MACROPHAGE CYTOPHILIC ANTIBODY TO SALMONELLA TYPHIMURIUM

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ABSTRACT

MACROPHAGE CYTOPHILIC ANTIBODY TO
SALMONELLA TYPHIMURIUM

By

Charles W. Stone

A new and more quantitative technique to study macrophage cytophilic antibody is described. It essentially consists of incubation of sensitized cells with antigen in suspension followed by millipore filtration. The filtrate and filter fractions are then counted separately giving the percent of antigen bound (filter) and unbound (filtrate). This technique was used to study the role of cytophilic antibody in *Salmonella typhimurium* adherence to peritoneal cells. Bacterial binding was significantly increased in the presence of immune rat serum. Studies to differentiate opsonic from cytophilic properties were of only limited success, but they did confirm previous findings that a large portion of the immunoglobulin fraction had opsonic properties and that adsorption with large numbers of macrophages ($\sim 1.5 \times 10^7$) did not remove a significant amount is cytophilic activity. Experiments designed to measure the cytophilic binding of free endotoxin by sensitized peritoneal cells showed very low levels of binding (<0.5%).

Charles W. Stone

This study was the first attempt to demonstrate the binding potential of free endotoxin to cytophilic antibody. Interspecies studies using rat serum and mouse peritoneal cells showed evidence suggestive of a higher level of binding with mouse cells than rat cells. This was the first interspecies study between the mouse and the rat with regard to cytophilic activity. It was also the first interspecies study of cytophilic activity with gram negative bacteria. Cumulatively, these data show that macrophage cytophilic antibody increases the rate of phagocytosis of gram negative bacteria by increasing adherence to macrophages. This is one of several ways in which macrophage cytophilic antibody exerts its antibacterial action.

MACROPHAGE CYTOPHILIC ANTIBODY TO *SALMONELLA TYPHIMURIUM*

By
Charles W. Stone

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DEDICATION

To my parents, Paul W. and Sara C. Stone
for their help and encouragement.

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INTRODUCTION

Macrophage cytophilic antibodies have been demonstrated in an association with a number of immunologic phenomenon (85,107). However, no clear-cut role has been ascribed to them. Part of the reason for this is that there is a considerable amount of confusion and controversy concerning the significance of their immunologic action. Another difficulty is that the usual methods used to measure them are semiquantitative at best (10,18,31).

Macrophage cytophilic antibody given passively or present on sensitized macrophages confers protection to *Salmonella typhimurium* in mice (95). A similar macrophage cytophilic antibody has been found in antisera to *Escherichia coli* and *Vibrio cholerae* (1, 81).

This thesis describes a more quantitative technique to study macrophage cytophilic antibody *in vitro* using viable *Salmonella typhimurium* SR11 and *S. typhimurium* endotoxin as antigens. In addition, a careful analysis of macrophage cytophilic antibody in general, its immunologic characteristics and its relation to phagocytosis and resistance to infection was made. Hopefully, this review has clarified

at least some of the confusion and controversy surrounding macrophage cytophilic antibody.

Using this experimental model, we first tested the ability of rat peritoneal cells sensitized with normal and immune rat serum to bind viable *S. typhimurium*. Second, normal and immune rat serum were absorbed with heat-killed *S. typhimurium* and the ability of this sera to sensitize rat peritoneal cells was examined. Third, immune rat serum absorbed with macrophages was tested for its ability to sensitize rat peritoneal cells to bind viable *S. typhimurium*. These later experiments were performed in an attempt to differentiate the opsonic and cytophilic properties of immune serum. Fourth, the ability of normal and immune rat serum to sensitive rat peritoneal cells to bind *S. typhimurium* endotoxin was tested. The purpose for this study was to determine the role of macrophage cytophilic antibody in the removal of free endotoxin *in vitro*. Fifth, the ability of normal and immune rat serum to sensitize mouse peritoneal cells to bind viable *S. typhimurium* was evaluated to test the interspecies potential of macrophage cytophilic antibodies to gram negative organisms.

LITERATURE REVIEW

Historical Background

The first relatively clear indication of the existence of macrophage cytophilic antibody was reported over 20 years ago. In 1954 Girard and Murray (40) detected a large amount of anti-*Salmonella* antibody associated with thoracic cavity macrophages occurring in inflammation caused by gum-arabic beef extract. Passively administered antibody appeared to be concentrated on or in macrophages. There had been some suggestion of this phenomenon in earlier work by Lurie (73) on the development of immunity to tuberculosis. Zinsser and Mueller (119) also found evidence suggesting macrophage-associated antibody in their work on the nature of bacterial allergies.

Boyden and Sorkin (18) observed that antibodies in certain rabbit antisera to human serum albumin (HSA) fixed to normal rabbit spleen cells. After washing, the cells were able to specifically adsorb ^{131}I-HSA. Boyden (16,17) later demonstrated antibodies specifically cytophilic for macrophages. Other investigators reported a similar phenomenon in different systems (1,10,89,113). Cytophilic antibody was defined by Boyden (16) as "a globulin component of

immune serum which becomes attached *in vitro* to certain cells in such a way that these cells are subsequently capable of specifically adsorbing antigen."

Investigation of macrophage cytophilic antibody was greatly facilitated by the development of several techniques to detect them *in vitro*. Boyden (16,17) introduced a rosette-forming reaction on macrophage monolayers usually referred to as the passive direct technique. Macrophages are allowed to attach to glass and form monolayers and washed. The monolayers are then sensitized with antiserum for a sufficient period of time and washed again. Antigenic particles, erythrocytes, proteins, bacteria, etc., are then incubated with the sensitized cells and washed to remove unbound antigen. Antigen-macrophage rosettes can then be observed and counted by microscope. This technique has several limitations: 1) sensitized cells have to be attached to glass, 2) the counting method is laborious and only semiquantitative, and 3) it does not exclude attachment of antigen to glass. Berken and Benacerraf (10) introduced the passive indirect technique. This procedure basically consists of sensitization of antigen with antiserum. The antigen-antibody complexes are then added to a macrophage monolayer. The monolayer is observed for rosette-formation as in the passive direct technique. A major drawback with this method is that it essentially measures the opsonizing

activity of antiserum, not its cytophilic activity. Jonas *et al.* (63) introduced an alternative assay method, the suspension-centrifugation technique. Antigen is mixed with passively sensitized cells in suspension for a sufficient period of time and then gently centrifuged. The resuspended pellet is examined microscopically for attached antigen particles. This technique does not require attachment to glass and is thought to be more sensitive.

The present study uses a more quantitative modification of the suspension-centrifugation technique somewhat similar to the method described by Del Guercio *et al.* (31).

These authors sensitized peritoneal cells which were suspended in phosphate buffer with serial ten-fold dilutions of guinea pig antisera to *S. typhimurium* at 20C for two hours. The cells were centrifuged, resuspended in phosphate buffer, and radiolabeled *S. typhimurium* was added in a ratio of 200 bacteria per macrophage. The bacteria-macrophage mixture was incubated overnight at 4C. The cells were centrifuged and the radioactivity measured in each fraction.

Several other techniques have been used to measure cytophilic activity and are of interest in this study.

Brumfitt *et al.* (19) studied the attachment of *Escherichia coli* to rat peritoneal macrophages. They used low temperature (9C, 20C), antibiotics in media and iodoacetate to selectively study attachment. Macrophages were

cultured *in vitro* with M-199 and the fate of *E. coli* was studied using streptomycin or neomycin in the medium. This procedure was thought to separate extracellular from intracellular growth and killing. Only extracellular organisms were killed and intracellular events could be followed without the complexities introduced by extracellular growth. Both reducing ambient temperature and iodoacetate decreased phagocytosis and a greater proportion of the bacterial population was destroyed under these conditions, implying a greater extracellular population.

Auzins and Rowley (6) described a technique which separated free bacteria from those attached to suspensions of mouse peritoneal macrophages. The bacteria, *Salmonella typhimurium* C5 were preopsonized with fresh pig serum. Macrophages were suspended in Hank's buffered saline solution in a siliconized two milliter syringe and a number of preopsonized *S. typhimurium* C5 were added at an approximate ratio of bacteria to macrophage of 5:1. Pieces of Whatman No. 1 filter paper were fitted exactly into the hub of a 25 gauge needle. Three drops of the macrophage-bacteria mixture were passed through the filter at each sampling at which time the filter was changed and the needle cleaned and dryed. The rate of association of opsonized and unopsonized bacteria to peritoneal macrophages was studied at 20C and 37C.

Levenson and Braude (72) studied the cytophilic properties of "late" and "early" antisera to *Salmonella typhi* Vi antigen using the passive indirect technique. The test antigen was *S. typhi* Vi adsorbed on erythrocytes. This method makes it possible to follow the temporal changes in cytophilic activity using a monospecific bacterial antigen without the complexities of viable bacteria.

Hsu and Mayo (53) described a method for detecting macrophage cytophilic antibody that is different both in the phenomenon observed and the functional significance. Macrophages with cytophilic antibody were found to clump *in vitro* in the presence of antigen. This phenomenon was species specific, could be elicited by either the O or H antigens of *S. typhimurium* SR11 and was thought to represent an enhancement of antibacterial immunity.

Vlaovic *et al.* (115) described a technique differentiating the leukocyte migration inhibition due to cytophilic antibody from that due to cell-mediated immunity by a modification of the capillary tube method.

There has been considerable controversy concerning the definition and mechanism of action of macrophage-cytophilic antibodies as those antibodies that attach to macrophages after antigen-antibody complexes form. These antibodies are detected by the passive indirect method which involves mixture of antigen and antibody and washing the antigen-antibody complexes formed. Macrophage are then "sensitized"

with the antigen-antibody complexes and examined microscopically. Rosette cells formed by this method are morphologically the same as those formed by the macrophage-monolayer techniques. Berken and Benacerraf (11) define cytophilia as "that property of opsonizing antibody which provides the receptors that permit the binding of the antibody to the macrophage cell membrane in preparation for phagocytosis." However, Parish (89), Tizard (106) and Nelson (85) suggest that these "opsonic adherence antibodies" are not cytophilic before combination with antigen and represent a different class of antibody than that described by Boyden (16). Parish (89) reported a distinct $\gamma 1$ fraction containing only cytophilic antibody (as defined by Boyden). Bovine gamma globulin and bovine plasma albumin were the antigens used. It was possible to remove all of the cytophilic fraction by absorption with macrophages. In addition Parish demonstrated only a slight increase in phagocytosis of protein antigen pretreated (opsonized) with the cytophilic fraction; presumably showing that the cytophilic fraction had little classical opsonic activity. This fraction was found to be a 19S macroglobulin.

