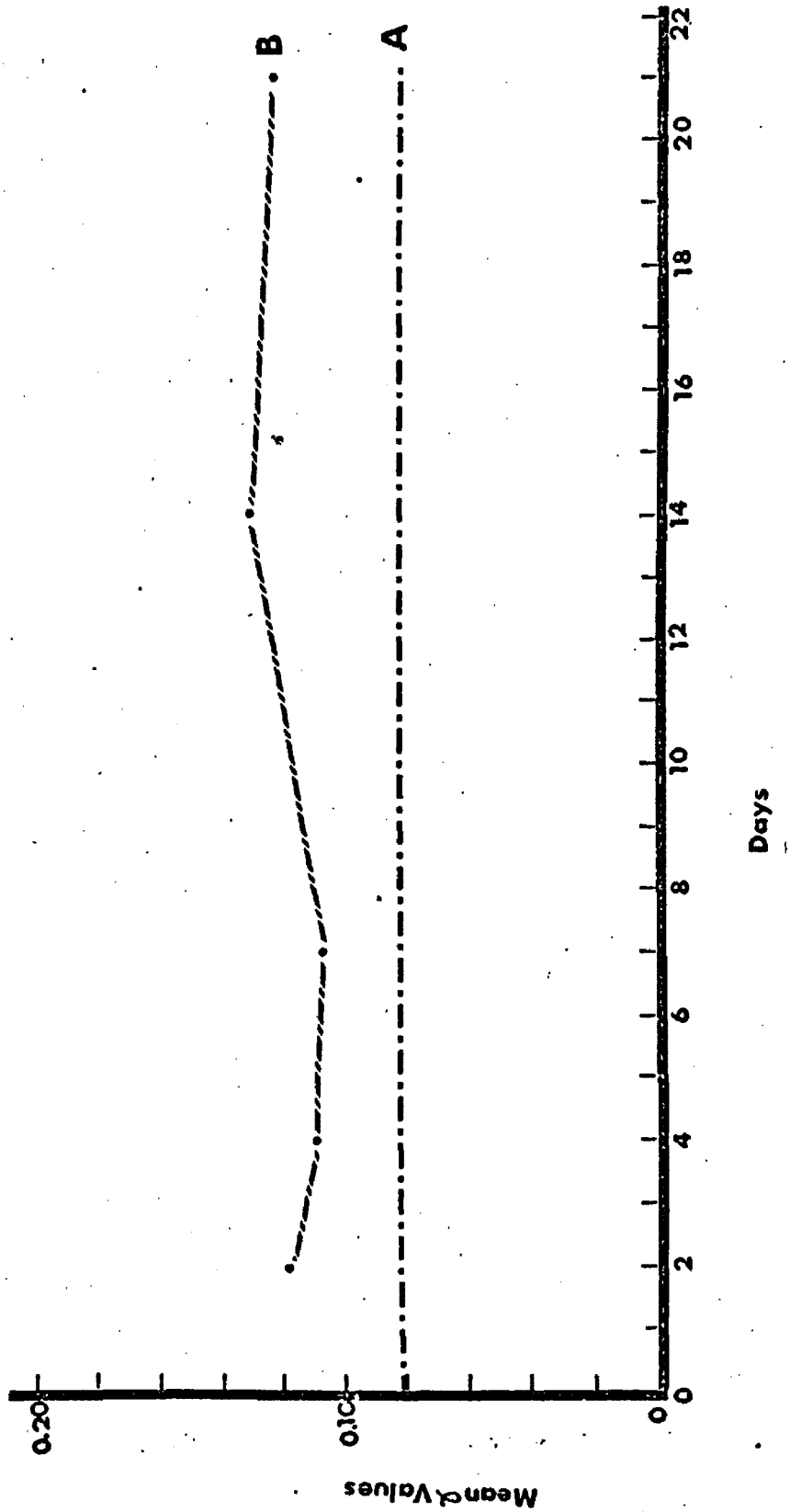


Table 7

Attempt to generate a pseudo-secondary response with silica (MUS) in LPS nonresponder mice (ie. C3H/HeJ mice).

GROUP	TREATMENT[a]	1° [b]	2° [c]	AB TITER[d]
	DAY 0	DAY 0	DAY 21	DAY 25
A	MUS	NONE	LPS	4.5
B	PBS	NONE	LPS	4.5
C	PBS	NONE	NONE	0
D	PBS	LPS	NONE	2
E	PBS	LPS	LPS	4.5

[a] 8.0 mg MUS i.v.
[b] 0.1 ug LPS i.v.
[c] 1.0 ug LPS i.p.
[d] Antibody titers were obtained by passive hemagglutination tests. Each value represents the antibody titer of sera pooled from 3 mice. Titers are expressed as values of x, derived from the equation, $x = \log_2 (HD/2)$, where HD was the reciprocal of the highest dilution of sera that produced hemagglutination.

Table 8

Attempt to generate a pseudo-secondary response with Seakem carrageenan (CAR) in LPS nonresponder mice (ie. in C3H/HeJ mice).

GROUP	TREATMENT[a]	1°[b]	2°[c]	AB TITER[d]
	DAY 0	DAY 0	DAY 21	DAY 25
A	CAR	NONE	LPS	4.5
B	PBS	NONE	LPS	4.5
C	CAR	NONE	NONE	0
D	PBS	NONE	NONE	0
E	CAR	NONE	CAR	0
F	PBS	LPS	NONE	4.5
G	PBS	LPS	LPS	4

[a] 5.0 mg CAR
[b] 0.1 ug LPS i.v.
[c] 1.0 ug LPS i.p.
[d] Antibody titers were obtained by passive heragglutination tests. Each value represents the antibody titer of sera pooled from 2 or 3 mice. Titers are expressed as values of x, derived from the equation, $x = \log_2 (HD/2)$, where HD was the reciprocal of the highest dilution of sera that produced hemagglutination.

CAR on day 0 and LPS on day 21 (group A), produced anti-LPS titers that were comparable to the primary titers of control groups (groups B, F, and G).

These data indicated that if mice did not possess the genetic capability of exhibiting secondary responsiveness to LPS, they could not generate pseudo-secondary responsiveness to LPS. Therefore, the genetic requirements for a pseudo-secondary response mimiced those requirements for a true secondary response.

Temporal kinetics for generating secondary responsiveness. Before the kinetics for generating pseudo-secondary responsiveness could be examined, it was necessary to re-establish the temporal requirements for generating a true secondary response. After this was established, kinetic comparisons of pseudo-secondary responsiveness with true secondary responsiveness could be made.

Groups of three mice were given primary injections of LPS on day 0 and secondary injections of LPS on varying days after the primary injections. Sera were collected 4 days after the final injections of LPS.

Data from Fig. 7 show that a second injection of LPS would not generate a secondary response until approximately the 9th day after the primary injection. Thereafter, anti-LPS titers increased until the end of the experiment, 21 days after the primary injection.

