Investigation of the mechanism of priming for a pseudo-secondary antibody response to LPS

Pamela M. Obey
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AN INVESTIGATION OF THE MECHANISM OF PRIMING FOR A PSEUDO-SECONDARY ANTIBODY RESPONSE TO LPS

By

Pamela M. Obey

B.S., University of Wisconsin-Madison, 1980

Presented in partial fulfillment of the requirements for the degree of

Master of Science

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ABSTRACT

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An Investigation of the Mechanism of Priming for a Pseudo-secondary Antibody Response to LPS.

Director; Jon A. Rudbach.

A typical secondary-type antibody response has been observed in mice given a single injection of lipopolysaccharide (LPS) from Escherichia coli 0113 21 d after treatment with either of the macrophage toxins, carrageenan (CAR) or microparticulate silica (MUS). The development of pseudo-secondary responsiveness has been shown to be dependent upon lysosomal damage induced by the toxins and to occur following immunization with extracts (presumably of lysosomal origin) of macrophages. Consequently, the theory has evolved that priming for a pseudo-secondary response was caused by a lysosomal component, released following lysosomal destabilization, which exhibited immunological cross-reactivity with LPS from E. coli 0113. The mechanism of priming for a pseudo-secondary response was investigated further in the present report. It was demonstrated that an immunogenic molecule(s), capable of sensitizing mice to LPS, was present in the serum after the administration of CAR or MUS. The appearance of the priming factor in serum was correlated with the onset of lysosomal destabilization and was prevented from occurring by stabilizing lysosomal membranes with the use of poly(-2-vinylpyridine-N-oxide) (PVPO). It was found that the same genetic restrictions applied to the generation of a pseudo-secondary as well as a true secondary response. C3H/HeJ mice, LPS-nonresponders, were unable to mount a pseudo-secondary response to LPS when initially injected with serum containing the priming factor. Nude mice, given the same treatment, also failed to respond in a pseudo-secondary fashion. This was interpreted to mean that the priming event was T-cell dependent. However, it was evident that the priming factor could be liberated from the lysosomes of both C3H/HeJ and nude mice, as serum harvested from these mice after treatment with CAR was able to sensitize known responders for a pseudo-secondary response. These data provided supportive evidence for the proposal that a lysosomal component which was antigenically similar to E. coli 0113 was responsible for priming mice for a pseudo-secondary anti-LPS antibody response.
ACKNOWLEDGMENTS

I would like to acknowledge with sincere appreciation Dr. Jon A. Rudbach for his guidance and financial support which made this study possible. Also, I would like to thank the members of my thesis committee, Drs. Walter Hill, Gary Gustafsen, and John Taylor for their encouragement and professional advice.
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ABBREVIATIONS

Ab         antibody
AcPh       acid phosphatase
Ag         antigen
B\textsubscript{M} memory B cell
C3         third component of complement
CAR        carrageenan
CPK        creatine phosphokinase
ddH\textsubscript{2}O distilled, deionized water
\(\beta\text{-Gal}\) \(\beta\text{-D-galactosidase}\)
\(\beta\text{-GlcU}\) \(\beta\text{-D-glucuronidase}\)
i.p.       intraperitoneal
i.v.       intravenous
LPS        lipopolysaccharide
MUS        microparticulate crystalline silica (Min-U-Sil)
NMS        normal mouse serum
NRS        normal rabbit serum
OCT        ornithine carbamyl transferse
PBS        phosphate buffered saline
PFC        plaque-forming cell
PHA        passive hemagglutination
PVPO       poly(-2-vinylpyridine-N-oxide)
RES        reticuloendothelial system

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<td>RML</td>
<td>Rocky Mountain Laboratory</td>
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<tr>
<td>RRBC</td>
<td>rat red blood cell</td>
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<tr>
<td>SRBC</td>
<td>sheep red blood cell</td>
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<tr>
<td>TD</td>
<td>T-cell dependent</td>
</tr>
<tr>
<td>TI</td>
<td>T-cell independent</td>
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<td>TT</td>
<td>colloidal thorium dioxide (Thorotrast)</td>
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I. INTRODUCTION

Historical background

Following an immunogenic stimulus, a series of cellular events occurs prior to the expression of a specific immune response. These events are often divided into two categories: (1) the afferent limb, which involves processing of antigen (Ag) by macrophages and leads to the activation of lymphocytes and (2) the efferent limb, which includes the proliferation and differentiation of specifically activated lymphocytes in conjunction with the elaboration of various effector molecules synthesized by these cells. The type of immune response which is expressed depends upon the nature of the Ag and may involve humoral or cell-mediated immunity, or both. Cell-mediated immunity is modulated directly by T cells or by T cell products whereas humoral immunity is mediated primarily by antibody (Ab), a product of B-cell activation.

As specific immune responses, humoral and cell-mediated immunity share three characteristic features: (1) specificity, (2) diversity and (3) memory. Specificity refers to the highly selective means by which the products of the immune response distinguish between different molecular species and react only with the Ag (or one very similar to it) which initiated the response. The diversity of a specific immune response simply reflects the ability of the host to recognize several
antigenic sites on a single immunogen and respond to each of them. The third characteristic of a specific immune response, memory, leads to the production of specific cell populations which, upon a second exposure to the immunogen, give rise to an enhanced response. In terms of humoral immunity, a memory response is therefore defined by comparison to the primary response obtained. Typically, a primary response exhibits a latent period of 7-10 d between the introduction of Ag and the appearance of specific Ab in the serum. Once Ab does appear, it is predominantly of the IgM class (with low levels of IgG production) and does not persist for long periods of time. A memory, or secondary, response, however, is characterized by a shortened latent period, a higher peak titer of Ab which persists for long periods of time, and a predominance of IgG antibody. Heightened IgM responses have also been observed and described as secondary responses.

The mechanisms underlying the development of immunological memory have been the subject of intense investigation for many years and researchers have often taken different approaches to the study of the problem. One method has been to investigate the signals required for the activation of memory B cells (B\textsubscript{M}) and the subsequent production of a specific memory Ab response. Since the mid 1970s, many researchers have adopted the viewpoint that two signals are necessary to activate B\textsubscript{M} cells during a secondary response. This theory was based primarily on the finding that T-cell independent(TI) Ags were capable of inducing typical primary responses without the aid of T cells, but were almost
universally incapable of generating secondary responses (9, 31, 44, 56, 93, 94). T-cell dependent (TD) Ags, on the other hand, were found to be effective in eliciting both primary and secondary responses (17, 37, 46, 47, 67). It was suggested that TI Ags failed to stimulate secondary responses because they provided the immune system with an antigenic signal only and lacked the ability to supply a necessary second signal which TD Ags acquired through helper T cell function. Bacterial LPS proved to be the major exception to the rule. As a TI Ag, LPS was shown to stimulate both IgM and IgG memory responses (93, 94, 95, 96). It was postulated that the unique Lipid A portion of the molecule substituted for T cell help and, therefore, was able to provide the second signal required for the activation of $B_M$ cells.

Other investigators have chosen to study the phenomenon of immunological memory in terms of the generation, rather than the activation, of $B_M$ cells. Klaus (49, 50) proposed that $B_M$ cell production in the mouse dependent upon the activation of complement and the subsequent deposition of Ag-Ab-C3 complexes in lymphoid follicles. It was suggested that TI Ags failed to induce $B_M$ cell production because these Ags were only able to elicit IgM antibodies, which did not activate mouse complement and consequently did not localize in follicles. Embling, et al. (28) have reported similar results in the human system.

In a third approach to the study of memory development, some researchers have attempted to define the structural requirements for Ag
in the generation of secondary Ab responses. Recently, Chen, et al. (21) made use of a synthetic, TD hapten-carrier system to demonstrate that a minimum of two antigenic sites recognizable by T cells had to be present on each molecule in order to successfully induce IgG memory.

In view of the complex nature of the mechanisms responsible for the generation of memory responses, it was of interest to note the curious display of immunological memory observed by Becker and Rudbach in 1978 (11). During the course of an investigation designed to clarify the role of macrophages in the induction of immunological responses in vivo, it was found that mice treated with the macrophage toxins, carrageenan (CAR) and microparticulate silica, Min-U-Sil(MUS), produced a secondary-type Ab response when immunized with a single dose of LPS 21 d later. It was determined that the response obtained was not due to contamination of the CAR or MUS preparations with LPS because two injections of these materials did not elicit a detectable Ab response.

The ability of toxin-treated mice to exhibit a secondary-type response to a single injection of LPS has since been termed a pseudo-secondary Ab response and further studies have revealed that the mechanisms by which CAR and MUS acted as macrophage toxins were essential for the development of such a response (76). Carrageenan, a high molecular weight, sulfated polygalactan, has been reported to exert its cytotoxic effects on macrophages by causing osmotic rupture of lysosomal membranes (1, 2, 3, 19). The proposal was made that CAR
was phagocytized and transported to secondary lysosomes in the same manner as other polysaccharides (22), but that, because of its complex molecular structure, CAR was not digested into osmotically inactive particles. Consequently, the affected lysosomes swelled and eventually ruptured. As a result, lysosomal digestive enzymes were released into the cytoplasm and cell death ensued. Microparticulate silica has also been shown to cause labilization of lysosomal membranes with the subsequent discharge of hydrolytic enzymes into the surrounding tissue fluid (3). However, instead of exerting an osmotic effect, silica was thought to induce permeability changes in the membrane by interacting with phospholipid or protein components of the membrane. A third inhibitor of macrophage function, Thorotrast (colloidal thorium dioxide) (TT), has been demonstrated to interfere with macrophage activity by inhibiting cell membrane synthesis rather than damaging lysosomes (98), thereby distinguishing its mode of action from that of CAR and MUS. When a comparison was made in mice between the Ab response obtained to a single dose of LPS and the macrophage toxin given 21 d earlier, it was found that TT, unlike CAR and MUS, could not sensitize for a pseudo-secondary response to LPS. Therefore, it was postulated that destabilization of the lysosomal membrane was a necessary prerequisite for the generation of a pseudo-secondary anti-LPS response (76).

With the knowledge that the development of a pseudo-secondary response was dependent upon lysosomal rupture, the hypothesis was formed that a lysosomal component, which was immunologically cross-reactive with LPS, was released following destabilization of the membrane.
and was then able to prime mice to later challenge with LPS (66, 76). A recent investigation (66), has provided supportive evidence for this theory. It was demonstrated that macrophage lysates generated by freeze-thaw fracture contained a distinct determinant, presumably of lysosomal origin, which cross-reacted immunochemically with LPS from *Escherichia coli* 0113, the particular antigenic variety of LPS used in the study of the pseudo-secondary response. Furthermore, macrophage lysates were shown to prime mice for a pseudo-secondary Ab response to LPS. In the same study, it was suggested that the products released from ruptured macrophages were specifically cross-reactive, immunochemically and immunologically, with LPS from *E. coli* 0113, because attempts to elicit a pseudo-secondary response in CAR-treated mice failed when LPS from *E. coli* K235 was used to challenge the mice.