Guinea pig antibodies responsible for passive cutaneous anaphylaxis or the sensitization of strips of ileum for reaction with antigen were found among the 7S $\gamma 2$ fraction (85).

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Tizard (106) showed that mouse anti-sheep erythrocyte cytophilic antibodies included IgM. The passive indirect method in contrast detects only IgG. Cytophilic antibodies attached only to macrophages. Erythrocytes opsonized by the passive indirect method attached to polymorphonuclear neutrophils, mast cells and macrophages. Electrophoresis showed cytophilic activity only in a region close to the origin with β and fast γ mobility. In contrast, opsonic activity was found widely distributed through the γ region. Cytophilic activity in antisera obtained during the primary response was demonstrated to be susceptible to freeze-thawing damage. It was possible to destroy activity by as few as two freezing cycles to -20C (Tizard, 107).

In contrast Uhr (113) found that guinea pig antisera to *S. paratyphi B* flagella produced without adjuvant only produced macrophage rosettes when a method similar to the passive indirect technique was used (opsonic adherence antibody). However, complete Freund's adjuvant is necessary for the production of macrophage cytophilic antibody in guinea pigs. Also the test antigen (whole bacteria) was different than the immunizing antigen (flagella). Apparently the antibodies which Uhr studied were not cytophilic in the manner described by Boyden (15).

Cytophilic and opsonic adherence antibodies have at least one common characteristic. Both activities are lost

after pepsin treatment. This enzyme destroys the Fc portions of immunoglobulin heavy chains. Apparently both the cytophilic and opsonic adherence sites are located on the Fc portion. It has been suggested that opsonic antibody becomes cytophilic by configurational changes in Fc following binding of antigen. Also, cytophilic antibodies may already have these changes preformed in the Fc portion (106).

The immunoglobulin class and/or subclass containing macrophage cytophilic antibody varies by species, antigen used, route of injection and time after immunization. Nelson (85) and Tizard (107) have prepared a detailed listing of these characteristics. However, it should be pointed out that only IgG_1 and IgG_3 bind to macrophages in humans. This is significant because IgM or other classes of IgG are cytophilic in other species including those on which most experimental models are based (55). The production of macrophage cytophilic antibody occurs independently from other antibodies and usually requires complete Freund's adjuvant, intravenous injection, or immunization with a live attenuated bacterial strain.

The antibody class in the primary response is usually IgM and in the secondary response IgG (85,107). Apparently cytophilic antibodies in mice and rats can be produced by heat-killed *E. coli* and *Salmonella typhimurium* without

using complete Freund's adjuvant (94,107). Rowley *et al.* (95) observed a mouse 19S macroglobulin cytophilic antibody to *Salmonella typhimurium*.

Cytophilic antibodies attach to macrophages with the Fc portion (106). The exact structure of the binding site is not known. Human $gamma_1$ myeloma proteins (cytophilic) and $gamma_2$ myeloma proteins (non-cytophilic) differ only by 4 peptides (peptide mapping) (45,57).

Opsonic antibodies bind to macrophages only after combination with antigen. Antigen-antibody complexing induces confirmational changes in the IgG molecule (58,84). It is likely that pre-existent changes in the unbound antibody molecule are responsible for cytophilic properties (107). Comparable changes are likely to occur in the Fc portion of homocytotropic antibodies (103).

The macrophage membrane receptor for cytophilic antibody in guinea pigs has been investigated by Howard and Benacerraf (50) and Davey and Asherson (30). The receptors were found to be resistant to trypsin, chymotrypsin, papain, ficin, pronase, reducing agents (mercaptoethanol) and enzyme poisons (azide fluoride and dinitrophenol). However, they were susceptible to phospholipase A, lecithinase C, *Naja naja* venom, reagents which react with free SH groups (iodoacetamide and p-chloromercurybenzoate) or with both SH and NH_2 groups (formaldehyde and isothionate) and oxidizing

agents (periodate and nitrite) (85). Mouse and guinea pig receptors for 7S IgG cytophilic antibodies in mice were found to be resistant to trypsin and pepsin (70). The macrophage receptors for 19S macroglobulin were sensitive to trypsin (86,106), and rabbit macrophage cytophilic receptors were sensitive to trypsin (101). Tizard (106) found that alveolar and peritoneal macrophages did not differ significantly in their capacity to adsorb cytophilic antibody. However, Berken and Benacerraf (10) reported that mouse, guinea pig and rabbit alveolar macrophages bind a greater number of cytophilic antibody molecules than peritoneal macrophages in the same species. It is unknown whether this is a difference in the total number of receptor sites on the alveolar and peritoneal macrophage or if the "alveolar macrophages are more reactive because of having fewer sites occupied (being in an area with very low concentrations of plasma proteins in the environment)" (85).

Macrophage cytophilic antibodies bind only to macrophages (17). It has been shown that neutrophils, eosinophils, lymphocytes, fibroblasts, peritoneal serosal cells, alveolar epithelium and mast cells are not sensitized by macrophage cytophilic antibody (50,63,106). Alveolar, splenic and peritoneal macrophages, Kupffer cells, microglia and blood monocytes bound approximately the same amount of antibody in the mouse (106). However, Keller and Sorkin (64) reported a much higher level of cytophilic binding in

rat mast cells and rat liver cells (obtained by passage through a steel mesh) with rabbit antisera to human serum albumin. This very large uptake by mast and liver cells was thought to be partly due to their large size. This topic will be discussed later in more detail. These findings have never been confirmed. Coulson *et al.* (28) found "that guinea pig small lymphocytes, transformed in mixed cell culture, may also take up macrophage cytophilic antibody." Kossard (68) showed "that rabbit anti-sheep erythrocyte antisera contained antibodies cytophilic for rabbit and guinea pig macrophages and small lymphocytes."

Several factors have a profound effect on the binding of cytophilic antibodies to macrophages. The first to be investigated was ambient temperature. Apparently, the effect of temperature on the binding of macrophage cytophilic antibody varies between species. A rise in temperature inhibits most adsorptive processes possibly due to significant entropy changes (107).

It was found that rabbit anti-HSA bound better at 0C than at 37C. In addition the bound antibody could be eluted at 37C. Kossard and Nelson (69) and Boyden (16) reported better binding of cytophilic antibody at 4C. Mouse IgG and IgM macrophage cytophilic antibodies were found to bind much better at 4C than at 37C (106,107). Jonas *et al.*(63) found that changes in temperature did not significantly

affect guinea pig macrophage cytophilic antibody adsorption. It was observed by Berken and Benacerraf (10,11) that cytophilic antibodies were bound better at 37C but were eluted rapidly.

Another factor affecting cytophilic antibody binding is competition for receptors by non-specific cytophilic immunoglobulins. Other cytophilic antibodies present in serum profoundly affect the binding of a specific cytophilic antibody. Usually this causes an inhibition of antibody adsorption. It is believed that non-specific cytophilic antibodies compete for available cell receptors with specific cytophilic antibodies (10,57,63).

There is an apparent heterogeneity of cell surface receptors. Adsorption of macrophage cytophilic antibodies is an example of adsorption of a solute onto a surface. This phenomenon has been expressed mathematically by the Langmuir adsorption isotherm which states that the amount of solute adsorbed by a surface is proportional to the amount available for adsorption until the surface receptors are saturated. Adsorption of IgG by mouse macrophage was found to deviate from the expected values. However, mouse IgM adsorbed in the expected fashion (108). The deviation from expected values could be due to interaction between receptors. Low levels of non-specific mouse cytophilic antibody does in fact increase adsorption *in vivo* and

in vitro. This would suggest cooperative interaction between receptors in inflammatory foci with increased antibody levels.

> The mechanism of the observed increase in receptor avidity may be due to simple neutralization of macrophage charge by adsorbed charged immunoglobulin molecules, which may allow other molecules or particles to be adsorbed. Alternatively it is possible that activation of high avidity receptors is brought about directly by the previous occupancy of low avidity receptors (108).
>
> Changeux *et al.* (23) have proposed a theory to account for such interaction between receptors, hypothesizing that receptors are linked to each other and that the efficiency of interaction is related to the degree of association between receptors (based on acetylcholine, postsynaptic receptors).

Mouse peritoneal macrophages may show a significantly increased uptake of antibody in inflammatory foci (108).

> Evidence is accumulating (Tizard, unpublished observation) that *in vivo* macrophage cytophilic antibodies exist entirely in solution. However, under conditions of raised immunoglobulin levels in the microenvironment of macrophages there may be a net movement of immunoglobulin onto cells.

Huber and Fudenberg (55) suggest that based on the available evidence IgG receptors and cytophilic receptors are similar or identical. Their function is to mediate the binding of antigen-IgG complexes. They also pointed out that in studies by Berken and Benacerraf (10) a large portion of the $\gamma 2$ fraction in guinea pigs showed the ability to bind to macrophage receptors. However, it should be pointed out that the definition of cytophilic antibody used by

Berken and Benacerraf is not significantly different from the usual definition of opsonic antibody.

Interaction of dendritic macrophages with lymphocytes in lymphoid follicles leading to the production of antibody has been studied extensively (7,88). The localization of antigen on the processes of dentritic macrophages is believed to be mediated by a cytophilic antibody. In fact, the binding of antigen has been correlated quite closely to antibody both in its time of appearance and physical location (7).

The dendritic processes of these cells are extensive and in close physical proximity to lymphocytes. The antigen remains attached for long periods of time (2). It is believed that antigen is presented to lymphocytes initiating antibody production. This process is usually known as antigen processing. It should be mentioned that dentritic cells are not typical macrophages and usually are not classified as such. The cytophilic antibody has not yet been characterized.