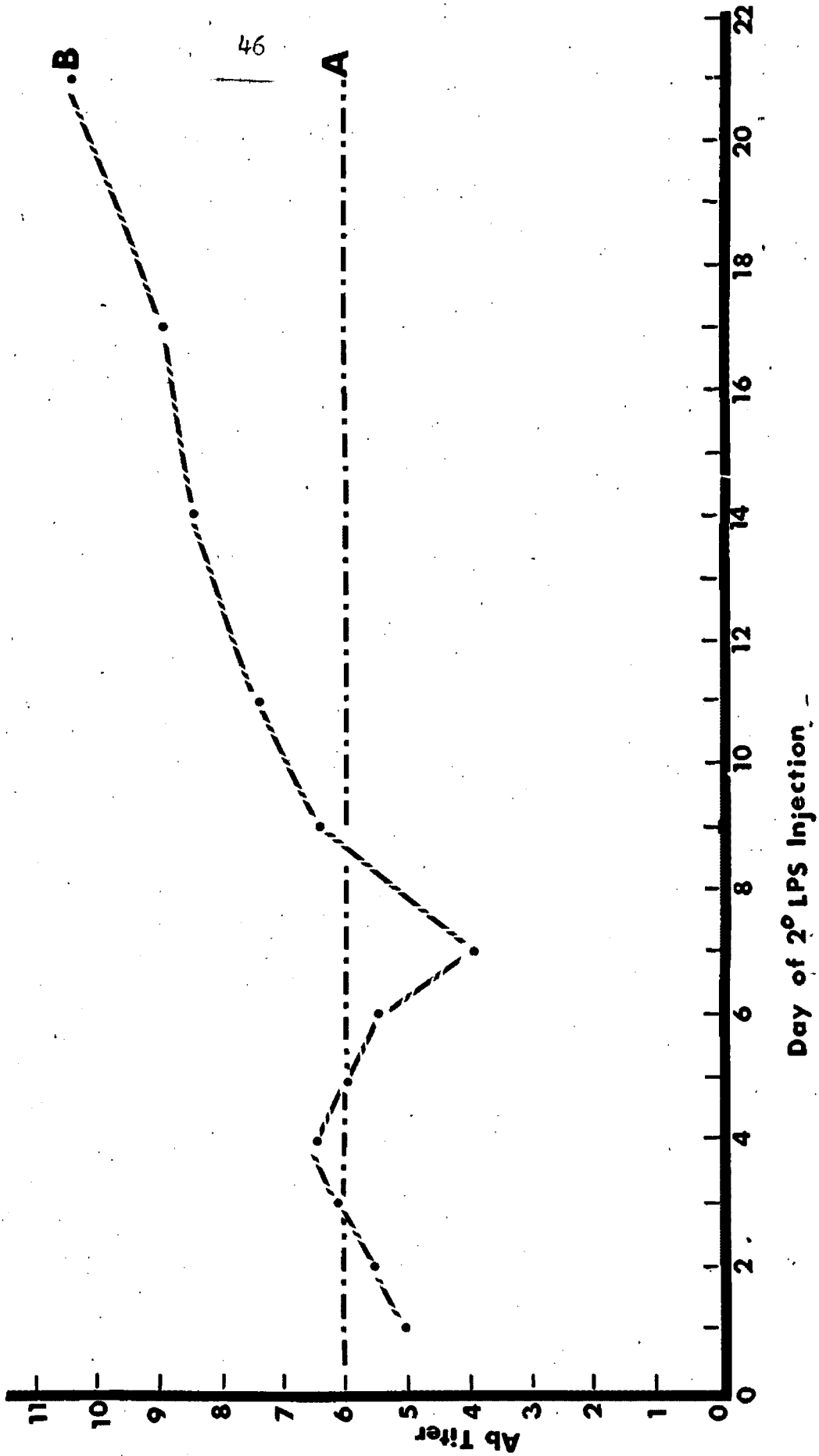
Kinetics of the pseudo-secondary response. Once the kinetics for generating a true secondary response were established, the kinetics of the pseudo-secondary response could be examined. These data might indicate a similarity in the mechanisms by which the

Fig. 7. The effect of varying the timing of the secondary injection of LPS with respect to the primary injection of LPS. 1.0 ug LPS was given i.p. on day 0. A second, 1.0 ug injection was administered i.p. on the days indicated. Control mice received only a single 1.0 ug dose of LPS on day 0. Mice were bled 4 days after the final injection of LPS. Antibody titers were obtained by passive hemagglutination tests. Each value represents the antibody titer of sera pooled from three mice. Titers are expressed as values of x, derived from the equation, $x = \log_2 (HD/2)$, where HD was the reciprocal of the highest dilution of sera that produced hemagglutination.

Symbols:

Line A: Control mice

Line B: Test mice



two responses were generated. Therefore, groups of four mice were given CAR on day 0. LPS was administered at three day intervals after the injection of the macrophage toxin and sera were collected 4 days after the injection of LPS. Fig. 8 shows that a pseudo-secondary response could not be mounted before approximately the 9th day after treatment with CAR. Peak secondary anti-LPS titers were seen on day 12 and thereafter. It was concluded that the kinetics of a pseudo-secondary response generated with CAR were similar to the kinetics of a true secondary response. Therefore, the manner by which the pseudo-secondary response was generated required timing that was similar to that required by the mechanism generating true secondary responsiveness.

Modulation of RES activity by CAR in athymic nude mice. Before the generation of pseudo-secondary responsiveness could be attempted in nude mice, it was necessary to determine if RES suppression could be achieved with the standard doses of macrophage toxins. MUS was found to be toxic to the nude mice at doses that suppressed RES function; therefore, MUS was omitted in the following investigati

Groups of mice were given the standard dose of CAR at time 0. Phagocytic activity was assessed by the ability of mice to clear colloidal carbon from the peripheral blood until 72 h after the administration of CAR. The phagocytic function of mice treated with CAR was compared with the phagocytic function of both nude mice that received PBS at time 0 and normal littermate mice that received CAR or PBS at time 0. On Fig. 9 it can be seen that the RES function of the nude mice that received CAR was initially suppres

Fig. 8. The effect of varying the timing of the administration of LPS with respect to the administration of Seakem carrageenan (CAR). CAR was given on day 0 and LPS was administered on the days indicated. Mice were bled 4 days after the injection of LPS. Antibody titers were obtained by passive hemagglutination tests. Each value represents the antibody titer of sera pooled from four mice. Titers are expressed as values of x, derived from the equation, $x = \log_2 (HD/2)$, where HD was the reciprocal of the highest dilution of sera that produced hemagglutination.

