



The immune and mitogenic response by Balb/c, C3H/HeJ, and nude mice to *Brucella abortus* bacterin and endotoxin

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A thesis submitted in partial fulfillment of the requirements for the degree of MASTER OF SCIENCE in Microbiology

Montana State University

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Abstract:

Brucella abortus cells and *Brucella* lipopolysaccharide have been the subject of conflicting discussion in the literature because of early confusion over which fraction in a phenol-water extraction technique contained the endotoxin, and because of divergent results about the role of T-cells in the immune response based on experiments using anti-theta serum or congenitally athymic mice. The responses of experimental animals to the *Brucellae* also seem to differ significantly from the responses elicited by the Enterobacteriaceae and their lipopolysaccharides. This paper investigates various parameters of the response to killed *Brucella abortus* 0119-3 cells and the purified endotoxin. Balb/c and nude mice were used to investigate the role of the thymus in the response to these antigens. The C3H/HeJ mouse strain, which exhibits an anomalous response to the lipopolysaccharides of *Escherichia coli* and *Salmonella* spp., was also included in this series of experiments to determine if the defect which limits the C3H/HeJ response would also limit its response to this particular endotoxin. It was demonstrated that in Balb/c and C3H/HeJ mice both the magnitude of the response to BA cells and the class of antibody synthesized over a prolonged period following a single immunization are highly dose dependent. Comparison of this data with that for nude mice demonstrates clearly that nude mice are significantly limited in their response; whether this is a direct or indirect effect of their athymic condition could not be determined from the experiments reported here. Balb/c, C3H/HeJ, and nude mice all make secondary responses to BA cells; IgG constitutes a major portion of the secondary responses. The BA-LPS was shown to be immunogenic over a dose range of 1 µg to 100 µg. This purified endotoxin elicits a primary response which appears to consist entirely of 2-ME-sensitive antibody in Balb/c, C3H/HeJ, and nude mice. The similarity of both magnitude and class of antibody of the primary response by both nude and Balb/c mice suggests that the BA-LPS may well be a thymus-independent antigen. Secondary responses, demonstrated in Balb/c and C3H/HeJ mice, consisted of 2-ME-sensitive and 2-ME-resistant antibody. Nude mice were not examined for secondary responses. Mitogen studies using spleen cell cultures from Balb/c, C3H/HeJ, and nude mice, and thymus cell cultures from Balb/c and C3H/HeJ mice revealed that both BA and BA-LPS are highly mitogenic for spleen cells. Minimal responses obtained in thymus cell cultures suggest that the BA cells and endotoxin lack mitogenic activity for T cells.

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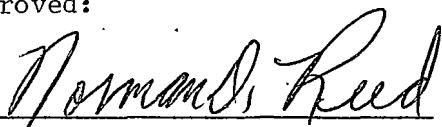
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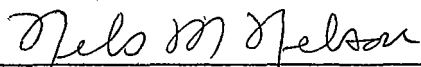
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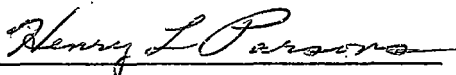
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December, 1976

ACKNOWLEDGEMENTS

I thank Dr. N.D. Reed for consultation and review of the manuscript. I also thank Dr. Lois Jones for supplying the Brucella abortus 0119-3 endotoxin, Dr. J.A. Rudbach for donating the Escherichia coli 0113 lipopolysaccharide, and Mr. Edward Sheehan for the Brucella abortus tube agglutination and ring test antigens. I wish to thank Dr. Dean Manning for encouragement to enter the field of immunology and for his time generously given in the early part of my training. Also I thank Dr. Judy Manning for consultations and for my introduction to Dr. Lois Jones. Fine technical assistance was provided by Ms. Pat Healow. I especially thank Mr. Craig W. Spellman for valuable consultation and help in the preparation of this paper.

This research was supported by U.S. Public Health Service Grant AI 10384.