More recent work has helped to clarify antigen processing (25). It was demonstrated that highly purified peritoneal macrophages from immune animals following exposure to antigen could cause transformation of immune lymphocytes (98). Thymocytes, L_2C leukemia cells, polymorphonuclear leukocytes and column purified lymph node lymphocytes were

not able to cause lymphocyte proliferation. It was found that peritoneal cells from non-immune animals were not able to bind enough antigen at very low concentration (μg/ml) to cause lymphocyte proliferation. Peritoneal cells from immune animals were able to do so at μg/ml concentrations of antigen. Non-immune peritoneal cells sensitized with serum from immune animals were subsequently able to stimulate lymphocyte proliferation. This effect could be blocked by hapten coupled with a heterologous antigen. Binding of antigen at very low concentrations could also be blocked by anti-immunoglobulin (25).

At higher antigen concentrations anti-immunoglobulin had no effect on peritoneal cell-induced lymphocyte proliferation. Thus, a cytophilic antibody dependent mechanism is only necessary at very low antigen concentrations. At high antigen concentrations the alternative non-antibody dependent mechanism operates. Although the studies were performed *in vitro*, it appears likely that cytophilic antibody concentrates sufficient antigen on macrophage surface to initiate a secondary immune response when antigen is present intermittently and in low concentration (25).

α_1-globulins have been found to be associated with cytophilic activity. However, there are a number of difficulties in studying and interpreting findings with this class of serum factors. They are: 1) lability to freezing

and thawing, 2) weak sensitization of macrophages, 3) nature of equilibrium between free and macrophage bound state, 4) nature of producing cells, 5) stability and half-life *in vivo*, and 6) attachment to lymphocytes and other cell types (85). A poorly characterized α_1-globulin was found in mice. Studies were performed in mice to better characterize their nature. The normal serum of a number of Swiss mice was tested for cytophilic activity. One pool showed a low titer (1:2) by the indirect test in a fraction containing albumin and a fast α_1-globulin. Red blood cells adhered to normal nonsensitized cultured macrophages of C57 Bl/6J mice and some Swiss mice. Using both the direct and indirect test, no cytophilic factors were found in normal C57 Bl/6J mouse sera to Sarcoma 1 cells. Sera raised by injection of complete Freund's adjuvant alone contained one pool which showed anti-sheep erythrocyte cytophilic activity. Mouse anti-sheep erythrocyte antisera contained cytophilic antibodies to sheep red cells and none to guinea pig and human erythrocytes. No hemagglutinating or complement-fixing activity has been found in the albumin-α_1-globulin fraction. The α_1-globulin factors appear to have the characteristics of antibodies (85). There have been a number of reports of antibody with more usual types of activity in the α-globulin fraction (4,20,26,32,104). Allen, Saba and Molnar (3) isolated and characterized an α_2-globulin which

stimulated *in vitro* Kupffer cell phagocytosis of test colloids and stimulated R.E.S. clearance of test colloids.

Electrophoretic studies show that α_1-globulins are not related to conventional immunoglobulins (85). The apparent immunologic specificity of α_1-globulin cytophilic antibodies suggest they are unrelated to acute phase proteins like C reactive protein and the Darcy α_1-glycoprotein that occur in serum after tissue injury. They also have different electrophoretic mobility than other unusual α-globulins such as: 1) α_1-macroglobulin virus inhibitors in normal human, monkey, bovine and ferret sera (12), 2) slow α_1-globulin appearing intermittently in adjuvant induced arthritis of rats (36), and 3) α_1-globulins in tuberculosis in humans (99) and guinea pigs (29).

Tizard (109) reported desensitization of macrophages for cytophilic antibody following intravenous injection of *E. coli* lipopolysaccharide. The response was described as being triphasic. First, a slight increase in adsorption occurred in the initial five minutes. This was followed by a significant depression of antibody adsorbance reaching a maximum level in 30 minutes. By fourteen hours there was a significant rebound of adsorption above normal levels. It is significant that the maximum depression occurred during the first hour after LPS injection. This is the time period in which the important pathogenic changes are

believed to occur in endotoxin poisoning (Moon, personal communication). Whether the preceding changes are major pathogenic events or merely a reflection of altered cell metabolism or membrane properties is not known.

Shortly afterwards, Tizard (110) investigated the mechanism of action of LPS on antibody adsorption. He found no relationship between surface charge or rate of anaerobic glycolysis and the changes in antibody adsorbance. A serum factor was postulated as the mediator of the LPS related events. This factor was not present in serum or whole blood thus excluding clotting factors or leukocyte pyrogens as mediators. It was also thought unlikely that the factor was an immunoglobulin because the triphasic response occurs in gnotobiotic mice. Tizard (110) thought it possible that the factor could be a lymphokine because of its similarity of action to macrophage inhibition factor. It is possible that interaction of the antigen (LPS) with sensitized lymphocytes would bring about release of a lymphokine. No further work on this topic has been reported. However, Kessel and Braun (65) reported a cell-bound antibody which protected macrophages from the toxic effect of endotoxins.

There has been a significant degree of controversy concerning the biologic role of cytophilic antibodies. Macrophage cytophilic antibodies may act as either classical or cell-bound opsonins. Mouse macrophages were sensitized

with anti-sheep erythrocyte serum at 4C, washed and antigen added. The mixture was then incubated at 37C. Both IgM and IgG cytophilic antibodies increased phagocytosis. The IgG fraction increased phagocytosis to a greater extent than the IgM-fraction (107). At 37C two processes are known to occur: 1) elution of macrophage cytophilic antibody, and 2) activation of phagocytosis. Whether Tizard observed elution of cytophilic antibody, subsequent opsonization and phagocytosis of antigen-antibody complexes or simply temperature activated phagocytosis of membrane bound antigen-antibody complexes (cell-bound opsonin) is not clear.

Rowley, Turner and Jenkin (95) presented convincing evidence that protective immunity to *Salmonella typhimurium* in mice can be transferred to normal animals by immune peritoneal macrophages. Resistance could also be transferred by sensitization of normal cells with immune serum. Trypsin digestion destroyed the ability to transfer immunity with immune cells. At 37C immune macrophages were found to elute a 2-mercaptoethanol sensitive material (macroglobulin). This eluted macroglobulin was found to protect normal mice. Tizard (107) suggests that this cytophilic macroglobulin acted as a classical opsonin. Parish (89) reported a similar macrophage cell-bound antibody for *E. coli*.

More recently, Mittal (81) demonstrated the production of macrophage cytophilic antibodies in rabbits and mice to

Escherichia coli. Cytophilic antibodies were found only in antisera from rabbits immunized with a live smooth (3662) strain of *E. coli*. Capsular and somatic antigens of strain 3662 attached to red blood cells did not produce cytophilic antibody. It was possible to passively sensitize rabbit peritoneal macrophages *in vivo* only with antisera to live strain 3662 of *E. coli*. Antisera to other strains were not effective. *In vitro* passive sensitization was possible with peritoneal macrophages, lymph-node cells and spleen cells, but not with polymorphonuclear leukocytes. A rough strain of *E. coli* (Lilly) did not produce cytophilic antibodies. Sensitization of spleen cells, macrophages and PMNs was by the passive direct method. Lymphocytes were tested by the suspension-centrifugation technique. Several reports implicating a similar cell-bound antibody in immunity have appeared in the literature.

Vi-coated erythrocytes formed rosettes on anti *S. typhi* Vi polysaccharide sensitized macrophages that were resistant to phagocytosis at 37C for long periods (72).

Actor and Pitkin (1) reported the presence of a cell-adherent immune factor in immunity to *Vibrio cholerae* by immune serum or immune peritoneal cells and normal peritoneal cells sensitized with immune serum. Immune spleen cells were able to transfer immunity but only if given at least fourteen days prior to challenge. The immunity

obtained by transfer of immune peritoneal cells was long-lasting; that transferred by sensitized normal cells was temporary.

All injections were intraperitoneal. Mice were immunized with a killed whole cell vaccine of *V. cholerae* Ogawa 41. It was felt that two mechanisms of protection were operating; one, cytophilic antibody and two, a non-cell-adherent factor. The reason for suggesting a non-cytophilic factor was that heterologous serum was capable of protection on passive transfer (1/60 efficiency of homologous serum), but was unable to sensitize macrophages.

Macrophage cytophilic antibodies have been found in trypanosomiasis and schistosomiasis. Tizard and Soltys (111) described cytophilic antibodies in the sera of rabbits infected with a mouse adapted strain of *T. brucei*. The titer of cytophilic antibodies reached a peak in one week and decreased steadily afterwards. They were associated with high titers of agglutinating antibody. The antibodies were resistant to 2-mercaptoethanol and heating to 56C for 30 minutes. Adherence occurred only to mononuclear phagocytes and not to polymorphonuclear leukocytes or small lymphocytes. They bind to cells better at 4C and 22C than at 37C.

Recently Capron *et al.* (21) and Capron *et al.* (22) have reported an IgE macrophage cytophilic antibody which

causes adherence of peritoneal cells to *Schistosoma mansoni* schistosomules *in vitro*. These antibodies were found in the serum of schistosome-infected rats. There was a highly significant difference in the number of adherent macrophages between normal cells and antisera sensitized macrophages.

Binding of antisera was destroyed by heating at 56C for 3 hours and could not be activated by fresh guinea pig serum. Adsorbance with *S. mansoni* antigens significantly decreased the ability of sensitized macrophages to bind to parasites. These were shown to be specific IgE antibodies by adsorption with goat anti-rat IgE serum specific for ε. *Chain* or $F(ab^1)_2$. Serum containing IgE myeloma antibodies and reaginic antibodies failed to sensitize macrophages for immune adherence.

Fluorescent staining with goat anti-rat IgE was positive in immune serum sensitized macrophages but negative in macrophages sensitized with antisera heated to 56C for 3 hours or adsorbed with $F(ab^1)_2$ anti-rat IgE. IgE binding to macrophages was confirmed by peroxidase-labeled IgG anti-rat IgE and ultrastructure studies. It was felt that these specific IgE macrophage cytophilic antibodies accelerate the killing of *S. mansoni* schistosomules and may be important in the immunity to the parasite in rats and possibly in humans (22).