Symbols:

Line A: PBS day 0; 0.1 ug LPS day 0

Line B: PBS day 0; 1.0 ug LPS day 21

Line C: PBS day 0; 0.1 ug LPS day 0; 1.0 ug LPS day 21

Line D: 5.0 mg CAR day 0; 1.0 ug LPS on the days indicated

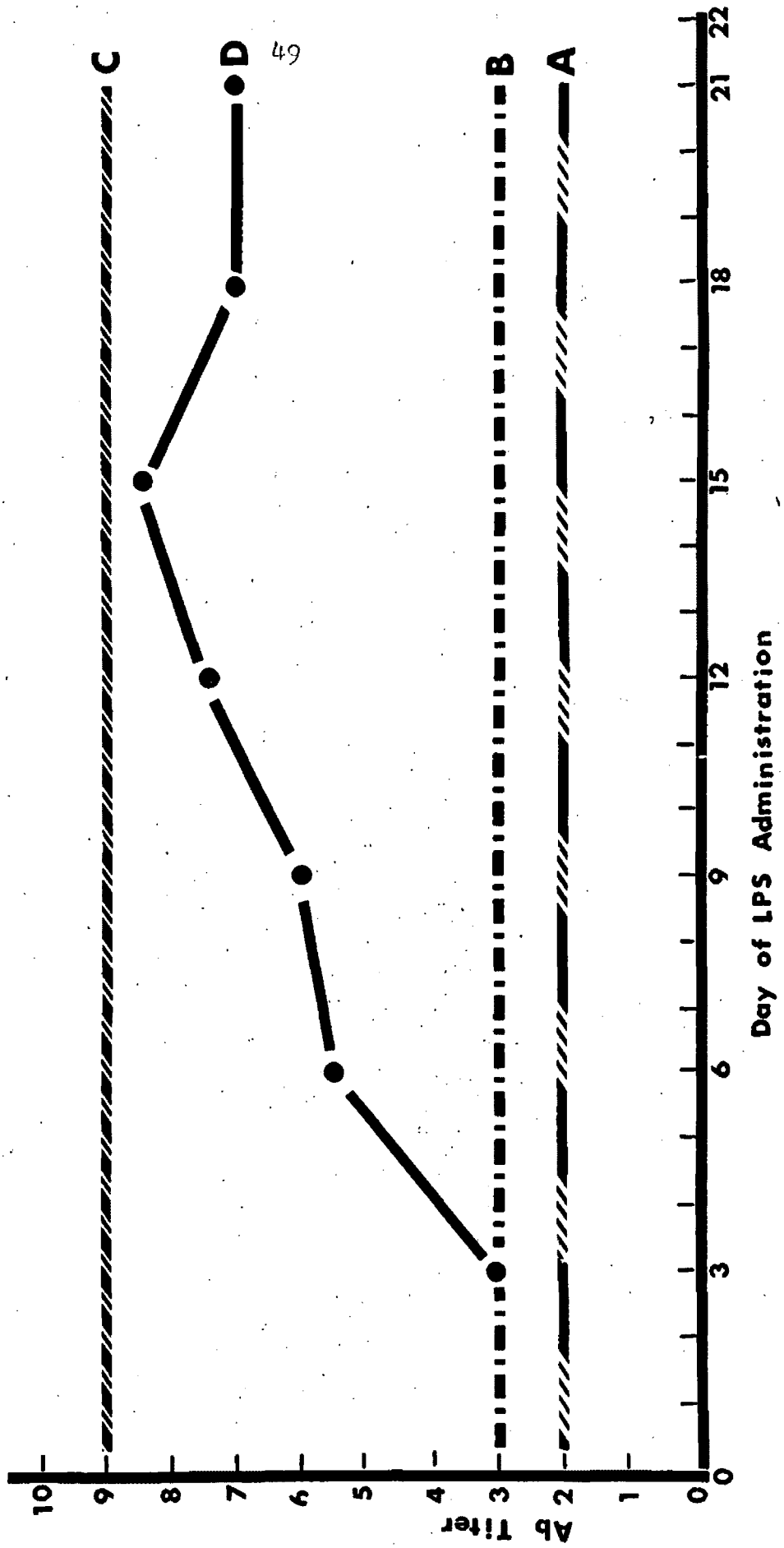


Fig. 9. Modulation of RES activity by Seakem carrageenan (CAR) in athymic nude mice and their normal littermates. RES activity was measured by the ability of mice to clear carbon particles from the peripheral blood. Mice were given 5.0 mg CAR i.p. at time 0 and were tested at the times indicated. Control mice received PBS at time 0. Each value represents the arithmetic mean of two mice.

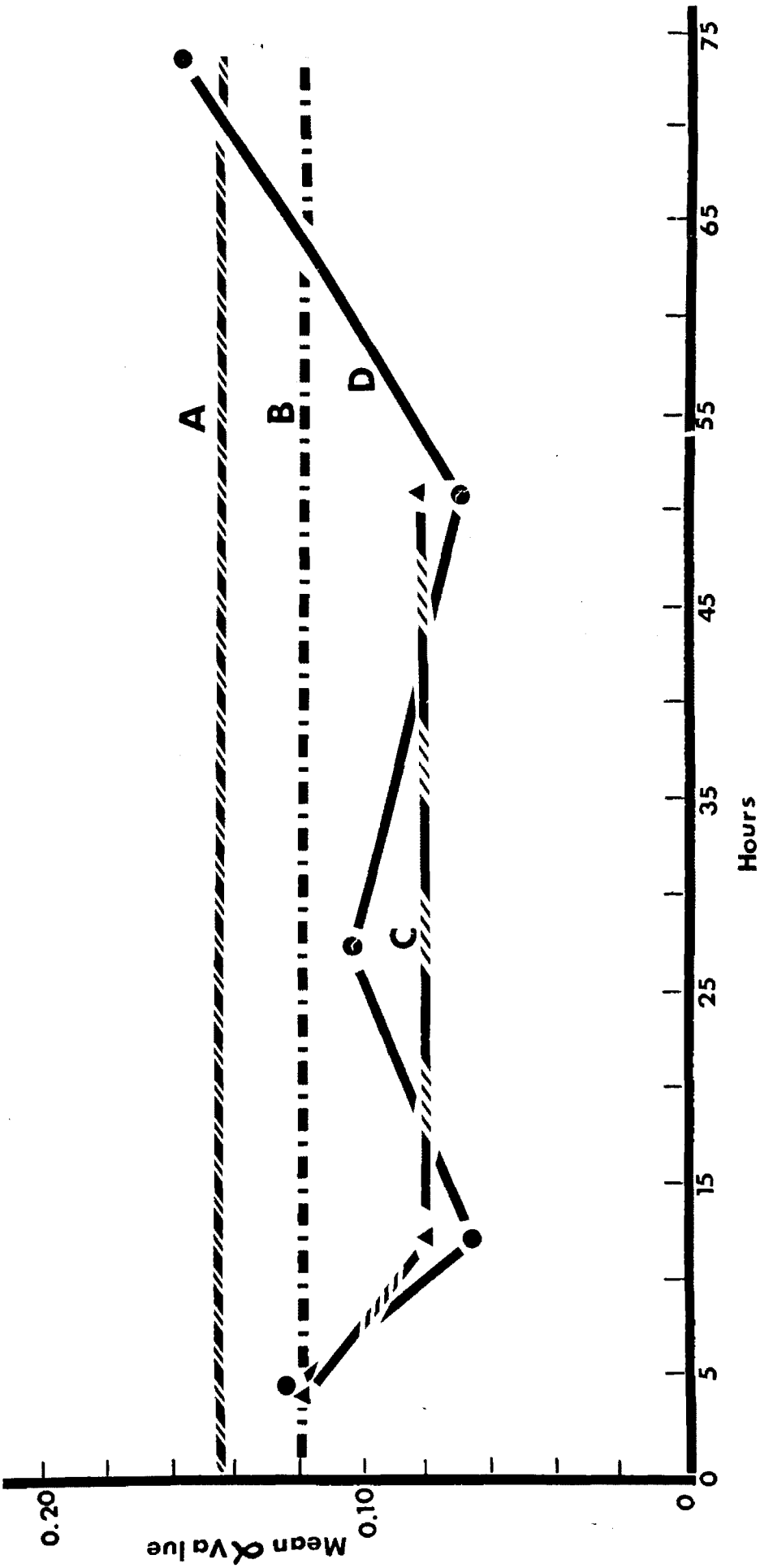
Symbols:

Line A: Nude control mice

Line B: Normal control mice

Line C: Normal test mice

Line D: Nude test mice



to a level below that suppression obtained in CAR-treated euthymic mice. By 70 h after treatment with CAR, the phagocytic activity of the nude mice had returned to normal.

From these data it was determined that the standard dose of CAR could suppress RES function in nude mice to a degree similar to the suppression achieved with CAR in normal mice. Therefore, 5 mg CAR was used in attempts to generate a pseudo-secondary response in nude mice.

CAR potentiates the endotoxicity of LPS (8), and this standard dose proved to be toxic to the nude mice in the long-term experiments that were required for determining pseudo-secondary responsiveness. Experiments were then designed to determine whether or not a dose of CAR could be found that would still produce effective RES suppression, but be less toxic for the nude mice. Nude mice were given varying amounts of CAR at time 0. RES activity was measured at the times indicated by the ability of mice to clear carbon particles from the peripheral blood. Fig. 10 and 11 show the RES function of nude mice and their normal littermates for 72 h after treatment with CAR. In both nude and in littermate controls 4.0 mg CAR was the lowest dose that would significantly suppress phagocytic activity. Therefore, this was chosen to be the standard dose of CAR in the following experiments with nude mice.

Attempt to generate a pseudo-secondary response in athymic nude mice. The requirement for T cells in the pseudo-secondary response was assessed by the use of congenitally athymic nude mice. LPS is a T cell independent antigen and can elicit good

Fig. 10. RES activity following injection of varying amounts of Seakem carrageenan (CAR) in athymic nude mice. Mice were given varying amounts of CAR at time 0 and RES activity was measured at the times indicated. Control mice received PBS at time 0. RES function was tested by the ability of mice to clear carbon particles from the peripheral blood. Each value represents the arithmetic mean of two mice.

Symbols:

Line A: 1.0 mg CAR i.p.

Line B: 2.0 mg CAR i.p.

Line C: 3.0 mg CAR i.p.

Line D: 4.0 mg CAR i.p.

Line E: Control mice

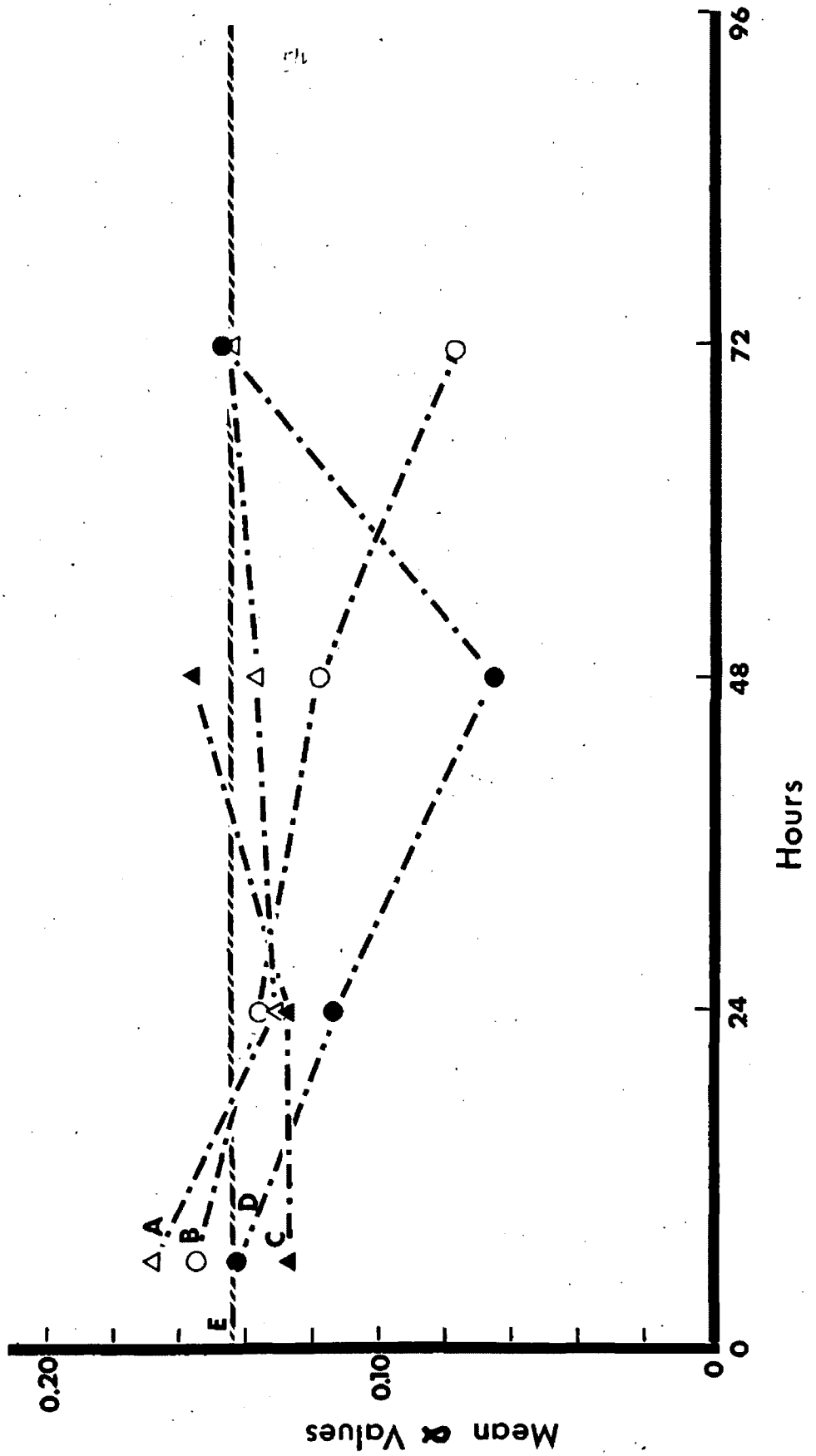


Fig. 11. RES activity following injection of varying amounts of Seakem carrageenan (CAR) in normal littermate mice. Mice were given varying amounts of CAR at time 0 and RES activity was measured at the times indicated. Control mice received PBS at time 0. RES function was tested by the ability of mice to clear carbon particles from the peripheral blood. Each value represents the arithmetic mean of two mice.