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ABSTRACT

Brucella abortus cells and Brucella lipopolysaccharide have been the subject of conflicting discussion in the literature because of early confusion over which fraction in a phenol-water extraction technique contained the endotoxin, and because of divergent results about the role of T-cells in the immune response based on experiments using anti-theta serum or congenitally athymic mice. The responses of experimental animals to the Brucellae also seem to differ significantly from the responses elicited by the Enterobacteriaceae and their lipopolysaccharides. This paper investigates various parameters of the response to killed Brucella abortus 0119-3 cells and the purified endotoxin. Balb/c and nude mice were used to investigate the role of the thymus in the response to these antigens. The C3H/HeJ mouse strain, which exhibits an anomalous response to the lipopolysaccharides of Escherichia coli and Salmonella spp., was also included in this series of experiments to determine if the defect which limits the C3H/HeJ response would also limit its response to this particular endotoxin. It was demonstrated that in Balb/c and C3H/HeJ mice both the magnitude of the response to BA cells and the class of antibody synthesized over a prolonged period following a single immunization are highly dose dependent. Comparison of this data with that for nude mice demonstrates clearly that nude mice are significantly limited in their response; whether this is a direct or indirect effect of their athymic condition could not be determined from the experiments reported here. Balb/c, C3H/HeJ, and nude mice all make secondary responses to BA cells; IgG constitutes a major portion of the secondary responses. The BA-LPS was shown to be immunogenic over a dose range of 1 μ g to 100 μ g. This purified endotoxin elicits a primary response which appears to consist entirely of 2-ME-sensitive antibody in Balb/c, C3H/HeJ, and nude mice. The similarity of both magnitude and class of antibody of the primary response by both nude and Balb/c mice suggests that the BA-LPS may well be a thymus-independent antigen. Secondary responses, demonstrated in Balb/c and C3H/HeJ mice, consisted of 2-ME-sensitive and 2-ME-resistant antibody. Nude mice were not examined for secondary responses. Mitogen studies using spleen cell cultures from Balb/c, C3H/HeJ, and nude mice, and thymus cell cultures from Balb/c and C3H/HeJ mice revealed that both BA and BA-LPS are highly mitogenic for spleen cells. Minimal responses obtained in thymus cell cultures suggest that the BA cells and endotoxin lack mitogenic activity for T cells.

INTRODUCTION

It has long been recognized that thymic influence and thymus-derived (T) lymphocytes are involved in several aspects of immunological responsiveness. One of these aspects, that of cell-mediated immunity, encompasses allograft rejection, graft vs. host reactions, contact hypersensitivity, and tuberculin-type skin reactions to many microbial antigens. The early work of Miller (61) showed that some sort of thymic influence was also essential to humoral immunity, at least in the mouse.

In the years between 1962 and 1967, experiments using neonatally thymectomized or adult thymectomized, lethally irradiated, bone marrow reconstituted (ATxBM) mice clearly illustrated that antibody production to such antigens as sheep erythrocytes (60) and serum proteins (97, 98) was significantly impaired in the absence of a thymus; subsequent implantation of a thymus gland could restore at least partial responsiveness. Claman demonstrated unequivocally that interaction between thymus-derived and bone-marrow derived (B) lymphocytes was essential to antibody production to sheep erythrocytes. Cell transfers into lethally irradiated mice resulted in antibody production only in those animals receiving both T and B cells (19).

Using mice that had been neonatally thymectomized, Miller (58, 59) was the first to show that a number of antigens evoked normal immune responses in spite of the absence of T cells.

However, the possibility existed that some T cells had already been seeded to peripheral organs prior to thymectomy. Therefore, it was necessary to confirm these observations in mice which, following thymectomy, were treated with anti-theta serum or to utilize ATxBM mice.

The response to some antigens appeared to be unaffected by any of the above treatments. These antigens included pneumococcal polysaccharide type III (SIII) (35, 36), polyvinylpyrrolidone (PVP) (3), polymerized flagellin (POL) (6, 24), and bacterial lipopolysaccharide (LPS) (35, 65). Further studies (28, 56, 78) utilizing congenitally athymic "nude" mice strengthened the concept that these antigens were indeed thymus-independent; i.e., that collaboration between B and T cells was not necessary for a humoral response.

These antigens were demonstrated to share certain features. Physicochemically, they could be described as polymeric molecular structures, with multiple identical subunits. Characteristically, the polymers show a progressive loss of immunogenicity with sequential reduction of subunit number. Apparently, the repetitive structure results in similar antigenic determinants being presented to the B cells in a sufficiently high concentration to trigger B cell proliferation and antibody production without T cell intervention. Immunologically, these antigens elicit an antibody

response which is almost entirely IgM in composition (10), an observation which has contributed to the idea that IgM production is less thymus-dependent than is IgG.

Early observations with Brucella abortus (BA) in chickens by Thorbecke et al. (100) and Rouse and Warner (82) were interpreted by some investigators to indicate that this antigen was also thymus-independent in its elicitation of a humoral response. These workers reported that neonatally thymectomized chickens treated with anti-thymocyte serum prior to injection of 20×10^9 killed BA cells produced very nearly normal anti-Brucella antibody responses. Cell transfer studies in mice by Takahashi et al. (96) and Thorbecke et al. (100) further implicated BA as a thymus-independent antigen; treatment of BA-immune cell populations with anti-Ig plus complement significantly inhibited their ability to transfer primary and secondary responses to syngeneic recipients which had received 600 r X-irradiation. Treatment of these cell populations with anti-theta plus complement prior to transfer did not alter the magnitude of the response to BA. These results were interpreted to mean that full responsiveness and memory to BA resided in the B cell population. Based on these studies, Mond et al. (66) used BA to examine development of B cell memory to a thymus-independent antigen.