Ivanyi (59) reported a suppression of the primary response to HSA by hyperimmune chicken anti-HSA. This suppression was abolished by repeated adsorption of the antisera with spleen cells. Apparently cytophilic antibodies in anti-HSA serum caused a suppression of the primary response.

For some time it has been thought that cell-bound antibodies might be at least partially responsible for delayed hypersensitivity. A number of investigators (9,14,42,48,49, 87) found no relation between macrophage cytophilic antibodies and intensity of delayed hypersensitivity. However, Boyden (17) did find a relation between the presence of macrophage cytophilic antibodies and delayed hypersensitivity in guinea pigs. The antigen in this case was sheep red blood cells and complete Freund's adjuvant was required for cytophilic activity. Boyden also suggested that unfractionated serum usually contains much more classical antibody than cytophilic antibody which could interfere with the action of cytophilic antibody. There is evidence that interference occurs *in vivo* between classical serum antibodies and the factors responsible for hypersensitivity to tuberculin and other types of cellular immunity (15,46).

Another group of investigators, Dupuy *et al.* (33), found that a cytophilic factor could take part in delayed hypersensitivity to PPD. They were able to transfer delayed

hypersensitivity to PPD to normal guinea pigs with 2-3 ml of fresh plasma from X-irradiated PPD hypersensitive donors. Similar plasma from nonirradiated PPD donors or from irradiated normal guinea pigs did not transfer delayed skin reaction. It was possible to transfer delayed hypersensitivity to PPD by incubating large numbers of nucleated spleen cells with plasma from irradiated sensitized donor and injecting into normal guinea pigs.

Hsu and Mayo (53) observed that immune macrophages clumped in the presence of antigen. This clumping was strong and could be obtained by both the H and O of *Salmonella typhimurium* SR11. They suggested this phenomenon was due to macrophage cytophilic antibody.

Granger and Weiser (43) demonstrated a cytophilic factor on peritoneal macrophages (mouse C57B1/K_s) which mediated binding of target cells (sarcoma I) to the peritoneal cells. This factor could be eluted at 56C. This same group later found that the factor was able to fix complement and kill target cells (44). Trypsin-treatment was found to remove this factor (116). It was also found that trypsin-treated macrophages could be activated by treatment with immune serum and eluate from immune cells. Similar results were reported by Gordon (41) and Hoy and Nelson (52).

Gill (38) reported the presence of marcophage cytophilic antibody in heterologous antilymphocyte serum. Cytophilic antibody was present as measured by both the passive direct and passive indirect technique. Macrophages sensitized with rabbit anti-mouse lymphocyte serum showed the presence of cell-bound antibody by immunofluorescent studies. Macrophages sensitized by normal serum did not. Alveolar macrophages obtained *in vivo* from rabbits producing anti-lymphocyte serum formed rosettes with thymocytes. Alveolar macrophages from normal rabbits did not. Antibody eluted at 56C from macrophages sensitized by the passive direct method was able to sensitize cells by the passive indirect method. Cytophilic activity was found in the IgG fraction by anion exchange chromatography. Pepsin treatment destroyed cytophilic activity. It was possible to remove almost all the cytophilic activity by repeated adsorption with macrophages (38). Taub and Lance (105) found a high uptake of chromium-51 labeled lymph node cells treated with ALS *in vitro* in the liver. It is possible that cytophilic antibody assisted Kupffer cell clearance in that situation (38).

Gill *et al.* (39) found a close relationship between cytophilic activity and the immunosuppressive effect of rabbit anti-mouse lymphocyte serum. In this study the titers of leukoagglutinating, cytophilic and indirect and

direct macrophage cytophilic antibodies were correlated with the survival time of CBA mouse skin grafts on balb/c mice. Simple linear regression analysis showed significant correlation with all types of antibody. Similarly multiple regression analysis showed significant correlation with all types of antibody. However, multiple regression analysis showed that titers of indirect and direct cytophilic antibody were the most efficient indicators of graft survival. It was suggested that leukoagglutinating and cytotoxic antibody played only a minor role (39).

Pokorna (92), Koprowski and Fernandez (67) and Gibbs *et al.* (37) reported suggestive evidence for cytophilic antibodies in autoimmune phenomenon.

Tizard (107) suggested two general ways in which macrophage cytophilic antibody carries out its role. The first mechanism would require combination of antigen with cell-bound antibody leading to fixation of the antigen. Following this the immune macrophage would exert its cytotoxic effect on the antigen or target cell. The second suggested mechanism would be combination of the antigen with the cell-bound antibody followed by activation of macrophage enzyme systems and altered macrophage activity. Tizard (107) listed several examples of cell "activation" by antigen cell-bound antibody. Rowley *et al.* (95) found a macrophage cytophilic antibody to *Salmonella typhimurium* capable of passive transfer of immunity. Urea extraction yielded a

minute amount of protein which was very active in sensitizing peritoneal cell. This protein was found to be a macroglobulin (95). Earlier work stated that immunity to *Salmonella* sp. was due to cellular factors and that antibodies played no role (35,96). Thus, the findings of Rowley's group raised the distinct possibility of the cooperative interaction of humoral and cellular factors in the immunity to *Salmonella*. Parish (89) reported an enhancement of phagocytosis of heat-killed *E. coli* with macrophages sensitized with antiserum to *E. coli*. This suggests that a similar cell-bound antibody enhances resistance to *E. coli*.

Ushiba *et al.* (114) reported that immunization with a live avirulent strain of *S. enteritis* did not produce 19S macroblogulins. However, they did find a 12.7S macroglobulin in the sera of mice immunized with the S-type killed vaccine similar to that reported by Rowley. The Japanese group used different strains of bacteria. They reasserted that immunity to *Salmonella* was due to cellular factors and not humoral factors.

Mackaness and his colleagues investigated the relative roles of humoral and cellular immunity in *Salmonella* infections. It was found that passive transfer of serum from immune animals or immunization with heat-killed cells increased clearance of bacteria but could not prevent multiplication of intracellular organism. Cellular immunity was

necessary for resistance to active infection (74). It is important to point out several differences between Mackaness's studies and Rowley's. First, Mackaness used actively infected animals while Rowley challenged animals with bacteria which were prophylactically given antisera. Second, Mackaness used intravenous injection leading to widespread dissemination rather than the intraperitoneal route which resulted in more local dissemination as used by Rowley. Significantly different phenomenon were observed in the two studies.

The Mackaness group went on to demonstrate that *Listeria monocytogenes*-infected mice had greater resistance to growth of *S. typhimurium* in the liver and spleen than did mice vaccinated with heat-killed *S. typhimurium*. Mice actively infected with *S. typhimurium* were totally resistant to intravenous challenge with *Listeria monocytogenes* (13). This was interpreted to mean that enhanced microbicidal activity (activation) of macrophages was necessary for resistance to active infection with *S. typhimurium* in mice. However, there is some question as to whether the Mackaness group actually studied cytophilic antibodies. Macrophage cytophilic antibodies to *S. typhimurium* can be most reliably produced with active immunization with avirulent organisms. Intravenous injection of heat-killed cells as used in the Blanden *et al.* (13) studies are not as

reliable as the other two methods. Recent evidence suggests that a shared antigen between bacterial species is responsible for the apparent "activation" of macrophages postulated by Mackaness (79).

More recently, Margolis and Bigley (78) have demonstrated a cytophilic macroglobulin in mice immunized with RNA-protein fractions of *Salmonella typhimurium*. This macroglobulin was specifically reactive with a protein complex comprising part of the bacterial cell wall. This same protein complex and RNA are the immunogenic agents. The cytophilic macroglobulin was eluted from peritoneal cells, washed spleen and lymph node cells by incubation at 56C for 1 hour. Peritoneal cells from animals immunized with live *S. typhimurium* RIA, phenol-extracted RNA-protein fraction and ethanol-extracted RNA-protein fraction all had significant amounts of cytophilic antibody.

Groups of normal mice were injected intravenously with macroglobulin and normal peritoneal cells sensitized with normal globulin and then 24 hours later injected intraperitoneally with 500 LD50 doses of *S. typhimurium* SR11. After 5 days there was a 100X decrease of viable bacteria in the livers and spleens of macroglobulin treated animals and 750X decrease in animals treated with sensitized normal macrophages. The survival time in both macroglobulin and sensitized macrophage treated animals was approximately

twice that of controls. *In vitro* studies showed a marked depression of bacteria extracellularly in cultures of peritoneal cells sensitized with macroglobulin. Intracellular bacteria were not affected. Immune peritoneal cells showed an almost complete elimination of bacteria both intracellularly and extracellularly after three hours (78).

Kurashiga *et al.* (71) and Mitsuhashi *et al.* (80) reported macrophage cytophilic antibodies to *Salmonella enteritidis* in mice immunized with attenuated *S. enteritidis* and on macrophages exposed to ribonucleic acid from immune monocytes. Collins and Mackaness (27) have shown that mice develop a delayed hypersensitivity reaction to a protein antigen in the cell wall. Smith and Bigley (100) found a similar protein-RNA complex which enhanced resistance to *Salmonella* and stimulated delayed hypersensitivity. The cytophilic macroglobulin was reactive with this protein complex (78).

More recently Eisenstein (34) demonstrated that protection in these ribosomal vaccines was due to contaminating O antigens. Mutants with alterations in the terminal sugars of O antigen were not able to confer protection. The RNA may function as an adjuvant in these vaccines.

Perhaps it would be helpful to review several aspects of the phagocytosis and killing of *Salmonellae* by phagocytes. First, there is a heterogeneity in the ability of phagocytes

to kill bacteria. In the initial stage most bacteria ingested are killed. The surviving bacteria are presumably in bacteriocidally incompetent phagocytes. Persistence of bacteria reflects the capabilities of only a fraction of the phagocytes. These bacteria multiply causing active infection. Also it is apparent that the interactions between macrophage and bacteria which occur during the initial period following introduction of bacteria are important in determining whether an active infection will result (60).