One of the potential criticisms of the pseudo-secondary response as a unique phenomenon has been that it was merely a representation of an enhanced primary response induced by the effects of CAR and MUS. This concern was based, in part, on several published reports which indicated that many macrophage inhibitors, in particular CAR, could serve as adjuvants (15, 78, 87, 88). In one such study (88), it was demonstrated that the adjuvant effect of CAR was due to the diversion of Ag from the liver to the spleen, a major site of Ab formation, during dysfunction of the reticuloendothelial system (RES). Three observations made by Remington and Rudbach (76), however, served to differentiate the development of a pseudo-secondary anti-LPS response
from that of a magnified primary response. The first was that the functional state of the RES was normal at the time of LPS challenge. This indicated it was unlikely that any adjuvant activity had occurred due to the inability of macrophages to sequester Ag in the liver and prevent an abnormal uptake of Ag by the spleen. The second and third observations involved the findings that the kinetic and genetic requirements for the generation of a pseudo-secondary response were the same as those for a true secondary response.

A second criticism of the theory underlying the development of a pseudo-secondary anti-LPS response has been that CAR and MUS, as opposed to some lysosomal product, were responsible for priming mice immunologically to LPS. This suggestion was interesting in view of the similarities that have been shown to exist between LPS, CAR and MUS. All three have been recognized as macrophage toxins which exert their effects by promoting lysosomal instability (1, 3, 19, 54, 57, 63, 82, 85, 91, 92). In addition, both CAR and LPS have been demonstrated to act as adjuvants (45, 64, 78, 86, 87, 88, 92), to cause immune suppression (7, 8, 27, 43, 60, 70, 84, 89) and to function as B cell mitogens (6, 35, 51, 69, 72). However, although CAR and LPS were also known to possess structural similarities (5, 58, 64), the proposal that priming to LPS occurred through the direct action of the macrophage toxins themselves was highly unlikely because MUS, which bore no immunochemical resemblance to LPS, was as effective as CAR in sensitizing mice for a pseudo-secondary response to LPS.
Statement of thesis and approach to the problem

Previous investigators have shown that mice treated with the macrophage toxins, CAR and MUS, produced a pseudo-secondary Ab response when challenged with a single dose of LPS from E. coli 0113 21 d later (11). To explain this phenomenon, the theory has been proposed that lysosomal rupture in macrophages, induced by CAR and MUS, lead to the liberation of a lysosomal component which was antigenically similar to LPS from E. coli 0113 and, therefore, was able to prime mice for a pseudo-secondary Ab response (66). The purpose of the present study was to examine the mechanism of priming to LPS (and the subsequent development of immunological memory) which resulted from treatment with CAR or MUS. To do so, an attempt was made to determine if, in vivo, lysosomal destabilization was followed by the release of an immunogenic molecule which was responsible for priming mice to LPS.

In order to estimate the times at which lysosomal destabilization occurred following administration of CAR, MUS and TT, mouse plasma was assayed for the presence of abnormally elevated levels of three lysosomal marker enzymes - acid phosphatase, $\beta$-D-glucuronidase and $\beta$-D-galactosidase. Then, by measuring the degree of general tissue destruction caused by the macrophage toxins, it was possible to determine when an increase in lysosomal enzyme activity indicated damage specifically to lysosomes located within macrophages. Creatine phosphokinase and ornithine carbamyl transferase, reliable enzyme markers of muscle and liver damage, respectively, were used to assess nonspecific tissue destruction.
Once the kinetics and specificity of lysosomal enzyme release had been established, serum, harvested from toxin-treated mice during periods of both lysosomal lability and stability, was tested for its capacity to prime recipients for a pseudo-secondary Ab response to LPS. In addition, an examination was made of the genetic requirements of the donors and recipients used to investigate the generation of a pseudo-secondary response by serum transfer. Furthermore, poly(-2-vinylpyridine-N-oxide) (PVPO), a known lysosomal stabilizer, was tested for its effect on the ability of serum from CAR and MUS-treated mice to prime recipients to LPS.

During the course of this study, it was also necessary to address the question of whether endogenous LPS, present within the lysosomes of macrophages, was responsible for inducing the pseudo-secondary anti-LPS response. This was accomplished by two methods. First, an experiment was designed to demonstrate the effect of CAR on the tissue distribution of a $^{51}$Cr-labeled LPS preparation which had been injected previously and allowed to accumulate in the phagolysosomes of macrophages. Then, the intestinal contents of several mice were cultured for the presence of _E. coli_, a potential source of endogenous LPS.

Finally, because CAR has been shown to act as a B cell mitogen (51, 72), it was important to determine if CAR caused the development of a pseudo-secondary anti-LPS response through polyclonal B cell activation.

The above study indicated that lysosomal destabilization was
followed by the liberation of a factor which was present in serum in an immunologically active form and was able to prime mice for a pseudo-secondary response to LPS. The release of this factor was blocked by the lysosomal stabilizing effect of PVPO and, from the evidence presented, it did not appear that LPS itself was responsible for inducing the pseudo-secondary Ab response obtained when mice exposed to CAR or MUS were subsequently challenged with LPS.
II. MATERIALS AND METHODS

Animals. All mice, with the exception of the athymic nude mice, were fed standard lab chow and given water ad libitum. The nude mice were maintained on autoclaved lab chow and water containing 10 mg tetracycline (Roche Laboratories, Nutley, NJ) per liter.

(i) ICR Swiss mice. Female Swiss outbred mice were purchased from Harlen-Sprague-Dawley (Indianapolis, IN) and used for experimentation when 4-8 weeks old. As outbreds, the Swiss strain has maintained nearly the same degree of genetic variation as natural murine populations (77).

(ii) RML mice. Both male and female Swiss-Webster derived mice were obtained from the Rocky Mountain Laboratory (RML) stock, Hamilton, MT. Mice used for experiments were 4-10 weeks old.

(iii) C3H/HeJ mice. Female C3H/HeJ mice were acquired from the Jackson Laboratory in Bar Harbor, ME and used for research when 5-6 weeks old.

(iv) Nude mice. Female Swiss outbred, nude mice were purchased from Harlen-Sprague-Dawley (Indianapolis, IN) and were used for investigation when 4-6 weeks old.

Macrophage toxins.

(i) Carrageenan. Calcium carrageenan (CAR) (Seakem 9, Marine Colloids, Springfield, NJ) was suspended in warm (37°C) phosphate
buffered saline (PBS) (0.15 M NaCl, 0.0033 M PO₄, pH 7.2) at a concentration of 10 mg/ml. Then 5 mg of the CAR was administered to each mouse intraperitoneally (i.p.). All suspensions were either used on the day of preparation or were discarded.

(ii) Microparticulate silica. Silica particles (Min-U-Sil, Whittaker, Clark and Daniels, Inc., New York, NY) of less than 5 μm in size were fractionated further according to the method of Becker and Rudback (11). Briefly, 50 g of silica was suspended in 500 ml of distilled, deionized water (ddH₂O) and subjected to ultrasonic vibration (Bronson, Danbury, CT) at room temperature for 30 sec. The suspension was diluted with an additional 500 ml of ddH₂O and placed in a 1 l graduated cylinder at room temperature. After allowing 24 h for settling to occur, the third fraction, corresponding to the 500-750 ml portion from the top, was removed and washed twice with ddH₂O. The sediment from fraction III was dried in a 60°C oven for 48 h. In preparation for injection of the silica, or Min-U-Sil (MUS), the dry powder was suspended in warm (37°C) PBS at a concentration of 20 mg/ml. Each mouse received 8 mg, intravenously (i.v.).

(iii) Thorotrast. Thorium dioxide (TT) (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 injected i.v. in a volume of 0.19 ml without dilution.

Lysosomal stabilizer. Poly(-2-vinylpyridine-N-oxide) (PVPO)
(Polysciences, Inc., Warrington, PA) was suspended in warm (37°C) PBS at a concentration of 16 mg/ml, with each mouse receiving 4 mg/0.25 ml, i.v.

Lipopolysaccharide. Lipopolysaccharide (LPS) from Escherichia coli 0113 (Braude strain) was supplied by J.A. Rudbach. It had been prepared as a phenol-water extract according to the method of Rudbach, et al. (80). Stock solutions were made in PBS at a concentration of 2 mg/ml and stored at -20°C. The appropriate dilutions were made just prior to use.

Collection of plasma for enzyme studies. Mice were anesthetized with ether (Mallinkrodt Chem. Works, St. Louis, MO) and exsanguinated by axillary incision. The whole blood was placed in conical centrifuge tubes containing 25 µl of sodium heparin (1000 units/ml) per mouse bled. The samples were then centrifuged at 900 x g for 10 min in a refrigerated centrifuge and the recovered plasma either assayed immediately or stored at -70°C.

Collection of sera for passive hemagglutination assay. Mice were exsanguinated by axillary incision as described above. The whole blood was collected in conical centrifuge tubes and allowed to clot at room temperature for 1 h, after which the clot was rimmed and the samples placed in a 4°C refrigerator overnight. The serum was removed from the clot, dispensed into 1.5 ml Eppendorf micro test tubes and centrifuged for 3 min in an Eppendorf centrifuge (model 5414,
Brinkman Instruments, Inc., Westbury, NY) to sediment any remaining red cells. Following heat inactivation of the sera (56°C for 30 min), the samples were stored in 4 ml vials at -20°C.

Collection of sera for passive transfer. Mice were exsanguinated by axillary incision and the whole blood collected in conical centrifuge tubes. The blood was allowed to clot for 10 min at room temperature and then placed in a 37°C water bath for 30 min. The clots were rimmed and centrifuged at 900 x g for 10 min. The sera were recovered and transferred immediately to recipient mice by the i.p. route.

Passive hemagglutination assay. Specific anti-LPS titers were measured by the passive hemagglutination assay (PHA). Sheep red blood cells (SRBC) (Colorado Serum Co., Denver, CO) were used as the indicator cells and were coated with LPS from E. coli 0113 which had been boiled for 2.5 h at a concentration of 1 mg/ml in 0.1 M PO₄ (pH 7.4). In the coating process, 9.0 ml PBS was combined with 1.0 ml LPS and 0.25 ml of the packed, washed SRBC. The mixture was placed in a 37°C water bath for 30 min and resuspended every 10 min. Following incubation, the cells were washed 3x in cold PBS and resuspended in microtiter diluent (1% normal rabbit serum)(NRS) to a concentration of 0.5%.

Normal SRBC were prepared in a similar manner. The packed, washed SRBC were resuspended in 1% NRS at a concentration of 0.5%.

A 50 µl amount of microtiter diluent was added to each well of a microtiter plate followed by the addition of 50 µl of antisera to
the first well of each row. Serial two-fold dilutions were made with 0.05 ml diluters (Cooke Engineering Co., Alexandria, VA). Finally, 50 µl of the 0.5% suspension of SRBC or sensitized SRBC was added to each well and the plates incubated in a humid chamber. The titers were read after 2 h at room temperature (20-22°C) and again after 24 h at 4°C. Titers were calculated from the equation, \( x = \log_2(\text{HD}/2) \), where \( x \) represents the titer and \( \text{HD} \) corresponds to the reciprocal of the highest dilution of antisera which produced hemagglutination. Thus, the titer was equal to the well number of the endpoint when the first well contained a 1/4 dilution of antiserum. Sera which failed to agglutinate the sensitized red cells were considered to have a titer of 0, i.e. a dilution of 1/2. All samples were tested in duplicate.