The humoral response to BA, however, contrasts sharply with

the responses seen in animals immunized with other thymus-independent antigens. As mentioned previously, SIII (9, 35), E. coli LPS (11, 70), and PVP (44), as well as the Vi antigen extracted from Citrobacter freundii (77), all elicit a response which is composed primarily of IgM. A second exposure to these antigens may result in a) tolerance, in the case of SIII (8) and PVP (47); b) a response identical in magnitude and antibody class (IgM) to the primary response, as with Vi (77), or c) a heightened IgM response as elicited by E. coli LPS (11, 63, 70). In none of these cases does a significant switch from 19S to 7S antibody production occur following a second immunization.

The response to Brucella abortus is noteworthy in two respects. Within twenty-one days following a single exposure to the bacterin, a 2-mercaptoethanol-resistant (2-ME-R) component of the humoral response, presumably IgG, becomes detectable (22). Reimmunization results in an increased production of antibody with a markedly increased 2-ME-R component (37).

Work by Kruger and Gershon (46) has further distinguished BA from other thymus-independent antigens. These investigators transferred thymocytes to lethally irradiated recipients and, following injection of antigen, measured DNA synthesis in the repopulated spleens. They found that the thymus-independent antigens PVP and SII stimulated little or no DNA synthesis. The transferred

thymocytes, however, were strongly stimulated by B. abortus.

(The interpretation of these results was complicated by the observation that recognized T-dependent antigens such as sheep erythrocytes and bovine serum albumin stimulated little DNA synthesis.)

The authors offered three possible explanations of their results:

1) The basis of T-independency of Brucella may differ from that of SII or PVO. Perhaps BA is such a good antigen because it stimulates B cells maximally in the absence of T cells; DNA synthesis in the thymocytes merely reflects "super antigenicity." 2) BA is such a good stimulator of T cells that it very efficiently utilizes the few that may remain following thymus deprivation. 3) The responses evoked by BA in thymus-deprived mice are not really normal, but studies thus far have not been adequate to demonstrate the deficiency.

Crewther and Warner (22) compared the primary anti-B. abortus responses of congenitally athymic "nude" mice to those of normal mice. They concluded that while the IgM response in the nude mice is not depressed, the IgG is considerably reduced, and thus must be dependent on functional T cells for full development.

Jacobson et al. (37) confirmed the studies by Crewther and Warner, stating that nude mice make significantly reduced primary responses to BA compared to those made by normal or littermate mice. Not only was the nude 7S antibody reduced; the 19S

production also appeared to be somewhat impaired. The defect in the 7S response, however, became much more apparent during a secondary response to B. abortus. If the nudes, however, received semi-syngeneic thymus cells four or five months prior to immunization with Brucella, the total secondary response was restored to normal; the 7S response differed only slightly from those of normal animals.

Tingle and Shuster (101) investigated more closely the role of thymus-derived lymphocytes in the development of a primary response to BA. They examined the dose-response relationship over an eight $\log_{10}$ dose range in both normal mice and in adult thymectomized, lethally irradiated, bone marrow reconstituted (ATxBM) mice. Their conclusions were that the presence or absence of T cells was highly determinative of both the class of antibody and the amount of antibody formed in response to a given dose of antigen; that the T cell played the role of both accelerator and amplifier of antibody production; and, therefore, that the thymus dependency or independency of BA was a function of the dose used for immunization.

The results of Tingle and Shuster correlated well with the reports of Droege (25) and Droege and Malchow (26) that neonatally thymectomized chickens had a reduced agglutinin response to BA. The response was most markedly affected at low doses of antigen, and the 2-ME-R antibody was more affected than was the 2-ME-

sensitive antibody.

As yet the antigenic moieties inducing the production of anti-*Brucella* antibodies have not been clearly identified. The major protein antigens present on the cell surface of *B. abortus* are the A (major antigen) and the M (minor antigen) antigens. Being a Gram-negative organism, *B. abortus* also possesses as part of its cell wall a lipopolysaccharide, a complex which contains both the O-somatic antigens and a lipid portion conferring what has been termed endotoxic activity.