Marecki *et al.* (77) using mice showed that *Salmonella typhimurium* SR11 was killed by both normal and immune phagocytes intracellularly and that the early immune response of the host was important in determining whether the infection would be contained locally or disseminated. The rate of extracellular growth of bacteria and phagocytosis were then the key factors in determining the outcome of infection. Antibody was found to be important in these extracellular events (54,117).

Rhodes and Hsu (93) found that the intracellular population of *Salmonella enteriditis* in guinea pigs was directly related to the extracellular population through phagocytosis. Immune serum was able to suppress the extracellular bacteria initially. Accumulation of intracellular bacteria actually reflected phagocytosis of an increasing population of extracellular bacteria rather than intracellular growth.

This emphasized again the importance of extracellular events (bacterial growth and subsequent phagocytosis) in interpreting intracellular events (bacteriocidal properties of macrophages). This source of misinterpretation was pointed out by Patterson and Youmans (90) in regard to the enhanced ability of "activated" macrophage to inhibit the growth of *Mycobacterium tuberculosis*. Thus, factors which influence the efficiency of the phagocytosis of bacteria are more important than previously thought in the resistance to infections with *Salmonellae*. Macrophage cytophilic antibody is one of these factors.

Although there has been some work on the role of macrophage cytophilic antibody in resistance to gram-negative bacteria, little work has been done on its role with endotoxin. Parish (89) coated red blood cells with somatic polysaccharide and exposed them to macrophages sensitized with antisera to *E. coli*. The erythrocyte polysaccharide complex adhered to the macrophages but no phagocytosis occurred. Berken and Benacerraf (10) found cytophilic antibody specific for *E. coli* polysaccharide or for the dinitrophenol hapten. This was demonstrated by the passive indirect technique using sera of guinea pigs immunized with heat-killed *E. coli* or DNP proteins in complete Freund's adjuvant. Dramatic binding (+3-+4 rosettes) could only be demonstrated with undiluted sera. There was some question as to whether

a sufficient amount of polysaccharide had been attached to the red blood cells. Because free endotoxin was not used in these studies, their relevance to *in vivo* situations is tenuous.

The role of Kupffer cells in the clearance of bacteria, viruses and other particulate matter from the blood stream has been well-studied (51,62,75,76). However, many subtle questions remain unanswered. Moon, Vrable and Broka (82) demonstrated using the perfused rat liver, a distinction between trapping and killing. Following gross removal of blood by perfusion with sterile M-199 "over 70-80% of a 10^9 to 10^{10} dose of viable *S. typhimurium* was trapped after a single pass, but no significant bacterial killing was observed. When blood or plasma was added to the perfusion medium, over 50% of the trapped bacteria were killed in 15 to 30 minutes. Phase contrast and election micrographs of perfused livers showed extensive extracellular trapping in the sinusoid." It is likely that at least part of this sinusoidal trapping of bacteria is due to cytophilic antibody. However, scanning electron micrographs of similar studies using *Candida albicans* showed no correlation between trapping and adherence to phagocytes (97).

It has been known for many years that Kupffer cells tend to occur at the junction of liver sinusoids (47). This strategic location would increase the trapping ability

of macrophages made "sticky" by cytophilic antibody.

Keller and Sorkin (64) found a high level of cytophilic antibody adsorption on liver cells. However, a mixed cell population was studied. In their study the adsorbance of cytophilic antibody (rabbit) was observed on rat tissue mast cells and cells from other tissues *in vitro*. Antisera were raised to human serum albumin and ^{131}I labeled-human serum albumin was used as the antigen. Mast cells, spleen cells, liver cells, mesenchymal lymph node cells and red blood cells had very low levels of adsorption when sensitized with normal serum (1-10 c.p.m.). The same cell types had higher levels of adsorption following sensitization with specific antisera (308-4820 c.p.m.). The three cell types with the highest levels were liver cells (4820 c.p.m), mast cells (3822 c.p.m.) and red blood cells (1622 c.p.m.). The ability of mast cells to adsorb homocytotropic antibodies is well-known and their role in allergies has been well-documented. However, liver cells with the highest levels of adsorption in this study have received very little attention. Part of the reason for this is that a mixed cell population was studied by Keller and Sorkin (liver fragmentation followed by passage through a stainless steel mesh). More importantly it has been very difficult to obtain and maintain either parenchymal cells or Kupffer cells *in vitro* in sufficient quantity and purity to make detailed studies

possible. Recently, much better techniques have been developed for this purpose (83, Zlydaszyk, personal communication).

After discussing this report of a very high level of cytophilic antibody on liver cells, perhaps it would be interesting to mention the stickiness of Kupffer cells observed by early workers. Beard and Rous (8) remarked, "Except for the immense circular membrane, no feature of the Kupffer cell is more striking than its stickiness,--which is equally remarkable whether the enveloping medium is serum or Tyrode solution. It has much interfered with the study of the cells *in vitro*. Whatever touches their surface tends to adhere, whether it be red or white cells or "bodies" that are floating by." "A sticky surface is evidently one of the special characters enabling the cell to perform its task of phagocytosis. The stickiness of leukocytes from the blood and from exudates, with both of which we have had much experience, is in comparison negligible."

Knisely *et al.* (66) after a lengthy and extensive study of selective phagocytosis *in vivo* noted that, "The filaments and coatings which are precipitated from frog plasma on India Ink are very sticky to the Kupffer cells which line the frog's hepatic sinusoids. They are so sticky to the sinusoid-lining cells that the moment a coated particle touches the surface of one it sinks into the cytoplasm of the sinusoidal lining, apparently as quickly as a small

globule of mercury rolling along a laboratory table top touches and merges with a larger one."

Although there has been a significant amount of research by well-qualified investigators there are still many unanswered questions. Much of the reason for this is the unusual nature of the antibodies themselves. Perhaps it would be helpful to list these difficulties (85):

1. Macrophages are weakly sensitized. Cytophilic antibodies are rapidly eluted from macrophages at 37C in most cases.

2. It is not known with absolute certainty how the equilibrium between free and macrophage-attached antibody functions *in vivo*.

3. The type of cells producing cytophilic antibody is not known with certainty.

4. The stability and half-life *in vivo* of cytophilic antibodies both in plasma and/or macrophages is not completely known.

5. The possibility exists that at least some cytophilic antibodies sensitize cells other than macrophage. Uhr (113) reported a low level of sensitization (~5%) of lymphocytes with antisera to *Salmonella* flagellar antigen.

6. It is possible that the biological consequences of attachment of antigen to 19S cytophilic antibodies on macrophages may differ according to the nature of the antibody and its eliciting antigen (113).

7. "Antibodies (of whatever class) which are present in relatively small amounts on the macrophage surface *in vivo* may be released either into the medium or into the interior of the cells during manipulations of the cells *in vitro*. This would provide an almost permanent excuse for failure to demonstrate a clear biologic function for them" (85).

In spite of these difficulties a number of roles have been suggested for macrophage cytophilic antibody *in vivo* in gram-negative infections. They are:

1. Parish (89) stated "*in vivo* cytophilic antibody sensitizes the macrophages lining sinusoids of lymph nodes, spleen, liver and other organs so that foreign material, for example bacteria or their toxins, are more rapidly removed from the circulation. Thus, its probable function is to act as a 'cell-bound' (or 'cell-fixed") opsonin."

2. Berken and Benacerraf (10) suggested, "It must be considered whether or not macrophages can transport and effectively deliver cytophilic, $\gamma 2$, opsonic or complement binding antibodies to sites of inflammation or tissue rejection that macrophages do travel from areas of high antibody concentration in serum or lymphoid organs to areas of low antibody concentration in various tissue spaces."

3. Increasing adsorbance of cytophilic antibody in areas of high antibody concentration (inflammatory foci) due to changing receptor avidity could be important in their biologic role (108).

4. Cytophilic antibodies may coat macrophages in all parts of the body both intra- and extra-vascularly and play a role in preventing infection.

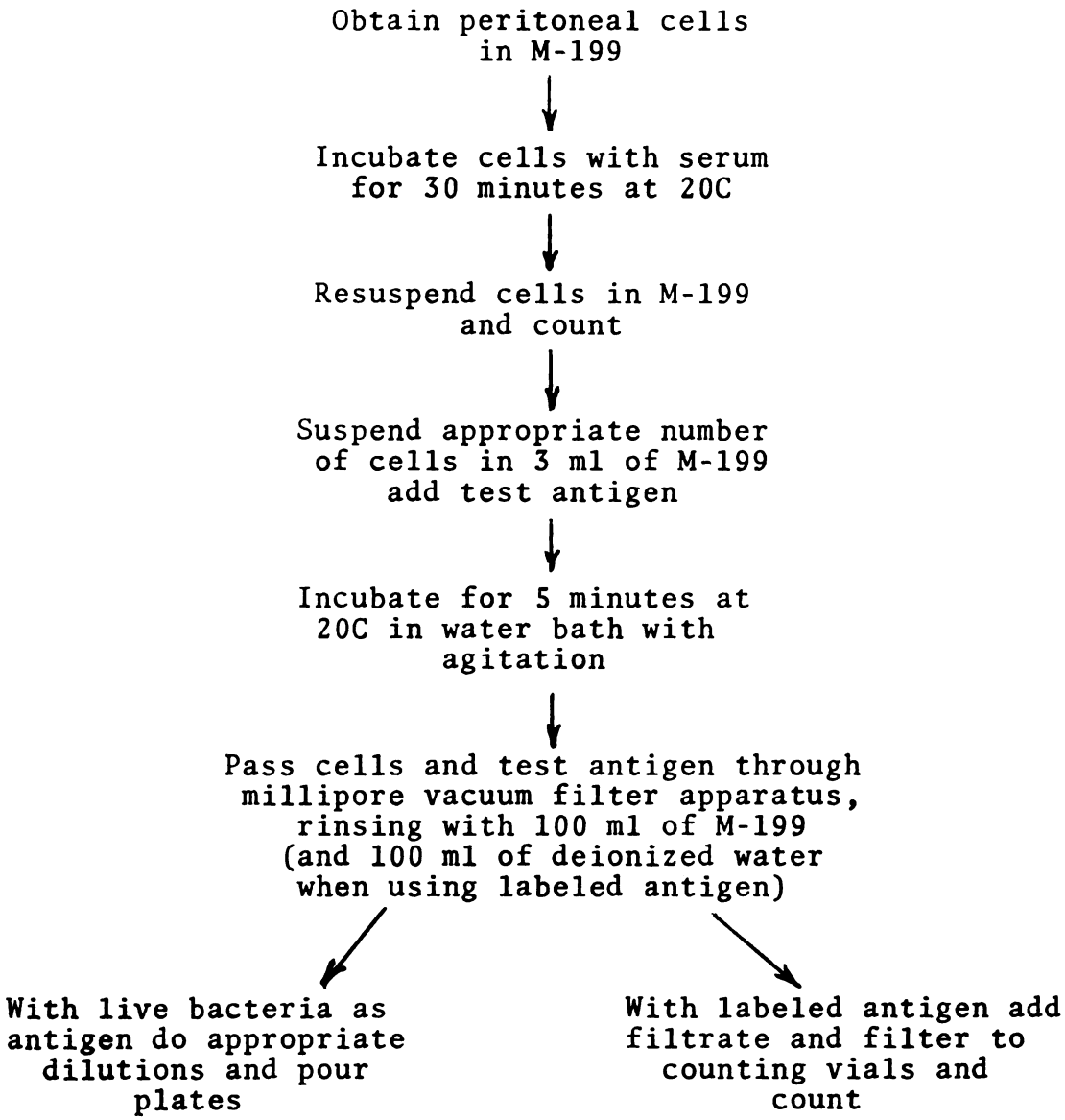
5. The significant decrease in adsorption of cytophilic antibody by macrophages in the first hour after endotoxin