Symbols:

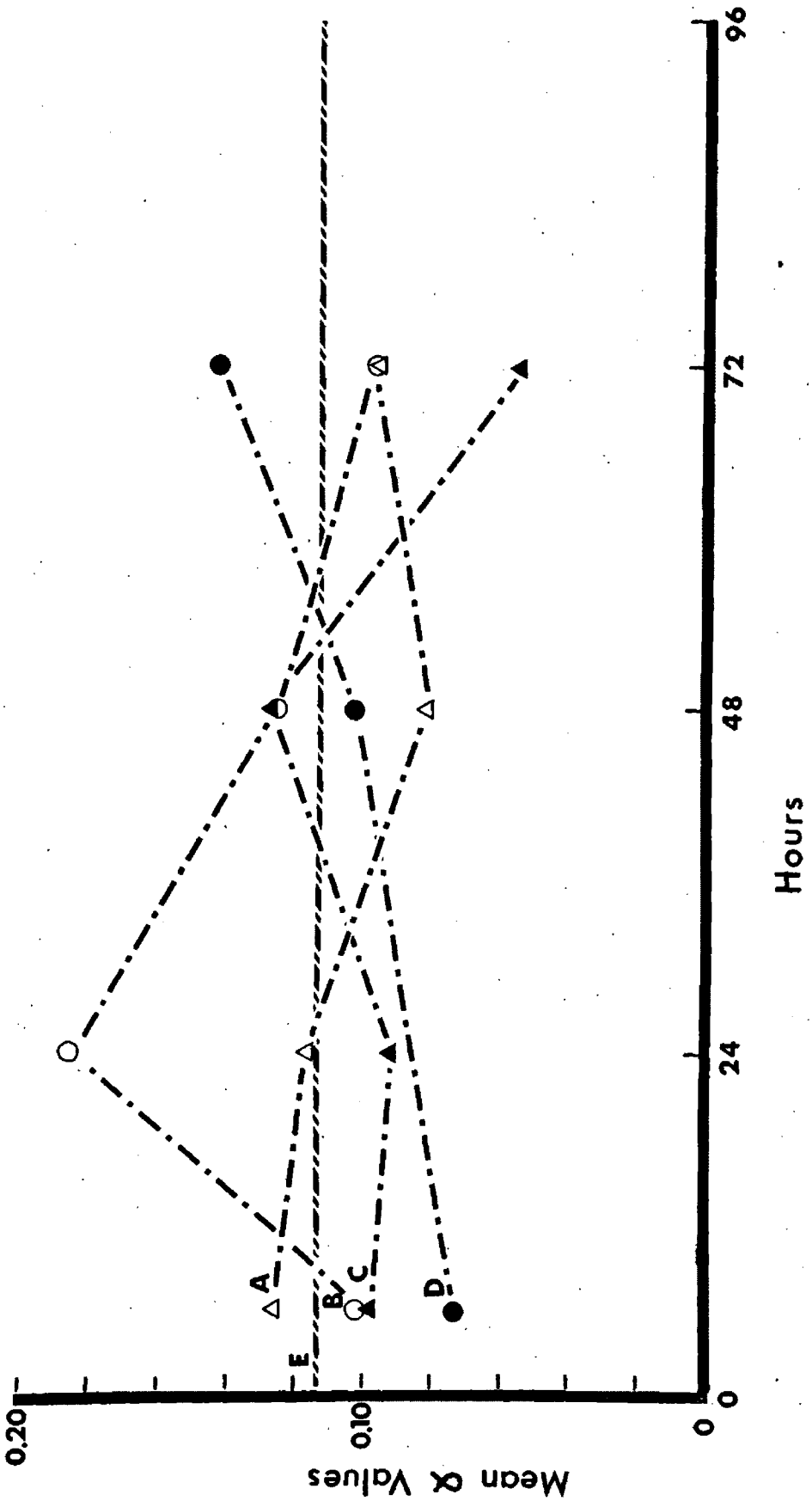
Line A: 1.0 mg CAR i.p.

Line B: 2.0 mg CAR i.p.

Line C: 3.0 mg CAR i.p.

Line D: 4.0 mg CAR i.p.

Line E: Control mice



antibody responses in the absence of T cells. Athymic nude mice were used to determine if a pseudo-secondary response could also be generated in the absence of T cells.

Nude mice and their normal littermates were treated with CAR on day 0. One or two injections of LPS were then given. Single doses of LPS were given either at 6 h after treatment with CAR or on day 21. Mice receiving two injections of LPS were injected at 6 h after treatment with CAR and again on day 21. Control mice were given PBS on day 0 and LPS at the times designated. Sera were harvested on day 25. Toxicity problems were encountered in this experiment, in spite of the reduced CAR dosage. By the time sera were harvested on day 25, toxicity deaths had reduced many groups to one or two mice. Therefore, any conclusions drawn from this data are tenuous. However, from Table 9 it can be seen that a pseudo-secondary response could not be mounted in the absence of T cells (group A). Mice with normal T cells could generate a pseudo-secondary response (group C); therefore the presence of T cells appeared to be a requirement for the generation of a pseudo-secondary response. Nude mice exhibited normal primary (compare groups E and F) and secondary (compare groups G and I) responses. Therefore, with only a minimal amount of data upon which to base this conclusion, priming for a pseudo-secondary response appeared to have a T cell requirement. However, T cells were not required for generation of a true secondary response.

Table 9

Generation of a pseudo-secondary response with Seakem carrageenan (CAR) in the presence and absence of T cells (ie. in athymic nude mice and their normal littermates).