Lipopolysaccharide (LPS) from Gram-negative bacteria has been shown to exert a wide range of biological effects. The best studied of the endotoxins is that of *Escherichia coli*. Biologic properties ascribed to these substances include lethality, inflammation, and pyrogenicity (62), along with many immunologic properties. These have been well characterized and may be briefly summarized as follows:

- 1) potent immunogen (48, 83), the response to which does not require thymus-derived cells (3, 56, 65, 78);
- 2) B-cell mitogen (4, 31);
- 3) stimulator of a polyclonal response (4, 5, 104);
- 4) adjuvant, enhancing the antibody production to various antigens (40, 41, 87);
- 5) inhibitor of induction of tolerance to protein antigens

(33, 50, 51);

- 6) substitute for helper T-cells, facilitating antibody production to thymus-dependent antigens in the absence of thymus-derived cells (64, 85).

Chemically, these endotoxins consist of three regions (52,55):

1) the O polysaccharide which is covalently linked to 2) the core polysaccharide covalently linked, by means of a trisaccharide of 2-keto-3-deoxyoctanoic acid (KDO), to 3) the lipid A structure. The specific antigenicity has been determined to reside in the O polysaccharide (54, 55, 91). The varied activities of lethality (52), toxicity (52), adjuvant activity (16), mitogenicity (16, 71), and the non-specific polyclonal effect (21) have been attributed to the lipid A moiety.

Studies of the Brucella endotoxin (BA-LPS) have resulted in ambiguous conclusions. BA-LPS differs significantly from the much investigated endotoxin of E. coli. When extraction of LPS from Enterobacteriaceae is carried out using the hot phenol-water method of Westphal (106), the LPS is recovered from the aqueous phase.

Redfearn, in 1960, first described the isolation of the Brucella endotoxin from the phenol phase (76), and it was subsequently discovered that endotoxins of Xanthomonas campestris (7, 34, 53) and Citrobacter freundii (72, 73, 74) also appeared in the phenol phase.

Consequently, much of the early literature concerning BA-LPS

includes fallacious conclusions, because the investigators explored the properties of those substances partitioning into the aqueous phase. Some of these erroneous impressions persisted long after Redfearn's work. Berger et al. (12), who examined only the aqueous phase of the Westphal extraction, reported in 1969 that BA-LPS could cause no dermal necrosis, elicit no Shwartzman reaction, and was much less pyrogenic and toxic than were the endotoxins extracted from E. coli and Serratia marcescens. However, for his extraction Berger used a rough strain of Brucella which lacks LPS. Rank et al. (75) published another bit of misleading information, stating that Brucella LPS contains no lipid A. Their references were to Berger et al. and to Freedman et al. (29), who used a commercial Boivin preparation of B. abortus from Difco, now known to be completely non-toxic for mice (42).

Other workers attempting to elucidate the differences between BA-LPS and the endotoxins from other Gram-negative organisms contributed further to the confused state of the literature concerning this organism. The preparation of the endotoxin becomes a recurrent problem in interpreting the data. Either the method of endotoxin extraction is not mentioned (109), or the validity of the preparative procedure is difficult to evaluate (1). Wilson et al. (108) attempted to study in mice the relationship of immunity and sensitivity to Brucella LPS using whole heat-killed or acetone-killed

cells, rather than extracted endotoxin. They reported that BA-LPS was not lethal in quantities comparable to the endotoxins of other Gram-negative organisms; furthermore, in their system, heat-killed BA 2308 injected into mice did not stimulate formation of any complement-fixing antibodies. Thirdly, they reported that killed BA cells did not induce decreased urinary nitrogen, as did the endotoxin of E. coli. These observations all pointed to the obvious conclusion that either there was something very different about BA-LPS or something very different about the mouse response to it.

Investigations by Kessel et al. (45) into the role of cell-associated antibody in endotoxin cytotoxicity substantiated the idea that mice respond differently to BA-LPS: A high percentage of peritoneal exudate cells (PEC) from normal animals died when incubated with E. coli endotoxin. No significant death of normal PEC occurred during incubation with BA endotoxin. If, however, the PEC were first incubated with anti-Brucella antibody, and then incubated with BA-LPS, a high degree of cytotoxicity was observed. Both Wilson et al. (108) and Kessel et al. (45) concluded that the typical endotoxin response seen to E. coli LPS is due to prior sensitization of the mouse to this ubiquitous organism. Because the mouse is an unnatural host for Brucella, the chances of previous exposure to this endotoxin is most unlikely. Therefore, no pre-formed antibodies were present, and no response to the endotoxin

was elicited.