Preparation of \(^{51}\text{Cr}-\text{labeled LPS.} \) LPS was labeled with \(^{51}\text{Cr} \) (New England Nuclear, Boston, MA) according to the method of Braude, et al. (18). A 2 mg sample of LPS was added to 0.907 ml PBS containing 300 µCi of \(^{51}\text{Cr} \). The preparation was incubated at 37°C for 2 d and shaken frequently during that time. The labeled LPS was then dialyzed against 0.001 M PO\(_4\) (pH 7.2) at 4°C until the counts per minute (CPM)/ml dialysate had stabilized, indicating that all the unbound \(^{51}\text{Cr} \) had been removed from the labeled LPS preparation. Generally 3 or 4 changes (1 liter each) of PO\(_4\) buffer were needed before the radioactivity in the dialysate diminished to background levels.
Lysosomal hydrolase activity. The activities in mouse plasma of three lysosomal hydrolases - acid phosphatase, β-D-glucuronidase and α-D-galactosidase - were determined by the method of Glaser and Sly (36). The assays were performed as described below.

(i) Acid phosphatase. A 200 μl amount of 8 mM p-nitrophenyl phosphate (Sigma Chem. Co., St. Louis, MO) in 0.1 M citrate, pH 4.8, was incubated with 25 μl of plasma for 1 h at 37°C. At the end of the incubation period, 1.5 ml of 320 mM glycine-200 mM carbonate buffer, pH 10.0, was added to stop the reaction. To ensure that the alkaline phosphatase present in the plasma was not activated by the addition of the glycine-carbonate buffer, the samples were then boiled for 3 min to denature the active proteins.

(ii) β-D-glucuronidase. For this assay, 400 μl of 10 mM p-nitrophenyl β-D-glucuronide (Sigma Chem. Co., St. Louis, MO) in 0.1 M acetate buffer, pH 4.8, was mixed with 25 μl of plasma and incubated at 37°C for 3 h. Then, 600 μl of the glycine-carbonate buffer, pH 10.0, was used to terminate the reaction and the samples were boiled for 3 min.

(iii) α-D-galactosidase. A 25 μl sample of plasma was added to 400 μl of 10 mM p-nitrophenyl α-D-galactopyranoside (Sigma Chem. Co., St. Louis, MO) in 0.1 M citrate buffer, pH 3.6, and the reaction allowed to proceed at 37°C for 3 h. Subsequently, 600 μl of the glycine-carbonate buffer, pH 10.0, was added to stop the reaction and the samples were boiled for 3 min.
Reagent blanks were prepared with 25 μl of ddH₂O instead of plasma.

In each case, centrifugation at 10,000 x g was necessary to remove denatured protein from the solutions before measuring the absorbance of the nitrophenol products at 420 nm. All measurements were made on a Coleman Junior II spectrophotometer (Model 6/20, Perkin-Elmer Corp., Coleman Instrument Div., Oak Brook, IL). All tests were performed in duplicate or triplicate. Activity was expressed as nmoles product formed/length of incubation time.

**Ornithine carbamyl transferase.** The level of ornithine carbamyl transferase (OCT) activity in mouse plasma was detected using a procedure (kit 108 purchased from Sigma Chem. Co., St. Louis, MO) based on the microdiffusion-Nesslerization method described by Reichard (75). The action of OCT in this procedure is to catalyze the reaction between citrulline and arsenate, forming ornithine and carbamyl arsenate. However, carbamyl arsenate is unstable and decomposes, liberating CO₂ and NH₃. The amount of NH₃ can be quantified and is proportional to the serum (plasma) OCT activity.

To initiate the assay, either 1.0 ml of 0.1 M citrulline-0.5 M arsenate buffer, pH 7.1, (Test) or 1.0 ml of 0.5 M arsenate buffer, pH 7.1, (Blank) was added to 1.0 ml plasma and incubated at 37°C for 24 h. Subsequently, 0.2 ml of 4 N perchloric acid was added to each sample and the resulting precipitate was sedimented by centrifugation at 10,000 x g for 10 min. Next, 1.0 ml of either the Test or Blank
supernatant was pipetted into the outer chamber of a Cuprak dish (Costar, Cambridge, MA) along with 3.0 ml of a 0.4 M borate buffer, pH 13.0. To the center chamber was added 1.5 ml of 0.01 N HCl. The dishes were incubated at 37°C for 2.5 h, during which time the ammonia liberated was trapped by the HCl.

To quantify the amount of ammonia formed, 1.0 ml of the HCl solution from the Test or Blank center chamber was added to a test tube containing a 3.5 ml ddH₂O and 0.5 ml Sigma Ammonium Color Reagent (Stock no. 14-2). After 10 min at room temperature, the absorbancies of the Blank and Test samples, with ddH₂O as a reference, were read at 420 nm on a Coleman Junior spectrophotometer. OCT activity was calculated from an established calibration curve and the plasma activity determined by subtracting the activity in the Blank from that of the Test. All assays were performed in duplicate.

Creatine phosphokinase. Creatine phosphokinase (CPK), an indicator of myocardial damage, was assayed for in mouse plasma by a modification (kit 520 purchased from Sigma Chm. Co., St. Louis, MO) of the procedure described by Hughes (42). In the test, phosphocreatine acts as a substrate for CPK and, in the presence of ADP, is converted to creatine. The amount of creatine formed is proportional to CPK activity.

Briefly, 0.5 ml of the phosphocreatine solution provided was incubated at 37°C for 15 min with either 0.1 ml of a 10-fold dilution of plasma (Test) or 0.1 ml of ddH₂O (Blank). To each tube, 0.2 ml ADP-glutathione was added and the reaction was allowed to proceed at 37°C
for exactly 30 min. Then, 0.2 ml of p-hydroxymercuribenzoate was used to stop the reaction. In order to induce color development, 1.0 ml α-naphthol, 1.0 ml of a diacetyl solution, and 1.0 ml of ddH2O were added to the reaction mixture and the tubes were placed in a 37°C water bath for 15 to 20 min. The absorbancies at 520 nm of Test samples were recorded with the Blank as a reference. CPK activity was then read directly from a calibration curve previously prepared.

**Plaque-forming cell assay.** The number of direct plaque-forming cells (PFC) specific for SRBC, rat RBC (RRBC) and LPS from *E. coli* 0113 and 0111 were quantified by a hemolytic plaque assay. SRBC were used as the indicator cells for the LPS antigens and were coated with either LPS from *E. coli* 0113 or 0111 as described in the PHA procedure. Spleens were removed from mice and single cell suspensions prepared by pressing the spleens through a 40 mesh stainless steel wire grid into cold 2x Eagle's Medium (Grand Island Biological Co., Grand Island, NY) buffered with 0.1 M Tris-HCl. The suspensions were allowed to settle in an ice bath in 15 ml conical centrifuge tubes for 5 min in order to remove cell debris. After transferring the supernatant fluid to fresh centrifuge tubes, the cells were washed once with 2x Eagle's and then suspended in 10 ml of 0.83% NH₄Cl and kept at room temperature for 5 to 10 min to lyse the erythrocytes. The cells were washed once with 2x Eagle's and then suspended in 2.0 ml of 1x Eagle's per spleen. The dilutions appropriate for plating were made in 1x Eagle's.
Trypan blue (0.04%) exclusion was used to indicate cell viability and the numbers of live spleen cells/ml media were calculated from counts obtained with a hemocytometer.

Individual tubes containing 2.0 ml of 1.0% agar (Bacto Agar, Difco Laboratories Inc., Detroit, MI) in 1x Eagle's were prepared and kept melted in a 45°C water bath. Just prior to plating, 0.2 ml of 1% DEAE-dextran (Pharmacia Fine Chemicals, Upsala, Sweden) was added to the agar, followed by 0.2 ml of the appropriate 30% red cell suspension and 0.2 ml of the spleen cell suspension. The tubes were quickly inverted 3x and the contents poured into 60 x 15 mm plastic petri plates. After the agar had solidified, the plates were placed in a humid chamber and incubated at 37°C for 2 h. Following incubation, 3.0 ml of guinea pig complement (Colorado Serum Co., Denver, CO), diluted 1:30 in a standard complement diluent (PBS with 9 x 10^-5 M CaCl₂ and 5 x 10^-5 M MgCl₂.H₂O), was added to each plate. The plates were then incubated at 37°C for an additional 45 min, after which the number of plaques/plate were counted. All tests were performed in duplicate and the average number of PFC was calculated. The PFC response was then expressed in terms of the number of PFC/spleen.

Co-agglutination test. Bacterial colonies, isolated from the intestinal contents of both male and female RML mice, which were composed of lactose-fermenting, Gram negative rods were analyzed for the ability to cross-react with E. coli 0113 by the co-agglutination test (30). This test makes use of the fact that the Cowen strain of
Staphylococcus aureus is rich in Protein A, which binds the Fc portion of IgG molecules. Consequently, the antibody-coated S. aureus becomes a reagent which agglutinates in the presence of homologous antigen.

A commercial S. aureus preparation (Pansorbin, Calbiochem-Behring Corp., La Jolla, CA) was coated with antiserum containing IgG antibodies specific for LPS 0113. A 10 μl sample of antiserum was added to 100 μl of the S. aureus suspension. The sensitized suspension was washed once with PBS in order to remove any unbound antibodies and then was diluted to 1 ml with PBS and stored at 4°C.

The test procedure was carried out by adding one drop of a heavy bacterial suspension to an equal volume of sensitized S. aureus. The samples were mixed thoroughly and read at the end of 2 min against a dark background. Agglutination was graded from 0 to 4 on the basis of the degree of clumping observed and was considered positive if the test wells appeared granular (+2) or exhibited a slight to heavy floccular appearance (+3 to +4) and both negative controls and staphylococcal reagent sensitized with other, unrelated, antibodies remained uniformly smooth and nongranular (0). A +1 reaction corresponded to a barely perceptible granular appearance. If nonspecific agglutination was observed, the test was considered positive only if there was a distinct difference (+4 versus +1 or +2) in agglutination between the test well and negative controls. Two reagents were used as negative controls. They included a nonsensitized S. aureus.
preparation and a staphylococcal suspension sensitized with NRS, which was devoid of antibodies to the antigens tested. In addition, samples of the \textit{S. aureus} suspension were sensitized with antisera directed against \textit{E. coli} 0111 in order to explore the degree of cross-reactivity, if any, observed. Anti-ovalbumin sera provided the source of unrelated antisera and a known culture of \textit{E. coli} 0113 was used to demonstrate positive agglutination.
III. RESULTS

Specificity of lysosomal rupture induced by various toxins for macrophages. According to the mechanism proposed for the generation of a pseudo-secondary response to LPS following treatment with CAR or MUS, the lysosomes of macrophages which had ingested the toxins were ruptured, leading to a discharge of lysosomal contents into the cell cytoplasm and, thus, into the surrounding tissue fluid (76). It was postulated that among the components released was a factor which was cross-reactive with LPS and therefore was able to prime mice to later challenge with LPS. The unsuccessful attempt to induce a pseudo-secondary response with TT was suggested to be the result of the inability of TT to destabilize the lysosomal membranes within phagocytizing macrophages and cause the release of the priming factor.