GROUP	MOUSE	TREATMENT[a]	1°[b]	2°[c]	AB TITER[d]
		DAY 0	DAY 0	DAY 21	DAY 25
A	nu/nu	CAR	NONE	LPS	3.5
B	nu/nu	PBS	NONE	LPS	3
C	nu/+	CAR	NONE	LPS	8
D	nu/+	PBS	NONE	LPS	4
E	nu/nu	PBS	LPS	NONE	2
F	nu/+	PBS	LPS	NONE	1
G	nu/nu	PBS	LPS	LPS	7.5
H	nu/+	CAR	LPS	LPS	8.5
I	nu/+	PBS	LPS	LPS	6
J	nu/+	CAR	NONE	NONE	0

[a] 4.0 mg CAR i.p.
[b] 0.1 ug LPS i.v.
[c] 1.0 ug LPS i.p.
[d] Antibody titers were obtained by passive hemagglutination tests. Each value represents the antibody titer of sera pooled from three mice. Titers are expressed as values of x, derived from the equation, $x = \log_2 (HD/2)$, where HD was the reciprocal of the highest dilution of sera that produced hemagglutination.

IV. DISCUSSION

During the course of a recent study of the role of macrophages in antibody responses, the phenomenon of pseudo-secondary responsiveness was discovered (7, 9). It was observed that one dose of LPS, given to mice that were treated with macrophage toxins 21 d previously, elicited a typical secondary type response. The present investigation revealed a strong similarity between true secondary responsiveness and the single antigen dose-pseudo-secondary response. In fact, it appeared that the macrophage toxins, CAR and MUS, primed animals for a secondary anti-LPS response.

The mechanism of pseudo-secondary responsiveness was investigated in the following manner. First, the mechanism by which various macrophage toxins elicited RES suppression was correlated with their capability to generate the pseudo-secondary response. It was determined that only those macrophage toxins that produced lysosomal destabilization and subsequent cytotoxicity could generate pseudo-secondary responsiveness. Therefore, the consequences of the lysosomal destabilization and cell death--either the release of factors upon macrophage autolysis or factors involved in the subsequent regeneration of the macrophage population--appeared to be responsible for priming activities.

Next, the functional state of the RES was observed, following treatment with various macrophage toxins, until day 21; this was the time at which the eliciting dose of LPS was administered. It was seen that by day 5 after treatment with macrophage toxin,

RES function was normal and that normal or enhanced phagocytic activity continued until day 21. Thus, macrophage function was at a normal or an enhanced level at the time the pseudo-secondary response was triggered. These functional macrophages could have participated in the generation of the immunological responsiveness that was observed.

Finally, the genetic and kinetic requirements, as well as the T cell dependency, of a true secondary response were compared to those requirements for a pseudo-secondary response. The genetic and kinetic requirements for pseudo-secondary responsiveness appeared to be identical to those requirements for true secondary responsiveness to LPS. However, one difference was discovered in that the true secondary response to LPS could be generated in the absence of functional T cells, whereas pseudo-secondary responsiveness could not. It must be remembered, however, that the data from the attempt to generate pseudo-seondary responsiveness in nude mice were tenuous. If further investigations can establish a dosage regimen of macrophage toxins that will not potentiate endotoxicity to too great an extent in nude mice, more valid experimental data can be obtained on the role of T cells in the pseudo-secondary response. However, if the above data, which indicated that pseudo-secondary responsiveness was T cell dependent, were valid, then perhaps the priming reaction in the pseudo-secondary responses was dependent on T cells. This interpretation would change the focus of the effect of the macrophage toxins away from the latter, triggering events in the secondary

response to a T cell dependent priming event.

The generation of pseudo-secondary responsiveness appeared to occur by a mechanism similar to that generating true secondary responsiveness. The role of the macrophage toxins in the pseudo-secondary phenomenon may have been to generate a population of memory cells that primed the mice for a specific secondary response, upon subsequent contact with LPS.

Macrophage toxins and the pseudo-secondary response. The use of the variouse isomers of carrageenan, MUS and TT in attempts to generate pseudo-secondary responsiveness revealed that the mechanism by which RES suppression was achieved was an important factor in the induction of the response. TT suppresses phagocytic function by RES blockade (68). No cell destruction was observed after TT treatment, and recovery of phagocytic activity was not dependent on the generation of a new cell population (68). Conversely, macrophage cytotoxicity was the means by which CAR and MUS produced RES suppression. Once phagocytosed, CAR and MUS were responsible for lysosomal destabilization, which led to rupture of the lysosomes with the subsequent release of digestive enzymes and eventual cell death (3, 15). Pseudo-secondary responsiveness was observed with MUS and the various isomers of carrageenan; however, a pseudo-secondary response could not be mounted in the presence of TT. Therefore, the autolysis of macrophages and/or their regeneration appeared to be a requisite for the generation of pseudo-secondary responsiveness. Merely suppression of RES function was not sufficient to prime for a pseudo-secondary response.

RES modulation and the pseudo-secondary response. Examination of RES function for 21 d in mice that had received CAR, MUS, or TT, had determined that at approximately 5 d following administration of the macrophage toxins, the suppressed phagocytic activity had returned to normal; in fact, it was even enhanced. Although the recovery from RES suppression by CAR, MUS, and TT appeared to be equally complete, it was apparent that the mechanisms of recovery could not have been identical. The macrophages present at day 21 in mice that had been given TT were those that had been suppressed on day 0; they had recovered their normal phagocytic function (68). Mice that were given CAR or MUS were depleted of their macrophage populations (3, 15). Therefore, the normal phagocytic activity seen after day 5 was the result of a new population of macrophages. The enhanced phagocytic activity that was seen following RES recovery in mice treated with MUS, CAR or TT was probably due to a rebound effect.

It had been postulated previously that pseudo-secondary responsiveness was the result of excessive stimulation of progenitor antibody-forming cells by a bolus of antigen that had not been sequestered in the macrophage-impaired mice (9). The present results showed that macrophage function was normal at the time the eliciting dose of LPS was administered. Therefore, antigen sequestration would have occurred normally, and the earlier postulate was not the means by which the pseudo-secondary response was generated.

As mentioned earlier, destruction of macrophages may have been required for generation of pseudo-secondary responsiveness.

The release of factors from the ruptured macrophage lysosomes or from the macrophages themselves could have served to prime B lymphocytes for a secondary anti-LPS response; these might have substituted for the LPS molecule. The immunodeterminant group of antigen alone apparently can stimulate unprimed B lymphocytes to produce a primary antibody response and to sensitize the animal for a secondary anti-LPS response (64). However, triggering of a secondary response required both the antigenic signal and a second, mitogenic signal; the latter is present in LPS as the lipid A portion of the molecule (64). The macrophage-derived factors may have been sufficient to substitute for the LPS molecule in the priming of B cells, so that when LPS was administered on day 21, the previously sensitized B lymphocytes mounted a secondary response.

It is unlikely that the macrophage toxins themselves primed the B lymphocytes. CAR and LPS:0113 do share come structural similarities, in that both contain galactose units (36, 40), and it could be postulated that this structural similarity would allow CAR to substitute for LPS in priming functions. However, the pseudo-secondary response is also observed with MUS, a material that bears no structural resemblance to LPS. Therefore, it is more reasonable that the consequences of MUS and CAR administration, rather than the macrophage toxins themselves, were responsible for priming for a secondary anti-LPS response.