injection may be evidence in a negative way for a significant role for cytophilic antibody in gram-negative infection.

6. It has been suggested by Jenkin and Rowley (60) that intracellular uptake of macrophage cytophilic antibody by phagocytosis or disruption of macrophage membranes leads to increased killing of *Salmonella*.

7. Macrophage cytophilic antibodies have been demonstrated in infection in many species of animals due to three different gram-negative organisms (*Salmonellae*, *E. coli*, and *Vibrio cholerae*) and two parasites (*T. brucei* and *S. mansoni*). It is likely that they exist in other types of infection. This would suggest evolutionary evidence for their importance in resistance to infection.

FLOW CHART EXPERIMENTAL METHOD



MATERIALS AND METHODS

Animals

Sprague Dawley Rats, 300-400 g, were obtained from Spartan Research Animals Inc., Haslett, Michigan. All animals were maintained under standard laboratory conditions. Purina Laboratory Chow and water were available *ad libitum*.

Bacteria

Salmonella typhimurium, strain SR-11, was used in all experiments. Overnight cultures of the organism, grown in brain heart infusion broth were centrifuged at 8000 rpm for 10 minutes. Cells were washed once in sterile deionized water and diluted to appropriate concentration. Pour plates of appropriately diluted samples were used for quantitation.

Macrophage Preparation

Sprague Dawley rats were anesthetized by intraperitoneal injection of 1.5 mg of sodium pentobarbital (Haver Lockhart Laboratories, Shawnee, Kansas). Sterile M-199 (50 ml) was injected intraperitoneally and the abdomen was gently agitated to insure mixture and freeing of peritoneal cells. After five minutes 15-25 ml of the M-199 was

withdrawn and placed in a Pyrex Plastic Centrifuge tube containing 400 μ of sodium heparin (200 μ/ml) and the rat serum under study. Following vortexing, the macrophage-rat serum preparation was mixed in a rotary shaker for 30 minutes at 20C. The mixture was centrifuged at 3500 rpm for five minutes and resuspended in 6 ml of M-199. The macrophages were then counted on a standard hemocytometer using methelene blue dye and 0.2% acetic acid. The cells were adjusted to 1-2 X 10^6 cells/ml and resuspended in 3 ml of M-199. At this time 0.1 ml of a 1:10 dilution of the *Salmonella* culture was added (bacteria macrophage ratio 10:1).

After vortexing this was placed in a rotary shaker bath for five minutes at 20C. The macrophage-bacteria suspension was then filtered on a 25 mm diameter millipore filter (Pore size 14 μM) and diluted with 97 ml of sterile M-199. The millipore filter was removed and blenderized in 100 ml of sterile deionized H_2O for one minute, diluted appropriately and pour plates were prepared.

The free bacteria in M-199 (filtrate) were diluted in sterile deionized water and pour plates were made. The bacteria were then counted on a Digimatic Colony Counter.

Heat-killed Cells

Salmonella typhimurium SR11 was grown for 24 hours in brain heart infusion broth and washed three times in

saline. The bacteria were then autoclaved for 10 minutes. The heat-killed cells were then suspended in saline to a concentration of 1 X 10^9/ml.

Immune Serum

Sprague Dawley rats were immunized by intraperitoneal injection by the following schedule: 1 X 10^8 heat-killed cells the first day, 1 X 10^9 heat-killed cells every four days for three weeks. The animals were bled by cardiac puncture. The serum was obtained by centrifuging at 10,000 rpm for 10 minutes. Tube agglutination titers were performed on the antisera.

Absorbed Serum

Large amounts of heat-killed cells ($\sim$2.0 X 10^{10}/ml of serum) were used. Absorption was done at 45C for 4 hours and stored overnight. The antiserum heat-killed cell mixture was then centrifuged at 10,000 rpm for 10 minutes and agglutination titers performed.

Normal Serum

Sprague Dawley Rats were bled by cardiac puncture. The serum was centrifuged at 10,000 rpm for 10 minutes. Tube agglutination titers were performed.

Radiolabeled Endotoxin Preparation

Two-tenths millicurie of $Na_2{}^{51}CrO_4$ in volume of 1.5 ml was added to 100 mg of *Salmonella typhimurium* endotoxin. The preparation was diluted to 10 ml with saline and incubated at 37C for 48 hours with constant stirring. The preparation was then dialyzed for 3-5 days at 4C against frequent changes of deionized water. The labeled endotoxin was centrifuged at 8,000 X g for one hour in the Sorvall Superspeed RC2-B automatic Refrigerated Centrifuge (Ivan Sorvall Inc., Newton, Connecticut). The supernatant was recentrifuged at 100,000 X g for eight hours in the Beckman Model L Ultracentrifuge (Beckman-Dickinson Co., Rutherford, New Jersey). The pellet containing the labeled endotoxin was resuspended in 4.0 ml of deionized water and stored at -70C. This material, according to Chedid *et al.* (24), contained the toxic, heavily labeled, high molecular weight lipopolysaccharide.

^{51}Cr-Labeled Endotoxin-Macrophage Experiments

Normal rat serum and immune rat serum and macrophages were obtained as described previously.

Five μ liter of ^{51}Cr-labeled endotoxin was pipetted into 1 ml of non-sterile M-199 and counted on the Packard Gamma Counter for 1 minute. Macrophages ($\sim$1-2 X 10^6) were then added and the total volume adjusted to 3.0 ml.

This mixture was incubated in a rotary shaker bath at 20C for 5 minutes. It was then filtered through a Millipore membrane filter and washed with 97 ml of non-sterile M-199 and 150 ml of deionized water, carefully rinsing the cell bottle and filter apparatus. The membrane filter was removed and placed in a gamma counter vial. Each of the two rinses of the filter apparatus were placed in gamma counter vials. The cell bottle, filter bottle and filtrate bottles were counted on the gamma counter and the percent recovery in each fraction was determined.

Statistics

Statistical evaluations were made by the White Rank Order method (118).

RESULTS

Characterization of Peritoneal Cell Population and Serum Tube Agglutination Titers

Tube agglutination titers were performed on all sera used in this study. The presence of cytophilic antibody and/or activity has been related to the presence of other types of antibody, especially agglutinating and complement fixing antibodies (18,85,111). All titers were performed as described in Materials and Methods.

Table 1. Tube agglutination titers.

Date	Serum Type	Titer
1/29/76	Immune #3	5120
1/29/76	Normal #2	40
2/20/76	Immune #4	2560
2/20/76	Immune #5	2560
2/26/76	Normal #3	80
3/12/76	Immune #3, #4, #5 Absorbed with ΔKC	640
3/31/76	Immune #4	5120
4/05/76	Normal #3 Absorbed with ΔKC	40
7/24/76	*Immune #9	5120

*Last immunization-subcutaneous in a vascular plexus.

Agglutination titers were in the range 2560-5120 for immune sera. Titers for normal sera were in the range 40-80. It was not possible to remove all of the agglutinating antibodies from immune sera or normal sera by absorption with homologous heat-killed cells.

Wright stain was done on most peritoneal cell samples used in this study. Standard Wright stain technique and reagents were used. The majority of cells present were macrophages or lymphocytes, occasionally a polymorphonuclear leukocyte was found (see Table 2).

Table 2. Peritoneal cell counts.

Cell Type	Percent of Cells	
	Macrophage	Lumphocyte
Mouse	66	34
Rat	54*,**	46

*Average 60 experimental determinations.

**Macrophage count ∿10% low because of technical difficulties.

The ratio of macrophage to lymphocyte was approximately 55:45. The macrophage count was about 10% low because of frequent macrophage disruption on fixed slides.

Experimental Apparatus Control

To check the capacity of the Millipore filter to allow free passage of viable bacteria and endotoxin several experimental determinations were performed using either bacteria or endotoxin alone. Using two different endotoxin preparations the percentage remaining on the filter was in the range of 0.36-0.88. The percentage remaining in the incubation tube was in the range 0.34-0.52. Therefore, 98-99% passed through the filter.

Using viable bacteria alone, the average recovery after incubation for three hours at 20C in M-199 or deionized water was 101.3%.

The effects of normal and immune serum on the ability of macrophages to bind viable gram negative bacteria and endotoxin was documented semiquantitatively by several investigators (10,19,72,89,113). A modification of the suspension-centrifugation technique was used in this study to observe this phenomenon in a more quantitative fashion. All experimental runs were performed as described in Materials and Methods.