In an effort to determine when the priming factor was available for interaction with the host immune system, a study was undertaken to monitor the periods of lysosomal destabilization following treatment with CAR and MUS. Abnormally elevated levels of the acid hydrolases—β-D-galactosidase (β-Gal), β-D-glucuronidase (β-GlcU), and acid phosphatase (AcPh)—in mouse plasma served to indicate when lysosomal rupture was taking place. For the sake of comparison, an assessment was also made of the degree to which lysosomal enzyme activity increased in mouse plasma after administration of TT.
The macrophage toxins described above have been shown to induce a variety of pathological effects (2, 27, 55). In addition, the degradation of various tissue components by lysosomal enzymes released from damaged macrophages has been suggested to occur as a secondary consequence of the phagocytosis of toxin (4, 25, 26). As a result, it was also necessary to assay for general tissue destruction in order to determine if the hydrolase activities detected were representative of damage specific for macrophages or if they were the result of damage to other tissues. Plasma levels of creatine phosphokinase (CPK), specific for myocardial and skeletal muscle injury, and ornithine carbamyl transferase (OCT), a sensitive measure of liver damage, were used to assess overall tissue destruction. Groups of three mice each were treated with the macrophage toxins or with PBS and were exsanguinated at various times after treatment. The blood collected was pooled and the plasma recovered for enzyme analyses. Fig. 1 illustrates the kinetics of lysosomal enzyme release after administration of CAR (Lines A-C). The acid hydrolases showed two peaks in activity; the first occurred for all three hydrolases at 6-8 h post-treatment. Failure to detect an increase in activity for OCT or CPK (Lines D and E, respectively) at this time suggested that the elevated hydrolase activity was due to the rupture of lysosomes specifically within macrophages. The second peak in hydrolase activity occurred from 12 (AcPh, Line A) to 16 h (β-Gal and β-GlcU; Lines B and C, respectively) and was accompanied by a dramatic increase in OCT plasma...
levels. (The CPK activity remained close to normal at all the times examined.) This implied that injury to hepatocytes may have resulted in the dysfunction or rupture of lysosomes within these cells and, therefore, that the lysosomal marker enzymes were not reliable indicators of damage to macrophages after 8 h of exposure to CAR. Similar results for MUS-treated mice are shown in Fig. 2. The activities of the acid hydrolases peaked for the first time from about 4-6 h after administration of the toxin. A corresponding rise in OCT or CPK plasma levels was not seen at this time, but by 16 h, as the lysosomal enzyme levels peaked again, there was a concomitant increase in both OCT and CPK activity. Fig. 3 illustrates the time course of lysosomal enzyme activity following injection of TT. Again each of the acid hydrolases showed a biphasic pattern of activity, although the peaks for each occurred at different time intervals (AcPh at 6 and 24 h, Line A; ß-Gal at 4 and 24 h, Line B; ß-GlcU at 4 and 12 h, Line C). Unfortunately, a limited supply of TT prevented evaluation of CPK and OCT activity.

A comparison of the kinetics of lysosomal enzyme release following the administration of CAR and MUS revealed that lysosomal destabilization took place earlier in MUS-treated mice than it did in mice given CAR. This observation confirmed a previous report which demonstrated that CAR exerted its cytotoxic effects on macrophages more slowly than MUS (3). It was also noted that mice exposed to MUS exhibited higher levels of hydrolase activity in their plasma than did
CAR-treated mice. This difference in the extent of lysosomal enzyme release was difficult to interpret. Some investigators have suggested that as the phagocytic load increased, the degree to which lysosomal enzymes accumulated in the surrounding tissue fluid also increased (24). Therefore, it was possible that MUS exceeded CAR in the capacity to induce the release of lysosomal enzymes because the dose of MUS that was given represented a greater phagocytic load to macrophages than did the dose of CAR. However, the route by which each of these toxins was administered may also have played a significant role in the extent to which lysosomal hydrolases accumulated in plasma. MUS was injected i.v., whereas CAR was administered by the i.p. route. Consequently, it was possible that treatment with MUS lead to a higher increase in hydrolase activity simply because it was more widely distributed throughout the tissues and was phagocytized by a larger number of macrophages which, in turn, released large amounts of hydrolytic enzymes following destabilization of lysosomal membranes.

The levels of hydrolase activities seen after stimulation with TT, especially during the first peaks, were lower than those detected in either MUS or CAR-treated mice. This was most likely the result of the different mechanism by which TT was thought to function as a macrophage toxin. CAR and MUS were thought to act on lysosomes directly, leading to rupture of these vesicles, while TT was postulated to interfere with cell membrane synthesis and not markedly affect lysosomal stability.
In general, it appeared that treatment with the macrophage toxins resulted in a biphasic pattern of lysosomal enzyme release. The first rise in hydrolase activity seemed to be specific for damage to macrophages. The second peak in activity, however, may have been the result of injury to other tissues, recycling of the macrophage toxins, or a combination of both. From these results, it must be assumed that the plasma levels of acid hydrolase activity were accurate markers for the destabilization of lysosomal membranes within macrophages during the first 8 h of exposure to the toxins, but not thereafter.

Effects of sera from mice treated with CAR, MUS, or TT on the generation of a pseudo-secondary Ab response to LPS. After having established that lysosomes, specifically located within macrophages, were damaged 6 h post-treatment with CAR or MUS, an experiment was designed to test the hypothesis that priming for a pseudo-secondary response to LPS was caused by a factor which was released from lysosomes following destabilization of the membrane by macrophage toxins. It was postulated that if a factor which was cross-reactive with LPS was released after lysosomal rupture, and if it was present in serum in an immunologically active form, then recipients of serum harvested from mice exposed to CAR and MUS should have exhibited a pseudo-secondary response upon challenge with LPS. Serum was transferred 6 h after treatment as well as at several other times in order to determine when, if at all, and for how long the priming factor was active in serum. Table 1 shows the
Figure 1. Kinetics and specificity of lysosomal enzyme release induced by treatment with CAR. Mice were injected, i.p., with 5 mg CAR or an equivalent volume of PBS (normal mice). Enzyme analyses of plasma pooled from 3 mice were performed post-treatment at the times indicated. For each enzyme studied, the level of activity present in the CAR-treated mice was expressed as \( x \) (enzyme activity in normal mice). Each value shown represents the mean of three experiments.

Symbols:

- **Line A**: Acid phosphatase
- **Line B**: \( \beta \)-D-galactosidase
- **Line C**: \( \beta \)-D-glucuronidase
- **Line D**: Ornithine carbamyl transferase
- **Line E**: Creatine phosphokinase

\( \uparrow \): Indicates time of injection of CAR
Figure 2. Kinetics and specificity of lysosomal enzyme release following the administration of MUS. Mice were injected, i.v., with 8 mg MUS or an equivalent volume of PBS (normal mice). Enzyme analyses of plasma pooled from three mice were performed post-treatment at the times indicated. For each enzyme studied, the level of activity present in MUS-treated mice was expressed as $x$(enzyme activity in normal mice). Each value shown represents the mean of three experiments.

Symbols:

Line A: Acid phosphatase  
Line B: $\beta$-D-galactosidase  
Line C: $\beta$-D-glucuronidase  
Line D: Ornithine carbamyl transferase  
Line E: Creatine phosphokinase  

↑: Indicates time of injection of MUS
Figure 3. Kinetics of lysosomal enzyme release induced by the administration of TT. Mice were injected, i.v., with either 50 mg TT or an equivalent volume of PBS (normal mice). Enzyme analyses of plasma pooled from three mice were performed post-treatment at the times indicated. For each enzyme studied, the level of activity present in TT-treated mice was expressed as $x$(enzyme activity in normal mice). Each value shown represents the mean of three experiments. A limited supply of TT prevented evaluation of CPK and OCT activity.

Symbols:

Line A: Acid phosphatase
Line B: $\beta$-D-galactosidase
Line C: $\beta$-D-glucuronidase

↑: Indicates the time of injection of TT
Table 1
Effects of sera from mice treated with CAR, MUS, and TT on the generation of a pseudo-secondary Ab response to LPS.

<table>
<thead>
<tr>
<th>GROUP</th>
<th>TREATMENT</th>
<th>Anti-LPS antibody titer</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Donor</td>
<td>Recipient</td>
</tr>
<tr>
<td>A</td>
<td>CAR</td>
<td>d.s. 6 h post CAR</td>
</tr>
<tr>
<td>B</td>
<td>CAR</td>
<td>d.s. 6 h post CAR</td>
</tr>
<tr>
<td>C</td>
<td>CAR</td>
<td>d.s. 6 h post CAR</td>
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<tr>
<td>D</td>
<td>CAR</td>
<td>d.s. 12 h post CAR</td>
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<tr>
<td>E</td>
<td>CAR</td>
<td>d.s. 48 h post CAR</td>
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<tr>
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<td>CAR</td>
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<tr>
<td>G</td>
<td>MUS</td>
<td>d.s. 6 h post MUS</td>
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<tr>
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<tr>
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<td>MUS</td>
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<td>CAR</td>
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<td>T</td>
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</tr>
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</table>

a. Antibody titers were obtained by passive hemagglutination tests and were expressed as values of \( x \) derived from the equation, \( x = \log_{10}(HD/2) \), where HD was the reciprocal of the highest dilution of sera that produced hemagglutination. Each experimental group value represents the titer of sera pooled from three to five mice.

b. 5 mg CAR, i.p.; 8 mg MUS, i.v.; 50 mg TT, i.v.; 0.30-0.35 ml donor serum(d.s.), i.p.

c. 10 µg LPS from E. coli 0113, i.p.
capacity of sera from mice treated with CAR, MUS, or TT to prime recipient mice for a pseudo-secondary antibody response to LPS. Serum taken from CAR-stimulated donors at 6 and 48 h (Groups B and E) was able to prime recipients for a pseudo-secondary response (as compared to a true secondary response in Group T). While the recipients of serum harvested 12 h, 16 h, and 5 d after exposure to CAR (Groups C, D, and F) did exhibit a higher anti-LPS antibody response than those which received normal mouse serum (Group P), there was not a great enough difference between the titers observed to consider the responses shown in Groups C, D, and F as pseudo-secondary responses. (Various groups must show at least a two tube difference in titer for them to be considered significantly different.) Serum from MUS-treated donors was able to prime recipients for a pseudo-secondary response when it was obtained 6, 12, and 16 h after administration of the toxin (Groups H, I, and J). By 48 h, serum from donors given MUS was no longer able to prime for an anti-LPS response that was statistically in the range of a pseudo-secondary response (Group K) and by 5 d, serum from the same donors was ineffective for sensitizing recipients to LPS (Group L). Serum obtained from donors treated with TT was unable to prime recipients for a pseudo-secondary response at any of the times examined (Groups N and O). Unfortunately, a limited supply of TT prevented a more thorough study. Groups A, G, and M demonstrated that the pseudo-secondary response observed in other groups was not a manifestation of residual antibody induced by the initial injection of serum from toxin-treated mice.
Comparison of serum vs. plasma in generating a pseudo-secondary Ab response. A series of experiments was set up to determine the effectiveness of plasma in generating a pseudo-secondary antibody response to LPS. This became necessary in view of the fact that the enzyme assays used to monitor the rupture of lysosomal membranes and the release of lysosomal products were performed with plasma while the passive transfers shown to be capable of priming mice for a pseudo-secondary response utilized serum. As a result of this variance in procedure, the following experiments were designed to test whether or not the plasma-serum systems were equivalent for the purposes of this study. Donor mice were given 5 mg CAR, i.p., and exsanguinated 6 h later. A pool was made of the blood collected and either the plasma or serum recovered. Transfers of serum, plasma, or serum treated with heparin were then made to recipient mice which were challenged with LPS 15 d later. As shown in Table 2, plasma (Group B), as well as serum (Group A), was able to prime mice for a pseudo-secondary response to LPS. In addition, heparin (Group C) did not interfere with the factor found in serum and plasma which was responsible for the priming event. Therefore, it appeared that the plasma-serum systems were equivalent.