Another aspect that must be considered is the population of new macrophages that arose after macrophage depletion by CAR

or MUS. This population was young and capable of optimal processing of antigen. Therefore, when LPS was introduced into the system, it could be processed very efficiently and presented to the antibody-forming cells. These young macrophages also may have been capable of increased production of IL-1 (56). The resultant proliferation of T cell clones may have aided macrophage derived factors in the priming of B lymphocytes.

The genetic requirements of the pseudo-secondary response. The pseudo-secondary response could not be generated in the LPS nonresponder, C3H/HeJ mice. C3H/HeJ mice have a defect in a single autosomal dominant gene (53) that is manifested by an inability of their cells, or the lack of a membrane component on their cells, to react with the lipid A portion of the LPS molecule (53, 67). Therefore, these mice could not respond to the second signal of LPS and could not mount a secondary response to LPS.

The fact that C3H/HeJ mice could not generate a pseudo-secondary response to LPS suggested that the genetic requirements for pseudo-secondary responsiveness were the same as those for true secondary responsiveness. Thus, mice must possess the genetic capability to respond to the second, mitogenic signal of LPS in order for pseudo-secondary responsiveness to be manifested. This, again, supported the hypothesis that the effects of CAR and MUS were exerted at the level of priming the B lymphocytes for a secondary anti-LPS response; in all probability, triggering of a secondary response occurred in a normal manner. In both cases, when the triggering dose of LPS was administered on day 21, the sensitized lymphocytes

were stimulated by the lipid A portion of the molecule to secondary responsiveness. Thus, upon their first exposure to LPS, mice treated with MUS or CAR reacted as if they had been previously sensitized to the LPS immunodeterminants.

The kinetics for development of the pseudo-secondary response. It was established that the kinetics for development of pseudo-secondary responsiveness followed those for generation of true secondary responsiveness. The present study confirmed earlier work (51) that had determined that a 10 to 14 d interval between primary and secondary injections of LPS was required for generation of a secondary response. Likewise, pseudo-secondary responsiveness required at least a 10 d interval between administration of the macrophage toxin and injection of LPS. It appeared that the pseudo-secondary response was generated by a mechanism that required the same timing as that mechanism producing a true secondary response. Again, it seemed reasonable to postulate that the pseudo-secondary response was triggered in the same manner as a true secondary response; the difference in the two responses occurred at the level of priming.

An alternative hypothesis could be suggested. The required interval between macrophage toxin-administration and injection of LPS was 10 d. RES function was shown to have recovered from suppression by MUS or CAR by day 5 following treatment with macrophage toxin. An additional 5 d may have been required for this young population of macrophages to have matured to the level that they could process and present antigen effectively. Once the

antigen handling capabilities had been established, these young macrophages may have been able to process and to present antigen much more efficiently than could an older population of macrophages. The result of increased antigen processing may have been manifested by a heightened antibody response to the single dose of LPS. However, other results of this study lent the most credibility to an hypothesis whereby treatment with macrophage toxins primed the B lymphocytes and the single dose of antigen triggered secondary responsiveness in a normal manner.

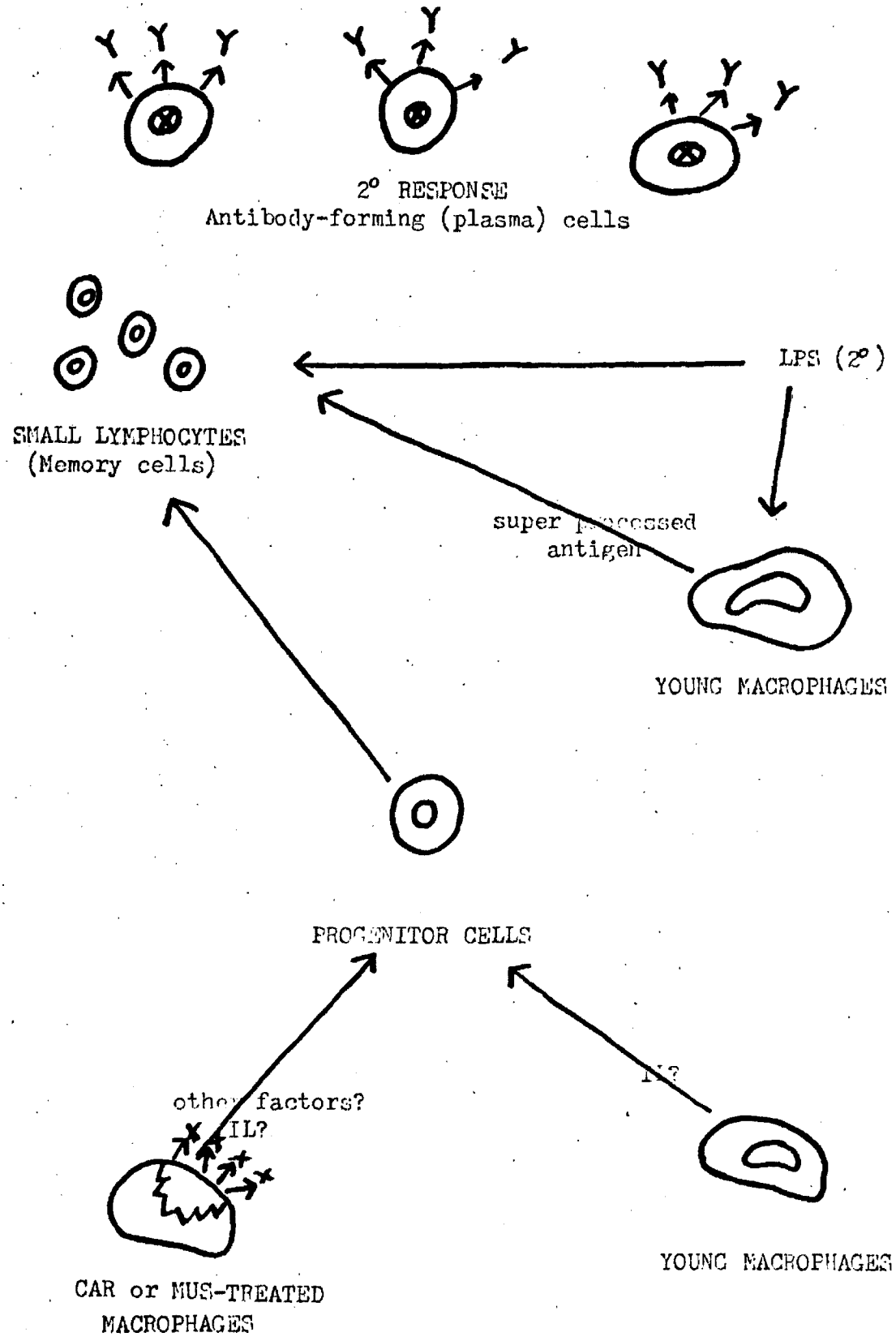
The requirement for T cells and the pseudo-secondary response. The potentiation of endotoxicity by CAR (8) was a problem in the experiments with nude mice. Amounts of CAR that would suppress RES function but would not potentiate endotoxicity to too great an extent in the short term experiments, still did potentiate endotoxicity too much for long term experiments. A non-potentiating, and yet RES-suppressing, dose of CAR was not found. Therefore, the lethality of the combined treatment reduced the numbers of mice in many of the experimental groups to the point that the data were not statistically sound. Any conclusions made from these data were tenuous and should be subjected to validation when an acceptable experimental system can be designed.