Effects of M-199, M-199-Normal Rat Serum and M-199-Immune Rat Serum on the Ability of Rat Peritoneal Cells to Bind Viable *Salmonella typhimurium* After Five Minutes

Rat peritoneal cells were treated with M-199 alone, M-199 normal rat serum or M-199-immune rat serum.

No statistically significant difference was found in the ability of peritoneal cells to bind viable bacteria after five minutes when treated with M-199 alone or M-199-normal rat serum. However, peritoneal cells treated with immune rat serum demonstrated statistically significant enhancement over M-199 alone or M-199-normal rat serum in their ability to bind viable bacteria after five minutes (see Table 3).

Table 3. Comparison of the effect of M-199, normal rat serum, and immune rat serum on the ability of rat peritoneal cells to bind viable *Salmonella typhimurium* after five minutes.[a]

Cells treated with:	Percent of Bacteria in the: Filtrate	Filter	Recovery
M-199	97.6[d] ± 19.6	12.0[d] ± 4.61	109.6 ± 14.9
Normal Rat Serum	80.0[b] ± 24.4	20.8[b] ± 12.4	105.5 ± 19.3
Immune Rat Serum	45.5[c] ± 24.6	43.9[c] ± 20.5	87.8[e] ± 21.5

[a]Average value from at least six experimental determinations.
[b]$P > 0.05$ - M-199 vs. Normal Rat Serum
[c]$P < 0.05$ - Normal Rat Serum vs. Immune Rat Serum
[d]$P < 0.001$ - M-199 vs. Immune Rat Serum
[e]$P \simeq 0.05$ Recovery, M-199 vs. Immune Rat Serum

Peritoneal cells treated with M-199 also bound 12.0% of the bacteria while 97.6% was in the filtrate. M-199-normal rat

serum treated peritoneal cells bound 20.8% of the bacteria with 80.8% free in the filtrate. Immune serum treated peritoneal cells bound 43.9% of the bacteria with 45.5% free in the filtrate. There was a statistically significant difference in total recovery between M-199 alone and M-199-immune rat serum ($P \simeq 0.05$).

Effects of M-199-Heat-killed Cell Absorbed Normal Rat and M-199-Heat-killed Cell Absorbed Immune Rat Serum on the Ability of Peritoneal Cells to Bind Viable *Salmonella typhimurium* SR11

Several milliliters of normal rat serum and immune rat serum were absorbed with large amounts of heat-killed *Salmonella typhimurium*. The effect of this serum on the ability of peritoneal cells to bind viable bacteria was studied. When compared with the results from nonabsorbed serum, the number of bacteria bound to macrophages was not statistically significant ($P > 0.05$). However, there was a statistically significant difference in the number of bacteria free in the filtrate ($P < 0.05$, $P < 0.05$) (see Table 4 on the following page).

Peritoneal cells treated with normal absorbed serum bound 21.1% of the bacteria with 105.4% free in the filtrate, Macrophages treated with immune absorbed serum bound 22.6% of the bacteria with 74.7% free in the filtrate. When compared with normal rat serum the peritoneal cells treated

Table 4. Comparison of the effect of absorbed and non-absorbed normal rat serum and immune rat serum on the ability of peritoneal cells to bind viable *Salmonella typhimurium*.[a]

Treatment	Percent of Bacteria in the: Filtrate	Filter	Recovery
Normal	80.0 ± 24.4	20.8 ± 12.4	101.5 ± 19.3
Normal Absorbed	105.4[b] ± 35.7	21.1[c] ± 18.7	121.3 ± 34.4
Immune	45.5 ± 24.6	43.9 ± 20.5	87.8 ± 21.5
Immune Absorbed	74.7[d] ± 23.6	22.6[e] ± 18.2	97.8 ± 11.95

[a]Average value from at least six experimental determinations.
[b]$P < 0.05$ Normal vs. Normal Absorbed
[c]$P > 0.05$ Normal vs. Normal Absorbed
[d]$P < 0.01$ Immune vs. Immune Absorbed
[e]$P > 0.05$ Immune vs. Immune Absorbed

with absorbed normal rat serum had a statistically significant increase ($P \leq 0.05$) in the number of bacteria free in the filtrate. Peritoneal cells treated with immune absorbed serum showed a statistically significant increase ($P < 0.01$) in the number of bacteria free in the filtrate when compared with immune serum treated macrophages.

Effect of Macrophage Absorbed Immune Serum on the Ability of Peritoneal Cells to Bind Viable *Salmonella typhimurium* SR11 After Five Minutes

One-half milliliter aliquots of antisera from three different pools were absorbed with 1-1.5 X 10^7 macrophages and the amount of bacterial binding was quantitated both before and after absorption. No statistically significant difference ($P > 0.05$) was found in the amount of bacterial binding between immune and macrophage absorbed immune serum (see Table 5).

Table 5. Comparison of the effect of immune serum and macrophage absorbed immune serum sensitized peritoneal cells to bind viable *Salmonella typhimurium* after five minutes.[a]

Cells treated with:	Percent of Bacteria in the: Filtrate	Filter	Recovery
Immune Serum	38.5 ± 20.7	47.1 ± 23	83.0[b] ± 19.8
Macrophage Absorbed Serum	51.6[b] ± 20.3	47.7[b] ± 15.7	99.3[b] ± 20.0

[a]Average value from at least six experimental runs.
[b]$P > 0.05$.

Effects of M-199, M-199-Normal Rat Serum and M-199-Immune Rat Serum on the Ability of Rat Peritoneal Cells to Bind ^{51}Cr-labeled *Salmonella typhimurium* Endotoxin After Five Minutes

Experimental runs were performed to observe the effects of M-199, M-199-normal rat serum and M-199-immune rat serum on the ability of rat peritoneal cells to bind ^{51}Cr-labeled endotoxin. There was no statistically significant difference ($P > 0.05$) between normal rat serum and immune rat serum treated cells. No statistically significant difference ($P > 0.05$) was found between M-199 and M-199-immune rat serum treated peritoneal cells (see Table 6).

Table 6. Comparison of the effect of M-199, M-199-normal rat serum and M-199-immune rat serum on the ability of rat peritoneal cells to bind ^{51}Cr-labeled endotoxin after five minutes.[a]

Treatment	Percent of Endotoxin in the: Filtrate	Filter
M-199	99.51 ± 0.295[d]	0.1797 ± 0.378[d]
M-199-Normal Rat Serum	99.11 ± 0.424[b]	0.315 ± 0.236[b]
M-199-Immune Rat Serum	98.45 ± 0.28[c]	0.47 ± 0.59[c]

[a]Average value from at least six experimental runs.
[b]$P > 0.05$ M-199 vs. M-199-Normal Rat Serum
[c]$P > 0.95$ Normal Rat Serum vs. Immune Rat Serum
[d]$P > 0.05$ M-199 vs. M-199-Immune Rat Serum

Effects of M-199-Normal and M-199-Immune Rat Serum on the Ability of Mouse Peritoneal Cells to Bind Viable *Salmonella typhimurium* SR11 After Five Minutes and Three Hours

Experimental runs were performed using mouse peritoneal cells treated with M-199-rat serum to determine their ability to bind viable *Salmonella typhimurium* SR11. A statistically significant difference ($P < 0.01$) was observed between M-199-normal and M-199-immune serum treated peritoneal cells. In addition, a statistically significant difference was found between five minutes and three hours with M-199-normal rat serum treated peritoneal cells (see Table 7).

Table 7. Comparison of normal and immune rat serum on the ability of mouse peritoneal cells to bind viable *Salmonella typhimurium* After Five Minutes and Three Hours.[a]

Cells treated with:	Percent of bacteria in the filtrate: 5 minutes	3 hours
Normal Rat Serum	99.6[a] ± 21.0	34.1[d] ± 26.1
Immune Rat Serum	22.0[b] ± 10.8	31.9[c,e] ± 24.3

[a]Average value from at least five experimental determinations.

[b]$P < 0.01$ IRS 5 minutes vs. NRS 5 minutes

[c]$P > 0.05$ IRS 4 hours vs. NRS 3 hours

[d]$P < 0.01$ NRS 5 minutes vs. NRS 3 hours

[e]$P > 0.05$ IRS 5 minutes vs. IRS 3 hours

Mouse peritoneal cells treated with M-199 NRS left 99.6% of the bacteria free in the filtrate while mouse cells treated with M-199-immune rat serum left 22% free in the filtrate after five minutes. After three hours mouse peritoneal cells treated with M-199-normal rat serum left 34.1% free in the filtrate and mouse cells treated with M-199-immune rat serum left 31.9% of the bacteria free in the filtrate. There was a statistically significant difference ($P < 0.01$) in the number of bacteria free in the filtrate between M-199-normal rat serum and M-199-immune rat serum treated cells after five minutes. In addition, there was a statistically significant difference in the number of bacteria free in the filtrate with peritoneal cells treated with M-199-normal rat serum between five minutes and three hours.

When compared with sensitized rat peritoneal cells treated with M-199-normal rat serum, sensitized mouse cells showed no statistically significant difference ($P > 0.05$) in the ability to bind viable bacteria after five minutes. However, mouse cells sensitized with M-199-immune rat serum had a statistically significant difference ($P < 0.05$) in their ability to bind viable bacteria after five minutes when compared with sensitized rat cells.

DISCUSSION

As mentioned earlier, there are several difficulties with earlier techniques for measuring cytophilic antibody, especially the macrophage monolayer technique. It is difficult to quantitate because not only each rosette-cell but also the number of antigen particles on each rosette must be counted. In addition, the counting must be performed in a short time period (3-5 minutes) following addition of antigen. Neither binding to glass or simple mechanical lodgement of bacteria next to the rosette-forming cell can be excluded. More importantly the number of macrophages with bacteria adherent are counted and not the percentage of bacteria added.