Stabilization of the lysosomal membrane with the use of poly(2-vinylpyridine-N-oxide). Previous studies (3) have shown that poly(2-vinylpyridine-N-oxide) (PVPO) protected macrophages from lysosomal
Table 2

Comparison of serum versus plasma for generating the pseudo-secondary response.

<table>
<thead>
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<th>Anti-LPS antibody titer</th>
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</tr>
<tr>
<td></td>
<td></td>
<td>7.5</td>
</tr>
<tr>
<td>F</td>
<td>-</td>
<td>PBS</td>
</tr>
<tr>
<td></td>
<td></td>
<td>LPS</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4.0</td>
</tr>
</tbody>
</table>

a. Antibody titers were obtained by passive hemagglutination tests and are expressed as values of $x$, derived from the equation, $x = \log_2(\text{HD}/2)$, where HD was the reciprocal of the highest dilution of sera that produced hemagglutination. Each experimental group value represents the titer of sera pooled from four or five mice.

b. 5 mg CAR, i.p.; 10 µg LPS from *E. coli* 0113, i.p.; 0.30 - 0.35 ml of either donor serum(d.s.) or donor plasma(d.p.), i.p.

c. 10 µg LPS from *E. coli* 0113, i.p.
disruption and the resultant cytotoxic effects of microparticulate silica (MUS) in vitro. It was suggested that PVPO blocked the action of silica by stabilizing the lysosomal membranes of affected cells. Based on this information, PVPO was used in an attempt to prevent lysosomal de-stabilization in vivo by CAR and MUS. Groups of three mice each were injected with PVPO 1 hr prior to administration of either the macrophage toxins or PBS. The mice were exsanguinated at various times post-treatment and the plasma collected from each group was pooled for enzyme analyses. The enzymes under study included the three lysosomal markers used previously to estimate lysosomal integrity - AcPh, β-Gal, and β-GlcU. The effect of PVPO on the ability of CAR and MUS to destabilize the lysosomal membranes of actively phagocytizing macrophages is illustrated in Figs. 4 and 5, respectively. In both cases, the marker enzymes exhibited normal levels of activity until after 8 h of exposure to the toxins. By 12 h there was a dramatic increase in activity for all three hydrolases studied. This represented a delay in the onset of lysosomal rupture since earlier results indicated that, without prior exposure to PVPO, lysosomal enzyme release was apparent 6 h after administration of CAR (Fig. 1) and 4 h after treatment with MUS (Fig. 2). These results suggested that PVPO acted in vivo to stabilize the lysosomal membranes within macrophages which ingested CAR or MUS, but that its effects were temporary and that lysosomal rupture did occur, albeit 6-8 h later than when the toxins were given alone.
Induction of a pseudo-secondary Ab response to LPS required lysosomal destabilization. The temporary ability of PVPO to stabilize lysosomal membranes against the destabilizing effects of CAR and MUS provided an excellent experimental situation for an investigation of the mechanism underlying the development of the pseudo-secondary response. By transferring serum during periods of both lysosomal stabilization and destabilization, it was possible to determine if lysosomal damage was specifically required for serum from toxin-treated mice to prime recipients to LPS. The procedure used in these investigations was as follows. In every case, donor mice received PVPO 1 h prior to a second treatment, which consisted of an injection of CAR, MUS, or PBS. Donor serum was then harvested for transfer to recipients at 6, 12, and 16 h after the second treatment. Recipient mice were challenged with LPS on day 15 and their sera titered 4 d later for anti-LPS antibody. Table 3 details the results. Mice given serum from donors 6 h post-administration of toxin (Groups A and D), which represented a period of lysosomal stabilization in PVPO-treated mice (see Figs. 4 and 5), were not primed to LPS and were unable to mount a pseudo-secondary response when challenged with LPS at day 15. In contrast, mice which received serum from donors at a time corresponding to lysosomal damage and rupture (Groups B, C, E, and F) were primed and did exhibit a pseudo-secondary response to LPS that was similar in magnitude to a true secondary response (Group J). Recipients of serum harvested from donors given PVPO plus a placebo injection of PBS (Group G-I) showed a typical primary response to a single injection of LPS at day 15. These controls served to demonstrate that PVPO was not
Fig. 4. Treatment with PVPO caused temporary stabilization of lysosomal membranes in mice exposed to CAR. Mice were injected with 4 mg PVPO, i.v., 1 h prior to the administration of CAR(5 mg, i.p.) or an equivalent volume of PBS. Enzyme analyses of plasma pooled from three mice were performed at the times indicated after treatment with CAR. For each enzyme studied, the level of activity present in mice given PVPO + CAR was expressed as x(enzyme activity in mice given PVPO + PBS). Each value shown represents the mean of two experiments.

Symbols:

Line A: Acid phosphatase
Line B: $\beta$-D-galactosidase
Line C: $\beta$-D-glucuronidase

↑: Indicates the time of injection of CAR
Fig. 5. Treatment with PVPO caused temporary stabilization of lysosomal membranes in mice given MUS. Mice were injected with 4 mg PVPO, i.v., 1 h prior to the administration of MUS (8 mg, i.v.) or an equivalent volume of PBS. Enzyme analyses of plasma pooled from three mice were performed at the times indicated after treatment with MUS. For each enzyme studied, the level of activity present in mice exposed to PVPO + MUS was expressed as x(enzyme activity in mice given PVPO + PBS). Each value shown represents the mean of two experiments.

Symbols:
Line: Acid phosphatase
Line B: β-D-galactosidase
Line C: β-D-glucuronidase
↑: Indicates the time of injection of MUS
Table 3

Induction of a pseudo-secondary Ab response to LPS required lysosomal destabilization.

<table>
<thead>
<tr>
<th>GROUP</th>
<th>TREATMENT</th>
<th>Anti-LPS antibody titer</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 0</td>
<td>Day 15</td>
</tr>
<tr>
<td>Donor</td>
<td>Recipient</td>
<td>Experimental Group</td>
</tr>
<tr>
<td>A</td>
<td>PVPO + CAR</td>
<td>d.s. 6 h post CAR</td>
</tr>
<tr>
<td>B</td>
<td>PVPO + CAR</td>
<td>d.s. 12 h post CAR</td>
</tr>
<tr>
<td>C</td>
<td>PVPO + CAR</td>
<td>d.s. 16 h post CAR</td>
</tr>
<tr>
<td>D</td>
<td>PVPO + MUS</td>
<td>d.s. 6 h post MUS</td>
</tr>
<tr>
<td>E</td>
<td>PVPO + MUS</td>
<td>d.s. 12 h post MUS</td>
</tr>
<tr>
<td>F</td>
<td>PVPO + MUS</td>
<td>d.s. 16 h post MUS</td>
</tr>
<tr>
<td>G</td>
<td>PVPO + PBS</td>
<td>d.s. 6 h post PBS</td>
</tr>
<tr>
<td>H</td>
<td>PVPO + PBS</td>
<td>d.s. 12 h post PBS</td>
</tr>
<tr>
<td>I</td>
<td>PVPO + PBS</td>
<td>d.s. 16 h post PBS</td>
</tr>
<tr>
<td>J</td>
<td>-</td>
<td>LPS</td>
</tr>
</tbody>
</table>

a. Antibody titers were obtained by passive hemagglutination tests and were expressed as values of $x$, derived from the equation, $x = \log_2(1/HD)$, where HD represents the reciprocal of the highest dilution of sera that produced hemagglutination. Each experimental group value represents the titer of sera pooled from four or five mice.

b. 4 mg poly(-2-vinylpyridine-N-oxide) (PVPO) were injected i.v. 1 h prior to either CAR(5 mg, i.p.), MUS(8 mg, i.v.), or PBS. Recipient mice received 0.30-0.35 ml donor serum(d.s.), i.p.

c. 10 μg LPS from E. coli 0113, i.p.
in itself responsible for inducing the pseudo-secondary response observed in other groups. From the above results, it was evident that serum transferred from CAR or MUS-treated mice was unable to prime the recipients to LPS unless lysosomal destabilization had occurred in the donors and that rupture or damage to lysosomes was a vital step in the generation of a pseudo-secondary response to LPS.

Genetic requirement of recipient mice for the generation of a pseudo-secondary Ab response by passive transfer of serum. Previous data showed that C3H/HeJ mice were unable to generate a pseudo-secondary antibody response when treated with toxins for macrophages (76), or with various fractions of macrophages (66), and later challenged with LPS. It was postulated that the mice were primed by the treatments mentioned, but, because C3H/HeJ mice lacked the genetic ability to respond to the Lipid A portion of LPS (40, 59, 81), there was no recognition of the signal required to trigger a secondary response. Nude mice were also shown to be incapable of mounting a pseudo-secondary response to LPS (76). The suggestion here was that nude mice, as responders to LPS, were not primed following treatment with the macrophage toxins because the priming event was T cell-dependent. As a corollary to these studies, an attempt was made to prime C3H/HeJ as well as nude mice to LPS by transfer of serum from CAR-treated mice. As seen in Table 4, serum from CAR-stimulated RML donors exerted an
Table 4

Genetic requirement of recipient mice for the generation of a pseudo-secondary Ab response by passive transfer of serum.

<table>
<thead>
<tr>
<th>GROUP</th>
<th>MOUSE STRAIN</th>
<th>TREATMENT</th>
<th>anti-LPS antibody titer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Donor : Recipient</td>
<td>Day 0</td>
<td>Day 15</td>
<td>Day 19</td>
</tr>
<tr>
<td>A</td>
<td>RML Swiss : C3H/HeJ</td>
<td>CAR</td>
<td>d.s. 6 h post CAR</td>
</tr>
<tr>
<td>B</td>
<td>RML Swiss : Nude</td>
<td>PBS</td>
<td>d.s. 6 h post PBS</td>
</tr>
<tr>
<td>C</td>
<td>RML Swiss : Nude</td>
<td>CAR</td>
<td>d.s. 6 h post CAR</td>
</tr>
<tr>
<td>D</td>
<td>RML Swiss : RML Swiss</td>
<td>PBS</td>
<td>d.s. 6 h post PBS</td>
</tr>
<tr>
<td>E</td>
<td>RML Swiss : RML Swiss</td>
<td>CAR</td>
<td>d.s. 6 h post CAR</td>
</tr>
<tr>
<td>F</td>
<td>RML Swiss : RML Swiss</td>
<td>PBS</td>
<td>d.s. 6 h post PBS</td>
</tr>
</tbody>
</table>

a. Antibody titers were obtained by passive hemagglutination tests and were 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. Each experimental group value represents the titer of sera pooled from three or four mice.

b. 5 mg CAR, i.p.; 0.30-0.35 ml donor serum (d.s.), i.p.

c. 10 µg LPS from E. coli 0113, i.p.
effect on the ability of C3H/HeJ mice to respond to a subsequent challenge with LPS. C3H/HeJ mice which received normal RML mouse serum at day 0 produced no detectable antibody response (Group B) when challenged at day 15 with LPS, while those which received serum from toxin-treated mice were found to have a mean titer of 4.3 (Group A). However, because this titer was much lower than that observed for the pseudo-secondary response produced in RML recipients (Group E), it probably represented an enhanced primary response made possible after priming of C3H/HeJ mice to some component of the LPS molecule had already taken place. Such results were consistent with earlier reports that C3H/HeJ mice were unable to mount a pseudo-secondary response to LPS. Previous work which demonstrated that nude mice were unable to generate a pseudo-secondary response was also confirmed. Nude mice which received serum from CAR-stimulated donors (Group C) were evidently not primed by such treatment and responded to a later challenge with LPS by producing a typical primary anti-LPS antibody response (as compared to control nude mice in Group D which were given a single injection of LPS 15 d after a placebo injection of normal mouse serum).