The data that were obtained did suggest, however, that a pseudo-secondary response could not be generated in the absence of T cells. This finding is contrasted to true secondary responsiveness, which could be generated in the absence of T cells. If true secondary and pseudo-secondary responses are identical at

the level of LPS triggering (the second signal), then the T cell dependency of the pseudo-secondary response occurred at the level of priming. Assuming that IL-1 production by young macrophages was important in priming B lymphocytes, then the lack of a pseudo-secondary response in nude mice would not be surprising. The IL-1 responsive IL-2 producing cells are lacking or nonfunctional in nude mice (56). Therefore, the interleukins would not function in the nude mice, and the mice could not have been primed, by treatment with CAR, for pseudo-secondary responsiveness to LPS. A simple T cell dependency of the macrophage-derived factors involved in priming also would have resulted in the abrogation of the pseudo-secondary response in nude mice. Thus, a T cell dependent factor that could prime B lymphocytes for a secondary anti-LPS response would not have been able to operate in the athymic nude mice. Also, the interleukins, which are responsible for the increased number of T cells that might aid B lymphocytes, could not function in the nude mice.

Conclusions. The treatment of mice with the macrophage toxins, CAR, or MUS, 21 d prior to the injection of LPS resulted in a typical secondary-type antibody response. The above results have led to the postulation of the following mechanism(s) for the generation of this response (see Fig. 12). Macrophages that were treated with CAR or MUS underwent lysosomal destabilization and subsequent autolysis. During autolysis, factors were released from the macrophages that were capable of signalling B lymphocytes to differentiate into memory cells. These macrophage-derived factors

FIG. 12. THE GENERATION OF PSEUDO-SECONDARY RESPONSIVENESS



could have functioned in a manner similar to the immunodeterminant group on the LPS molecule; they provided the necessary signal to B lymphocytes. When LPS was administered on day 21, the primed B lymphocytes were able to respond in a secondary fashion, as if they had encountered LPS previously, when in fact, this was their first exposure to the antigen. As the macrophage population recovered from depletion by CAR or MUS, young, healthy macrophages may also have contributed to the pseudo-secondary responsiveness. Interleukin production by young macrophages could have caused the proliferation of clones of amplifier T cells which, in turn, may have driven B lymphocytes to further proliferation. Operating alternatively to or in conjunction with the above reactions, young macrophages may have processed and presented antigen more effectively, thus causing a heightened antibody response. This could explain why a single dose of LPS can, indeed, generate a secondary-type antibody response.

The data generated in this study have shown that the mechanism by which the pseudo-secondary response was induced operated at the level of priming. The triggering activities required for pseudo-secondary responsiveness appeared to be identical to those required for a true secondary response. Therefore, the early theory that suggested a mechanism whereby progenitor antibody-forming cells were excessively stimulated by a bolus of antigen that had not been sequestered in the macrophage-impaired mice (9) was proved to be in error, and interest was focused at the priming step.

By centering the investigation of the mechanism of pseudo-

secondary responsiveness at the priming step, many questions have arisen. Do macrophages contain substances that could replace the LPS immunodeterminant group in sensitizing lymphocytes? What, if any, role does the destabilization of macrophages play in generating this substance(s)? Do interleukins play a part in generating the pseudo-secondary response? Will interleukins prime mice for a pseudo-secondary response? Answers to these questions will result in a clearer understanding of the mechanism by which pseudo-secondary responsiveness is generated.

V. SUMMARY

Previous work had established that a typical secondary type response could be generated by a single dose of LPS that had been administered to mice treated with the macrophage toxins, CAR or MUS, 21 d previously (7, 9). The mechanism of this phenomenon was investigated and it was determined that the requirements for pseudo-secondary responsiveness paralleled those for true secondary responsiveness. The requirement for T cells was the only exception; pseudo-secondary responsiveness required the presence of T cells and a true secondary response could be generated in their absence. It was postulated that the pseudo-secondary response was generated in a manner similar to the generation of a true secondary response. The differences between the two responses probably occurred at the level of priming. Priming for true secondary responsiveness was achieved by previous contact with the molecule of LPS, but pseudo-secondary priming was dependent upon lysosomal destabilization of macrophages by CAR or MUS.

Attempts to generate pseudo-secondary responsiveness with i-, κ-, and λ-carrageenans were successful. However, attempts to generate the phenomenon with TT, a macrophage toxin that suppressed the RES by physiological blockade (68), rather than by lysosomal destabilization and autolysis of macrophages, failed. From this it was determined that lysosomal destabilization and cytotoxicity of macrophages was a requirement for the generation of the response.

Phagocytic activity was followed for 21 d after treatment

with CAR, MUS, and TT by determining the ability of mice to clear colloidal carbon from the peripheral blood. It was found that RES function had returned to normal by 5 d after treatment with the macrophage toxins. Normal or enhanced phagocytic activity continued until day 21. Therefore, macrophages were functionally at a normal or an enhanced level at the time the pseudo-secondary response was generated.

C3H/HeJ mice, mice that lack the genetic capability to mount secondary responsiveness to LPS, were also unable to generate a pseudo-secondary response to LPS. This indicated that the genetic requirements for pseudo-secondary responsiveness paralleled those for true secondary responsiveness.

Likewise, the kinetics of the pseudo-secondary response followed those of true secondary responsiveness. A second injection of LPS would not elicit a secondary response until at least 10 d after the primary injection of LPS. At least 10 d were also required between treatment with macrophage toxins and the injection of LPS for the generation of pseudo-secondary responsiveness.

Results based on relatively meager data suggested that, unlike true secondary responsiveness, pseudo-secondary responsiveness required the presence of T cells.

A mechanism for pseudo-secondary responsiveness was postulated that attributed priming for the pseudo-secondary response to the consequences of the macrophage toxins. Substances capable of B lymphocyte priming were released from macrophages treated with MUS or CAR. Thus, a population of sensitized B lymphocytes were

available when LPS was administered on day 21. These cells responded to the antigen in a secondary fashion, mounting a secondary response to their first encounter with LPS.

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