For these reasons a relatively simple, more quantitative method was developed using Millipore vacuum filtration of a suspension of sensitized cells and antigen. In addition, the mechanical stress applied to the macrophages during the experimental procedure allows only those bacteria relatively tightly bound to be counted. It is hoped that this will alleviate some of the difficulties encountered with earlier methods.

The statistically significant difference found between the ability of normal and immune rat serum to sensitize peritoneal cells to bind viable *S. typhimurium* (cf. Table 1). This was not unexpected in view of the known ability of sensitized peritoneal cells to bind gram-negative bacteria *in vitro* by less quantitative methods (81,89). Parish reported phagocytosis, not binding, of heat-killed bacteria with antisera to somatic polysaccharide antigen, not whole bacteria as was done in this study. He did not report the percentage of phagocytosis. Mittal found a much higher percentage of binding (91-95%) than was found in the present study (∿44%). He used the arbitrary number of two bacteria per macrophage to indicate cytophilic binding. Again, only semiquantitative measurements of the number of macrophages with adherent bacteria were made and percentage of the total bacteria bound was not reported. Both studies used *Escherichia coli* as the test bacteria.

Another important technical point is the route and method of immunization of the eliciting antigen. This is important because there is considerable variation in the amount of true cytophilic antibody formed with different techniques. Careful consideration of these facts is necessary for accurate interpretation of data on cytophilic antibody. Immunization with a living attenuated strain of *Salmonella* sp. is the best and most reliable method of

obtaining macrophage cytophilic antibody (85,95). This method also approximates an *in vivo* infection more closely than other methods. Heat-killed cells given with complete Freund's adjuvant is the second best way to obtain macrophage cytophilic antibody.

Intravenous injection of heat-killed cells is the third best method (107). The use of heat-killed cells given intraperitoneally leads to a less satisfactory cytophilic antibody response in mice and presumably also in rats. Unfortunately, this method of immunization was used in this study. It is likely that the results obtained reflect a minimal level of cytophilic activity and would be more striking if a more reliable method of immunization had been used.

It is interesting to note the level of cytophilic binding in each set of experimental runs. When M-199 alone was used, 12% of the bacteria were bound. It is likely that this represents non-specific attachment of bacteria to the macrophage surface due to non-specific cytophilic antibody or specific cytophilic antibody produced in low titer by bowel flora in normal serum that reaches the peritoneal cavity by diffusion or otherwise. Peritoneal cells directly sensitized with M-199-normal rat serum had a slightly higher level of bacterial binding ($\sim$21%). Although not statistically significant it is likely that this represents

non-specific and/or specific cytophilic antibody found in normal serum in a greater concentration than that which is reached in the peritoneal cavity. Macrophages sensitized with immune rat serum showed a much higher level of bacteria binding (∿44%). It is likely that this reflects specific macrophage cytophilic antibody for *Salmonella* found in immune serum. Auzins and Rowley (6) found only a slightly higher level of phagocytosis (∿50%) of *S. typhimurium* C5 which had been preopsonized with fresh pig serum.

Both normal and immune sera were absorbed with very large numbers of heat-killed *Salmonella typhimurium*. A statistically significant difference was found in the free-unbound fraction (filtrate) of bacteria with both normal ($P < 0.05$) and immune ($P < 0.01$) rat serum (cf. Table 2). The difference found in the cell-bound fraction (filter) was not statistically significant. It is likely that this lack of significance with cell-bound bacteria was due to phagocytosis of bacteria by immune sera sensitized macrophage (Recovery--88%). Capron *et al.* (1975) reported a dramatic decrease (95% to 5%) in macrophage adherence to *Schistosoma mansoni* schistosomules following absorption of rat immune sera by soluble *S. mansoni* antigens. It has been reported by another group of investigators that a large portion (89-95%) of the total amount of immunoglobulin present can be removed by similar procedures (94).

They also found absorption of antibody across almost the entire immunoglobulin range. These findings are supportive of the speculations of Berken and Benacerraf (10) on the nature of cytophilia; that is, cytophilic antibodies are really opsonizing antibodies for whole bacterial cells which also have the ability to attach to macrophages either before or after combination with antigen.

Because of the large number of bacteria used (10^{12}) it is possible that the adsorption was due to mechanical or non-specific factors rather than specific immunologic adsorption. Also, it should be mentioned again that the method of immunization used in this study and Berken and Benacerraf (10) is less than optimal for the production of cytophilic antibody. It is likely that the sera in both studies contained mostly classical opsonic antibody rather then cell-bound antibody. This point is very important because there is still controversy over the differentiation of cytophilic and opsonic properties. Much of the controversy appears to be due to the differences in method of immunization and subsequent antibody produced. This study is the first attempt to evaluate the residual cytophilic binding properties of sera absorption with gram-negative bacteria.

Immune serum was absorbed with peritoneal cells and the percentage of binding before and after absorption was compared. Aliquots of immune sera from three different

pools were used. The difference in binding was not statistically significant ($P > 0.05$). However, it is possible that phagocytosis occurred with immune-sera-sensitized macrophages (recovery 91%) or that not enough macrophages ($\sim 1.2 \times 10^7$/run) were used. Berken and Benacerraf (10) adsorbed guinea pig $\gamma 2$-globulin onto lung macrophages. They found that uptake of antibody was maximal at a serum dilution of 1:2 and quantitatively estimated at 7-8 $\mu g/10^7$ cells. The amount of antibody adsorbed each time was relatively constant. The results in this study suggest similar findings. Their interpretation was that cytophilic activity was present in a large portion of the $\gamma 2$-globulin fraction. The results found in both studies suggest a preponderence of opsonic-cytophilic antibody in the antisera used and have only limited value in differentiating opsonic from cytophilic antibodies.

The ability to bind ^{51}Cr-labeled endotoxin to macrophages sensitized with M-199, normal rat serum and immune rat serum after five minutes was tested. Very little endotoxin was bound in any experiments. Parish (89) reported adherence of erythrocytes coated with *E. coli* somatic polysaccharride to macrophages sensitized with antisera to *E. coli* somatic polysaccharride. This same antiserum promoted the phagocytosis of killed *E. coli* (presumably at 37C). The cytophilic antibody was found to be a 19S macroglobulin.

Berken and Benacerraf (10) reported cytophilic binding of *E. coli* polysaccharride or its dinitrophenol hapten by the passive direct technique with guinea pig antisera produced by heat-killed *E. coli* or DNP proteins in complete Freund's adjuvant. They found it was necessary to use undiluted antisera to obtain 3+ to 4+ rosette formation. This phenomena was attributed to insufficient coating of the red blood cell surface with polysaccharride. It is possible that this caused the very low levels of binding found in this study.

Levenson and Braude (72) reported adherence (33-40% of macrophages) of erythrocytes coated with *S. typhi* Vi antigen in the presence of early antisera (3-4 days) to Vi antigen at 37C. Normal red cells and red cells coated with *S. typhi* O antigen (Boivin antigen) did not bind to macrophages in the presence of Vi antisera. Peritoneal macrophages sensitized by the passive direct method failed to bind erythrocytes coated with Vi antigen. Late antisera (7-8 days) enabled macrophages to phagocytose antigen coated red blood cells. In summary, macrophages sensitized with early sera (3-4 days) bound *S. typhi* Vi coated erythrocytes. Macrophages sensitized with late antisera (7-8 days) phagocytosed *S. typhi* Vi coated erythrocytes.

It is likely that the failure to demonstrate cytophilic binding of endotoxin in this study was due to a lack of

specific cytophilic antibody for endotoxin in antisera obtained from intraperitoneal injection of heat-killed *S. typhimurium*. However, Robbins *et al.* (94) reported IgG antiendotoxin antibody in rabbits immunized with heat-killed *S. typhimurium*. They used the intravenous route of injection. This study was the first to observe the cytophilic binding of free endotoxin.

Mouse peritoneal cells were sensitized with normal and immune rat serum. A comparison of the ability of mouse and rat peritoneal cells to bind viable *S. typhimurium* indicates that mouse cells sensitized by immune rat serum had a statistically significant increase ($P < 0.05$) in the ability to bind viable *S. typhimurium* after five minutes. A comparison of binding of bacteria to mouse cells between normal and immune rat sera indicates a significant statistical difference ($P < 0.01$). There was a significant statistical difference ($P < 0.01$) between five minutes and three hours with mouse cells sensitized with normal rat serum, but no difference was found with immune rat serum ($P > 0.05$) (cf. Table 7). There was no statistical difference at three hours between normal and immune rat serum sensitized mouse cells ($P > 0.05$).

Other studies of interspecies activity of cytophilic antibody have shown high levels of binding with homologous cells and lower levels of binding with heterologous cells

(10,69). The close genetic relation between the mouse and the rat would account for the significant binding found in both species. Perhaps the increased binding with mouse cells was due to the fact that the mouse is the normal host for *S. typhimurium*.

The clearance of bacteria at three hours is due to binding and/or phagocytosis. It is interesting to note the mouse cells sensitized with both normal and immune rat serum had similar levels of free bacteria (filtrate) (34.1% and 41.9%), after three hours. This suggests that over a period of time both normal and immune serum sensitized peritoneal cells have the ability to bind and/or phagocytose 70-75% of the total bacteria present. This was the first comparison of heterologous cytophilic activity between mouse and rat peritoneal cells.

This thesis represents a partial reevaluation of studies similar in some ways to the present one using more quantitative and clearly defined techniques.

The ability of sensitized peritoneal cells to bind viable bacteria has been demonstrated in a way that is not only more quantitative but is based on the total bacteria used, not on the number of cells forming rosettes with an arbitrary number of bacteria or antigen particles. As mentioned earlier the differentiation between opsonic and cytophilic antibodies is not resolved and the importance of

using reliable methods for producing antisera for these studies is emphasized. Furthermore, previous studies on the ability to bind endotoxin were not clearly reported and did not use free endotoxin, as would exist *in vivo*.

The level of cytophilic activity measured in this study very likely reflects a minimal level of activity. More striking results would have been obtained by using a live attenuated strain of bacteria for immunization and using a lower titer of cytophilic antibody for sensitization. In addition, the antibacterial effect of macrophage clumping due to cytophilic antibody was not measured in these studies.

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