**Genetic requirement of donor mice for priming to a pseudo-secondary Ab response by passive transfer of serum.** In view of the evidence presented by other investigators concerning the inability of C3H/HeJ and nude mice to generate a pseudo-secondary response to LPS, it was of interest to explore whether these strains of mice could act as donors, rather than
recipients, in generating a pseudo-secondary response by passive transfer of their sera following treatment with CAR. Table 5 shows that serum from CAR-stimulated C3H/HeJ mice was able to prime Swiss-Webster recipients for a pseudo-secondary antibody response (Group A) that was nearly equal quantitatively to that observed for a true secondary response (Group E). Normal C3H/HeJ serum did not have a significant effect on the anti-LPS antibody titer obtained in Swiss-Webster mice (Group B vs. Group F). Nude mouse serum, however, exhibited the capacity to prime recipients for an anti-LPS response that resembled a true secondary response in magnitude, whether or not the donors had been treated with CAR (Groups C and D).

Serum transferred from mice treated with CAR or MUS did not contain residual toxic particles capable of inducing destabilization of lysosomal membranes in recipient mice. Electron microscopy studies have demonstrated a similarity in structure between extracellular and intracellular CAR (19), suggesting that this material was not readily digested and that it was extruded from impaired macrophages in a nearly or completely intact form. Furthermore, studies with microparticulate silica (MUS) have shown that, once released from killed macrophages, it was as cytotoxic as the original preparation (3). Clearly then, the possibility existed that the serum transferred from mice treated with CAR or MUS contained residual or recycled toxin and that the pseudo-secondary response to LPS generated from such transfers was the result of the effects of the toxins themselves, rather than the...
Table 5

Genetic requirement of donor for priming for a pseudo-secondary Ab response by passive transfer of serum from CAR-treated mice.

<table>
<thead>
<tr>
<th>GROUP</th>
<th>MOUSE STRAIN</th>
<th>TREATMENT</th>
<th>Anti-LPS antibody titer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Donor : Recipient</td>
<td>Day 0</td>
<td>Day 15</td>
<td>Day 19</td>
</tr>
<tr>
<td>C3H/HeJ : Swiss-Webster</td>
<td>Donor</td>
<td>Recipient</td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>CAR</td>
<td>d.s. 6 h post CAR</td>
<td>LPS</td>
</tr>
<tr>
<td></td>
<td>PBS</td>
<td>d.s. 6 h post PBS</td>
<td>LPS</td>
</tr>
<tr>
<td>B</td>
<td>Nude : Swiss-Webster</td>
<td>CAR</td>
<td>d.s. 6 h post CAR</td>
</tr>
<tr>
<td></td>
<td>PBS</td>
<td>d.s. 6 h post PBS</td>
<td>LPS</td>
</tr>
<tr>
<td>C</td>
<td>None : Swiss-Webster</td>
<td>CAR</td>
<td>d.s. 6 h post CAR</td>
</tr>
<tr>
<td></td>
<td>PBS</td>
<td>d.s. 6 h post PBS</td>
<td>LPS</td>
</tr>
</tbody>
</table>

- Antibody titers were obtained by passive hemagglutination tests and were 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. Each experimental group value represents the titer of sera pooled from three or four mice.
- 5 mg CAR, i.p.; 10 μg LPS from E. coli 0113, i.p.; 0.30 ml donor serum (d.s.), i.p.
- 10 μg LPS from E. coli 0113, i.p.
demonstration of some unique factor found in the serum of mice exposed to the toxins. To determine if residual or recycled toxin was present in the serum transferred, the level of AcPh, β-Gal, and β-GlcU activity was assessed in plasma from recipient mice. It was assumed that if the toxins were present, damage to macrophages (or more specifically lysosomes) would have occurred within recipient mice and the activity of the lysosomal marker enzymes would have risen above normal levels. Groups of three mice each received an injection of either normal mouse serum (NMS) or serum derived from mice treated previously with CAR or MUS. At 6, 12, and 24 h post-transfer of the serum, recipient mice were bled and the plasma recovered from each group pooled for enzyme analyses. Mice which received serum from toxin-treated mice exhibited normal levels of acid hydrolase activities as compared to mice which received NMS, at all the times examined (specific data not shown). Therefore, it was concluded that the serum transferred from mice treated with CAR or MUS did not contain residual toxic particles capable of exerting damage to lysosomes in recipient mice.

Effect of CAR on the tissue distribution of $^{51}$Cr-labeled LPS in mice. Considerable controversy has surrounded the question of whether endotoxins were able to escape from the gut. Some investigators have shown absorption of endotoxin from normal intestine (52, 73) and from surgically implanted ileal segments (34), while others have claimed that no such absorption occurred (12, 83). In relation to the present
study, the question of absorption of endotoxin became important in that such LPS might be taken up by macrophages and stored within the lysosomes. (It has been suggested that lysosomes served as the intracellular targets for endotoxin on the basis that endotoxins induced lysosomal instability and caused the release of lysosomal enzymes into tissues) (13, 16, 32, 57, 82, 91, 92). Consequently, it may have been that, upon CAR or MUS-induced lysosomal rupture, LPS itself rather than an LPS-like molecule, was released and was responsible for priming mice for what has been called a pseudo-secondary response. Under these conditions, however, the response observed after challenge with LPS would have been a true secondary response.

Although no efforts were made to investigate further the issue concerning escape of endotoxin from the gut, an experiment was designed to test the proposal that treatment with CAR could have caused the release of LPS from tissue where it was presumably bound within the lysosomes of macrophages. The same dose of $^{51}$Cr-labeled LPS was administered, i.v., to all mice in the study and 24 h was allowed for the LPS to be cleared from the peripheral blood supply by the reticuloendothelial system (RES), primarily Kupffer cells, and stored within the lysosomes. Then, either CAR or PBS was injected, i.p., and 6 h later, at the time corresponding to the rupture of lysosomes specifically within macrophages, the mice were exsanguinated and the various tissues assayed for the presence of $^{51}$Cr-labeled LPS. The results are shown in Table 6. Following treatment with CAR, there was a significant
Table 6

Effect of CAR on the tissue distribution of Cr-labeled LPS in mice.

<table>
<thead>
<tr>
<th>GROUP</th>
<th>ORGAN</th>
<th>b. ng LPS</th>
<th>% LPS injected</th>
<th>c. p (as compared to normal)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>Liver</td>
<td>29000 + 1250</td>
<td>58.0 + 2.5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Spleen</td>
<td>265 + 60</td>
<td>0.5 + 0.1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Blood</td>
<td>476 + 143</td>
<td>0.9 + 0.3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Serum</td>
<td>678 + 171</td>
<td>1.4 + 0.3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>PF</td>
<td>4 + 6</td>
<td>&lt; .1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Carcass</td>
<td>11700 + 1990</td>
<td>23.4 + 4.0</td>
<td></td>
</tr>
<tr>
<td>CAR</td>
<td>Liver</td>
<td>25000 + 1040</td>
<td>49.9 + 2.1</td>
<td>.001</td>
</tr>
<tr>
<td></td>
<td>Spleen</td>
<td>257 + 67</td>
<td>0.5 + 0.1</td>
<td>.2</td>
</tr>
<tr>
<td></td>
<td>Blood</td>
<td>836 + 134</td>
<td>1.7 + 0.3</td>
<td>.05</td>
</tr>
<tr>
<td></td>
<td>Serum</td>
<td>961 + 153</td>
<td>1.9 + 0.3</td>
<td>.01</td>
</tr>
<tr>
<td></td>
<td>PF</td>
<td>245 + 141</td>
<td>0.5 + 0.3</td>
<td>.001</td>
</tr>
<tr>
<td></td>
<td>Carcass</td>
<td>27400 + 2370</td>
<td>54.7 + 4.7</td>
<td>.001</td>
</tr>
</tbody>
</table>

a. All mice received 50,000 ng Cr-labeled LPS i.v. at -24h. At time 0, 5 mg CAR or an equivalent volume of PBS was injected, i.p. At 6h, mice were exsanguinated and the various organs extracted for assay.

b. Nanograms LPS were calculated by the following formula: ng LPS = (50,000 ng LPS) x (counts/organ) / (counts/50,000 ng LPS). All samples were counted for 1 min and were corrected for background and decay. Each value represents the mean of six mice/group + the standard deviation about the mean.

c. p values were calculated by the Student's t-test.

d. 1.0 ml blood was counted. The amount of LPS was determined by assuming a circulating blood volume of 1.5 ml.

e. 0.2 ml serum was counted. Calculations were made assuming 0.7 ml recoverable serum from 1.5 ml blood.

f. 1.0 ml peritoneal fluid (PF) was extracted and counted. No further adjustments were made.
decrease (p<0.001, as compared to normal) in the amount of $^{51}$Cr-LPS found in the liver. At the same time, there was a corresponding increase in the amount of $^{51}$Cr-LPS detected in blood (p<0.05), serum (p<0.01), peritoneal fluid (p<0.001), and the carcass (p<0.001). No significant change (p>0.2) in the percent of $^{51}$Cr-LPS located in the spleen was detected after administration of CAR. These results seemed to indicate that, if present, LPS could have been liberated from lysosomes (following rupture of the lysosomal membrane by CAR) and redistributed to other tissues where it would have been able to sensitize LPS-responsive B cells for a secondary response.

Demonstration of E. coli as part of the normal gut flora of resident mice. Although Escherichia coli, a lactose fermenting, Gram negative rod, has been identified as part of the normal intestinal flora of many animal species, previous work (66) indicated that E. coli was absent from the intestinal tract of Swiss-Webster derived RML mice. Because the presence of indigenous LPS, originating from E. coli in the gut, could have affected the immune response to exogenously administered LPS, another attempt was made to isolate and identify the Gram negative, lactose positive intestinal flora of RML mice. Both male and female mice were used in the study and samples from nine mice were cultured. The entire intestinal contents from individual mice were suspended in 2 ml sterile PBS and streaked onto EMB and McConkey's agar. After 18-24 h incubation, six well isolated, lactose positive colonies were
chosen from either plate and restreaked on a blood agar medium. An
effort was made in choosing colonies to pick those colony types which
were morphologically distinct from one another. Since several of the
isolates were morphologically identical, only 2-3 of the colonies (per
mouse cultured) which were chosen for reisolation were further identi­
ified by standard biochemical tests (IMVIC series, TSI and SIM media,
decarboxylase reactions). All mice were shown to carry E. coli as
part of their normal gut flora. Details concerning the number of
isolates positively identified as E. coli vs. the number tested are
shown in Table 7. The only other lactose fermenting, Gram negative
rod identified was found to belong to the genus Klebsiella. Non­
lactose fermenting, Gram negative rods were also isolated from a few
mice, but no further characterizations were made of these organisms.

After determining that E. coli was indeed a normal inhabitant of the
gut of RML mice, it was necessary to investigate whether any of the
Gram negative organisms isolated would cross-react immunologically with
E. coli 0113, the particular antigenic variety used in the study of the
pseudo-secondary response. Nearly all the colonies which were
transferred to blood agar medium were tested for cross-reactivity with
E. coli 0113 and 0111 by the co-agglutination test. Antibody specific
for the 0113 or 0111 determinant was adsorbed to formalinized
Staphylococcus aureus and one drop of the sensitized S. aureus was
added to an equal volume of a heavy bacterial suspension. Care was
taken to boil the bacterial samples for 20-30 min prior to testing in
Table 7

Isolation and identification of Escherichia coli from RML mice.

<table>
<thead>
<tr>
<th>ANIMAL</th>
<th>Colonies isolated</th>
<th>Identified as E. coli/ Tested</th>
<th>a. Isolates cross-reactive with E. coli 0113 / E. coli 0111 / Tested</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>4</td>
<td>2/2</td>
<td>0/4 0/4</td>
</tr>
<tr>
<td>B</td>
<td>4</td>
<td>2/2</td>
<td>2/4 2/4</td>
</tr>
<tr>
<td>C</td>
<td>6</td>
<td>2/2</td>
<td>0/6 0/6</td>
</tr>
<tr>
<td>D</td>
<td>6</td>
<td>2/3</td>
<td>0/6 0/6</td>
</tr>
<tr>
<td>E</td>
<td>6</td>
<td>2/2</td>
<td>0/6 0/6</td>
</tr>
<tr>
<td>F</td>
<td>6</td>
<td>1/3</td>
<td>0/6 0/6</td>
</tr>
<tr>
<td>G</td>
<td>6</td>
<td>2/3</td>
<td>0/6 0/6</td>
</tr>
<tr>
<td>H</td>
<td>6</td>
<td>2/2</td>
<td>0/6 0/6</td>
</tr>
<tr>
<td>I</td>
<td>6</td>
<td>2/2</td>
<td>0/6 0/6</td>
</tr>
</tbody>
</table>

The intestinal contents of nine RML mice were cultured individually on EMB and McConkey's agar for isolation of Gram negative, lactose positive colonies.

a. The biochemical tests used in the identification of lactose positive colonies included: IMVIC series, motility, urease production, decarboxylase and TSI reactions.

b. Isolates were determined to be cross-reactive with E. coli 0113 and 0111 by the co-agglutination test.
order to unmask any O-somatic antigens which may have been present. Only two of the fifty isolates examined proved to be cross-reactive with \textit{E. coli} 0113 (Table 7). These same two isolates also showed cross-reactivity with \textit{E. coli} 0111 and were identified as \textit{E. coli} themselves. Since these samples were derived from the same mouse, and cultures from eight other mice showed no such cross-reactivity, it was concluded that resident RML mice, while they did harbor \textit{E. coli} as part of their normal gut flora, did not possess an \textit{E. coli} population which exhibited cross-reactivity with either the 0113 or 0111 antigenic determinants of \textit{E. coli}.

\textbf{Effect of CAR on the polyclonal activation of mouse spleen cells.} In the work of Kolb \textit{et al.} (51, 72), CAR has been described as a B-cell mitogen for the mouse system. While the induction of cell proliferation has not been found to correlate directly with the ability to stimulate Ig synthesis (48, 61), most B cell mitogens have also been shown to function as polyclonal activators of Ig synthesis (23, 62). It was of interest to determine if CAR induced nonspecific stimulation of Ab production because the presence of polyclonal Ab in the serum used to prime mice for a pseudo-secondary response could have potentially influenced the specific response obtained to a subsequently administered Ag (33, 53). Three groups of six mice each were treated with CAR and their spleens were excised for analysis 6 h, 2 d, and 5 d later. Each spleen was assayed individually and the number of direct
plaque-forming cells (PFC)/spleen specific for SRBC, rat RBC (RRBC), and LPS from *E. coli* 0113 and 0111 was quantified by a hemolytic plaque assay. Groups of untreated mice and mice given LPS (a known polyclonal cell activator) were included as controls. As indicated in Table 8, polyclonal activation of mouse spleen cells by CAR was apparent 2 d after treatment (Group D). The number of PFC detected at this time was well above background (Group A) for all four Ags tested and was equal, or nearly equal, in magnitude to the number of PFC observed following stimulation with LPS (Group B). Spleen cells taken from mice 6 h (Group C) and 5 d (Group E) after treatment with CAR showed background numbers of PFC.
Table 8

Effect of CAR on the polyclonal activation of mouse spleen cells.

<table>
<thead>
<tr>
<th>GROUP</th>
<th>TREATMENT</th>
<th>Time of Assay</th>
<th># PFC/spleen</th>
<th>Anti-0113</th>
<th>Anti-0111</th>
<th>Anti-SRBC</th>
<th>Anti-RRBC</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>NONE</td>
<td>12 ± 5</td>
<td>15 ± 10</td>
<td>75 ± 23</td>
<td>48 ± 17</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>LPS</td>
<td>2 d 10,750 ± 848</td>
<td>8,675 ± 601</td>
<td>3,825 ± 248</td>
<td>3,350 ± 141</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>CAR</td>
<td>6 h 68 ± 11</td>
<td>42 ± 3</td>
<td>128 ± 11</td>
<td>55 ± 9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>D</td>
<td>CAR</td>
<td>2 d 5,458 ± 529</td>
<td>4,575 ± 417</td>
<td>3,300 ± 482</td>
<td>2,900 ± 278</td>
<td></td>
<td></td>
</tr>
<tr>
<td>E</td>
<td>CAR</td>
<td>5 d 81 ± 38</td>
<td>77 ± 21</td>
<td>68 ± 18</td>
<td>78 ± 25</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The number of direct plaque-forming cells (PFC)/spleen specific for sheep RBC (SRBC), rat RBC (RRBC) and LPS from E. coli 0113 and 0111 was determined by a hemolytic plaque assay. The CAR values represent the mean of six mice/group. The untreated and LPS values represent the mean of two groups of two mice each in which the spleens were pooled.

a. 20 μg LPS from E. coli 0113, i.v.; 5 mg CAR, i.p.
IV. DISCUSSION

From the data presented, it was evident that a factor, which was able to sensitize mice for a pseudo-secondary antibody response to LPS, was released to the serum following destabilization of lysosomal membranes within macrophages. The release of this factor was inhibited by administering a lysosomal stabilizing agent, PVPO, prior to treatment with CAR or MUS. Furthermore, it was unlikely that the pseudo-secondary response was induced by LPS itself because there did not appear to be a suitable source of endogenous LPS.

Generation of a pseudo-secondary response by passive transfer of serum from CAR and MUS-treated mice. Previous investigators have proposed that priming for a pseudo-secondary response to LPS was caused by a factor liberated from lysed macrophages, or more specifically from ruptured lysosomes (66, 76). However, it was not until the current study that the presence of such a factor in the mouse system was demonstrated in vivo. In this report it was shown that serum, harvested from donors treated with MUS or CAR, was able to sensitize recipient mice to LPS. In particular, mice were primed for a pseudo-secondary response by serum which was collected from donors 6, 12, and 16 h after exposure to MUS and 6 and 48 h after treatment with CAR.

A preliminary analysis of the kinetics and specificity of lysosomal enzyme release had revealed that damage to lysosomes within macrophages
occurred 6 h after the introduction of either CAR or MUS. Thus, it was possible to correlate the discharge of lysosomal products from macrophages at 6 h with the ability of serum harvested at that time to prime mice for a pseudo-secondary response. An attempt to establish any further temporal relationships between elevated lysosomal enzyme activities and the ability of serum to sensitize recipients to LPS proved to be of little value. Serum obtained during a second phase of lysosomal damage induced by CAR (12-16 h) was unable to prime mice for a pseudo-secondary response, while serum collected during the ensuing period of lysosomal stability (48 h) was able to sensitize mice to LPS. In addition, serum from donors exposed to MUS for 12 as well as 16 h was capable of inducing a pseudo-secondary response to LPS, yet the levels of hydrolase activities detected at these times varied from nearly normal (12 h) to greatly elevated (16 h). Consequently, although it appeared that the initial rupture of macrophage-derived lysosomes lead to the release of a lysosomal component which was able to prime mice to LPS, it was apparent that the subsequent immunological activity of this component in serum was not related to the attendant levels of lysosomal hydrolase activities.

Several factors may have influenced the immune expression of this LPS-like substance and explained why, after the first 6 h of exposure to the toxins, such an inconsistent pattern was observed between the periods of lysosomal instability and the times at which serum from CAR- and MUS-treated mice was able to prime recipients for a pseudo-
secondary response. Although a number of researchers (97, 71) have reported that, in general, macrophages which interiorized large numbers of particles were rendered less active in phagocytosis, it has been suggested (10, 14) that a small population of macrophages which survived the toxic effects of CAR-ingestion were activated and exhibited enhanced, rather than diminished, phagocytic properties. If CAR-treatment resulted in the activation of some surviving macrophages, it was possible that these cells could have rapidly cleared the priming factor from the serum in spite of overt RES impairment, thereby accounting for the inability of serum harvested at 12 and 16 h to prime mice for a pseudo-secondary response. Activation of macrophages has not been reported following the administration of MUS, and in fact, one investigator has claimed that uptake of MUS always initiated cell death (25). Therefore, the prolonged (up to 12 and 16 h) immunological activity of the priming factor in serum derived from MUS-treated mice may have been attributable to the overall depressed state of the RES and the inability of a phagocytic cell population to sequester the Ag.

There remained one other plausible explanation for why serum obtained 16 h following treatment with MUS was able to prime mice for a pseudo-secondary response. Since this period of time corresponded to a second rise in lysosomal hydrolase activity, it may have been that recycled toxin or general tissue damage lead to further lysosomal labilization which was, in turn, followed by the re-emission of the component responsible for sensitizing mice to LPS. CAR-stimulated
mice also exhibited a second peak in lysosomal enzyme activity (16 h), yet serum taken from donors at this time did not prime recipients for a pseudo-secondary response. One reason for this may have been that the priming factor was not released for a second time in mice exposed to CAR. The increase in hydrolase activity detected at 16 h could have been a consequence of either secondary tissue damage or the recycling of CAR in smaller doses to newly matured macrophages. Both of these events could have resulted in the secretion of hydrolytic enzymes from lysosomes without rupture of the vesicles or discharge of the priming factor if it was bound to the membrane. If the form in which the priming factor was liberated in vivo played a role in its immunological activity, then perhaps this LPS-like molecule was present in the serum after the second period of CAR-induced lysosomal damage, but, because of the abnormal functional state of the RES and the overall systemic damage that was apparent at 16 h, it may not have been converted into a form in which the LPS-like immunodeterminant(s) was exposed to and recognized by the immune system of recipient mice. This may then have accounted for the ability of serum collected 48 h after the administration of CAR to prime recipients to LPS. By 48 h, RES function in CAR-stimulated mice would have just begun to improve (11) and recovering or newly matured macrophages may have been able to process the priming factor into the proper immunogenic form. It was apparent, though, that once RES activity had returned to normal levels (5 d after treatment with either CAR or MUS) serum from toxin-treated mice
no longer contained a component which was able to prime recipient mice for a pseudo-secondary response.

Finally, in a related experiment, it was shown that serum from mice given CAR or MUS did not contain residual toxic particles capable of causing lysosomal instability in recipient mice. Therefore, the mechanism of priming for a pseudo-secondary response by various sera was not effected or affected by contamination with CAR or MUS.

When viewed in toto, the above information indicated that lysosomal destabilization, induced by CAR or MUS, lead to the appearance in serum of a substance which was antigenically similar to LPS and, thus, capable of sensitizing mice for a pseudo-secondary response to LPS. In addition, the immunological activity of this substance in serum was potentially influenced by a variety of conditions which undoubtedly varied with respect to the macrophage toxin that was initially injected.

**Induction of a pseudo-secondary Ab response to LPS required lysosomal destabilization.** Two additional experiments were performed in order to corroborate or disprove the theory that the mechanism by which serum gained the capacity to prime recipients for a pseudo-secondary response to LPS involved lysosomal destabilization. In one experiment it was shown that serum from mice injected with TT, a macrophage inhibitor which has not been demonstrated to alter lysosomal stability, was unable to sensitize mice to LPS. In a subsequent investigation, PVPO was employed to protect lysosomes from the labilizing effects of CAR and MUS. Since the protection afforded by PVPO-treatment
proved to be temporary, it was possible to obtain serum from donor mice during periods of both lysosomal stability and instability. Mice which received serum harvested before lysosomal damage occurred did not exhibit a pseudo-secondary antibody response when later challenged with LPS. On the other hand, a pseudo-secondary response was observed in mice which had received serum collected while lysosomal rupture was taking place. From these results, it was evident that lysosomal destabilization was necessary for serum to acquire the ability to immunize mice for a pseudo-secondary response.

Genetic requirements for the generation of a pseudo-secondary response. An investigation was made into the genetic requirements of the donor and recipient mice used in the study of the generation of a pseudo-secondary antibody response by passive transfer of serum. It was found that nude mice which received serum from CAR-stimulated, RML mice (serum from the RML strain had already been shown to contain the priming factor) were not primed by such treatment and produced a typical primary antibody response when challenged with LPS 15 d later. The failure of nude mice to respond in a pseudo-secondary fashion was interpreted to mean that the priming event was T cell-dependent. These results were supported by the data of a previous study (76) in which a single injection of LPS was unable to elicit a pseudo-secondary response in nude mice that had been treated directly with CAR or MUS 21 d earlier. Likewise, when initially exposed to toxins for macrophages (76) or various fractions of macrophages (66),
C3H/HeJ mice have also been shown to be incapable of mounting a pseudo-secondary response to LPS. It was suggested that priming of the mice took place, but that, because C3H/HeJ mice were genetically unresponsive to the Lipid A portion of LPS, there was no recognition of the signal necessary to trigger a secondary response when these mice were subsequently challenged with LPS. The work presented herein has substantiated these claims. The anti-LPS antibody titer that was observed in C3H/HeJ mice, which were injected with serum from CAR-treated donors and later immunized with LPS, was significantly higher than that obtained in C3H/HeJ mice originally given normal mouse serum, but the response was not considered to be a pseudo-secondary response because it did not reach the same order of magnitude as the pseudo-secondary response manifested by RML mice which were given identical treatment. In order for C3H/HeJ mice to have exhibited a secondary-type response to any of the immunodeterminants of LPS, the mechanism for the activation of the appropriate $B_M$ cells would have had to have been independent of the recognition of the second signal provided by Lipid A. If an alternate pathway for $B_M$ cell activation was involved in the mechanism for the generation of a pseudo-secondary response, then the anti-LPS antibody titer obtained in C3H/HeJ mice would have been expected to be similar quantitatively to that obtained in RML mice. Since the level of antibody production was much lower in C3H/HeJ mice, it seemed likely that the response observed in these mice was merely a representation of an enhanced primary antibody response.
Although C3H/HeJ mice were unable to serve as recipients for the generation of a pseudo-secondary response by passive transfer of serum, it was shown that, following the administration of CAR, serum harvested from these mice was able to sensitize Swiss-Webster mice for a pseudo-secondary response. Nude mouse serum, however, proved to be effective in priming recipients to LPS whether or not the donors had been treated with CAR. This was an interesting observation in view of the proposal (20, 65) that nude mice have compensated for the lack of thymus-derived immune functions by developing a more highly activated RES. Because activated macrophages have been found to secrete high levels of lysosomal enzymes (as compared to resting macrophages) (68), it was possible that a lysosomal product, capable of sensitizing mice for a pseudo-secondary response, was present as a normal constituent of nude mouse serum. With the demonstration that serum from a variety of different strains of mice treated with CAR (responders as well as non-responders to LPS) was capable of priming recipients to LPS, it was conceivable that the factor which was responsible for the priming event was a lysosomal component present in all mice.

Endogenous LPS and the pseudo-secondary response. Several investigators have suggested that bacterial endotoxins were able to escape from the gut (34, 52, 73). When this possibility was viewed in conjunction with the finding that LPS was stored undegraded within the lysosomes of macrophages (13), it became important to determine if labilization of lysosomal membranes by macrophage toxins could have liberated LPS
itself, rather than a molecule which was antigenically similar to LPS, from damaged cells.

In one approach to the study of this problem, a standard dose of $^{51}$Cr-labeled LPS was administered to several mice and 24 h was allowed for the LPS to accumulate within the lysosomes of actively phagocytizing macrophages. Then, CAR was injected and 6 h later, at the time corresponding to the rupture of lysosomes specifically within macrophages, various tissues were removed from the mice and assayed for the presence of $^{51}$Cr-LPS. The results of this experiment showed that if LPS were present within lysosomes, then it could have been released to the serum (as well as other tissues) following destabilization of lysosomal membranes. Considering that very small doses ($10^{-9}$ to $10^{-11}$ μg) of LPS have been demonstrated to prime animals for a secondary response (74, 79), it was evident that even if a seemingly negligible quantity of LPS was released from lysosomes in situ, that amount of LPS probably would have been sufficient to prime mice for what has been called a pseudo-secondary response. However, the likeliness of such a priming event depended on whether there was a suitable source of endogenous LPS. In an earlier report (66), it was proposed that the pseudo-secondary response was specific for LPS from E. coli 0113. Therefore, a characterization was made of the Gram negative, lactose positive intestinal flora of resident mice. While all of the mice cultured were found to carry E. coli as part of their normal gut flora,
it was demonstrated that these mice did not, in the great majority of
cases, harbor an *E. coli* (or *Klebsiella*) population which was cross-
reactive immunologically with *E. coli* O113. Two sources of LPS that
were not examined for cross-reactivity with *E. coli* O113 included the
Gram negative anaerobic and Gram negative, non-lactose fermenting,
aerobic flora. It was unlikely that an anaerobic bacterium would have
cross-reacted with *E. coli* because these groups have not been related
serologically. Many of the lactose negative, aerobic genera, on the
other hand, have exhibited antigenic similarities to *E. coli*
serotypes (29), but it was doubtful that an LPS molecule derived from
a lactose negative species could have induced the pseudo-secondary
response because bacteria of this type were not isolated from every
mouse cultured. A final consideration included the possibility that
an immunologically unrelated LPS serotype was taken up by macrophages,
processed within the lysosome, and converted into a form which exhibited
antigenic similarities to LPS from *E. coli* O113. However, the
generation of a pseudo-secondary response was shown to be T cell
dependent and, based on this as well as the above information, it
appeared that indigenous LPS could not have been responsible for in-
ducing the pseudo-secondary response.

**Polyclonal activation of mouse spleen cells by CAR.** The intracellular
pathways culminating in blastogenesis of B cells and in polyclonal
Ig synthesis have generally been considered as independent, but closely
linked events (38, 39, 48, 61) and most B cell mitogens have also been identified as polyclonal B cell activators (23, 62). CAR has been demonstrated to function as a B cell mitogen in the mouse system (51, 72) and, therefore, the possibility existed that it also stimulated polyclonal activation of murine B cells. The importance of nonspecific Ab production by CAR, in relation to the present study, was rooted in the work of Forni, et al. (33) who showed that polyclonal IgM Abs directed against SRBC were able to induce, in the absence of Ag, an Ab response of the same specificity as the injected Abs. Based on Forni's report, which other investigators have not supported (41), it was conceivable that, if CAR treatment resulted in the production of a heterogenous population of anti-LPS Abs, these Abs would have been present in the serum transferred to other mice and could have activated B cell clones of the same specificity to synthesize Ab and differentiate into memory cells. As a result, the so-called pseudo-secondary response obtained after challenge with LPS would not have been a demonstration of some unique factor found in the serum, but rather the effects of an Ab population induced by polyclonal activation with CAR.

As indicated from the data presented in this study, polyclonal activation of mouse spleen cells by CAR was apparent 2 d after treatment. In view of this evidence alone, it may have been plausible that the pseudo-secondary response generated by serum collected 2 d following administration of the toxin could have been induced by polyclonal Abs.
However, serum harvested 6 h after CAR-treatment was also capable of priming recipients for a pseudo-secondary response to LPS and polyclonal activation was not detected in mice exposed to the toxin for 6 h. Consequently, the original proposal - that priming to LPS was caused by a factor present in serum following lysosomal destabilization - appeared to be the more likely explanation for the generation of a pseudo-secondary response by serum transfer from CAR-stimulated donors.

The ability of serum from MUS-treated mice to prime recipients to LPS also argued against the possibility that the pseudo-secondary response was induced by CAR through the action of polyclonal Abs. To date, MUS has only been shown to affect B lymphocytes indirectly (55) and therefore, it was highly unlikely that the administration of MUS resulted in nonspecific stimulation of murine B cells and the accumulation of polyclonal Abs in the serum. Since the body of evidence concerning the means by which serum from MUS-treated mice was able to sensitize recipients for a pseudo-secondary response has pointed toward the presence of a unique substance released from labilized lysosomes, it was doubtful that CAR, which also caused lysosomal destabilization, would have failed to liberate the same substance.
V. SUMMARY

The phenomenon of pseudo-secondary responsiveness to a single injection of LPS was originally described by Becker and Rudbach (11). It was noted that mice which had been treated with the macrophage toxins, CAR and MUS, produced a secondary-type Ab response when challenged with LPS from \textit{E. coli} 0113 21 d later. A subsequent investigation (76) revealed that the generation of a pseudo-secondary response to LPS followed the same kinetic and genetic requirements as a true secondary response and that priming to LPS seemed to be dependent upon lysosomal destabilization within macrophages. In a more recent report (66), it was shown that murine macrophages contained a component, presumably of lysosomal origin, which was cross-reactive immunologically with LPS from \textit{E. coli} 0113. Therefore, the theory has evolved that priming for a pseudo-secondary response was caused by a molecule(s) released from lysosomes following MUS or CAR-induced lysosomal rupture within macrophages. The intent of the current study was to determine if the presence of an immunogenic molecule, capable of sensitizing mice to LPS, could be detected \textit{in vivo} after the administration of CAR or MUS. It was shown that serum harvested from mice given these toxins contained a factor which was able to prime recipient mice for a pseudo-secondary response. The appearance of the priming factor in serum was correlated with the onset of lysosomal
destabilization and could be prevented from occurring by artificially stabilizing lysosomal membranes with the use of PVPO or by administering TT, a macrophage inhibitor which did not affect lysosomal stability. In addition, it was demonstrated that the priming factor was liberated from the lysosomes of C3H/HeJ and nude mice, although neither type of mouse was able to respond in a pseudo-secondary fashion to a single dose of LPS when initially injected with serum containing the priming factor. Finally, it appeared that LPS itself was not responsible for sensitizing mice for a pseudo-secondary response because representatives of the mice used in this study did not possess an endogenous bacterial flora which exhibited immunological cross-reactivity with \textit{E. coli} 0113.


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