Leong et al. (49) in 1970, with endotoxin isolated using the Redfearn modification (76) of the Westphal extraction procedure, examined some of the structural and physiological properties of B. abortus and B. melitensis. Chemically, the endotoxins from these two species were characterized as protein-lipopolysaccharide-2-keto-3-deoxy-octulosonic acid complexes which were heat stable and Pronase resistant. The complexes stimulated high agglutinin titers in rabbits, and produced severe leukopenia and leukocytosis in mice. Brucella LPS was indeed a much less potent pyrogen for rabbits than was E. coli LPS; however, in mice endotoxins from Brucella and E. coli were equally toxic. Leong et al. also carried out hybridization studies and demonstrated some homology between endotoxins from Brucella and some of the Enterobacteriaceae.

Subsequently, attempts were made to further characterize the LPS of Brucella. Renoux et al. (80) confirmed the absence of KDO in the aqueous phase of the extraction, and the presence of KDO and heptoses in the phenol phase. Furthermore, they found that the major antigenicity resided in the phenol phase extract, and that in guinea pigs the aqueous phase was completely non-immunogenic. They proposed a structure consisting of lipid A, a core polysaccharide, and side chain structures which differ from those of the Enterobacteriaceae.

Bowser et al. (13), using cation exchange chromatography, performed an amino sugar profile on hydrolysates corresponding to the LPS of Leong et al. The main amino sugars present were glucosamine and quinovosamine; trace amounts of galactosamine and a fourth unidentified sugar could be detected.

Immunological characterization of the phenol-extracted BA endotoxin has not proceeded rapidly. In the literature, conflict concerning the properties of the LPS remains apparent. Renoux et al. (79) examined the influence of BA-LPS on the initiation of antibody forming cells in mice immunized with sheep erythrocytes. They worked with products from B. abortus and B. melitensis extracted by three procedures. Trichloroacetic acid-extracted LPS from both Brucella strains, as well as phenol-phase LPS from B. melitensis, enhanced the specific anti-sheep erythrocyte response only at high doses (100 μ g) of endotoxin. These investigators speculated, as others had before them, that normal animals respond poorly to endotoxins from Brucella and cannot be sensitized so that typical endotoxin responses follow the injection of killed cells or Brucella endotoxins. Specifically, they stated that the polyclonal effects induced in mice by endotoxins are dependent on prior exposure of the animals to the organism or to the endotoxin itself.

Many of the investigators of the immunologic properties of the E. coli and Salmonella adelaide endotoxins have utilized mice of the

C₃H/HeJ strain. These animals have been shown to be refractory to many of the effects of these endotoxins. This strain, along with the high-LPS responder C₃H/HeN strain, were derived from the C₃H/He, and has been maintained separately at Jackson Labs, Bar Harbor, ME. since 1948. It seemed possible that use of this strain in investigations of the properties of the BA-LPS might elucidate further similarities or differences between this endotoxin and those extracted from the Enterobacteriaceae.

Sultzzer was the first to report the resistance of the C₃H/HeJ strain to the lethality of endotoxin (93). He further demonstrated that these mice gave an abnormally high infiltration of mononuclear cells in proportion to the number of polymorphonuclear cells in response to an intraperitoneal stimulus by LPS of either E. coli or Salmonella typhosa (92). Further studies revealed that these mice were resistant to stimulation by LPS as an immunogen, responding transiently to a restricted range of doses (87, 102). Additionally, their responses to the mitogenic (81, 87, 95, 102) and polyclonal (103) effects of LPS were negligible. Neither the ability of LPS to interfere with the induction of tolerance to deaggregated human gamma globulin nor the ability of LPS to act as an adjuvant in mice immunized with soluble bovine serum albumin could be demonstrated in this strain (87).

CWB mice are high responders to LPS. Genetic studies using backcross F_1 ($C_3H/HeJ \times CWB$) $\times C_3H/HeJ$ animals suggested that the defect in responsiveness may be due to a single gene, since the abilities to respond to immunogenic, mitogenic, and polyclonal stimuli by LPS appeared to segregate together, along with the prevention of LPS interference with tolerance induction. Furthermore, no correlation was shown between the lack of responsiveness to LPS and the loci governing either H-2 type or immunoglobulin heavy chain allotype; neither was the trait found to be sex-linked (102).

The cellular mechanism of C_3H/HeJ unresponsiveness to endotoxin has been investigated. Spleen cells from C_3H/HeJ mice, though refractory to the mitogenic properties of LPS, respond normally to other B cell mitogens (84, 95). The defect does not appear to affect the amount of LPS which binds to the B cells, since radiolabelled LPS was found to bind equally well to C_3H/HeJ and normal C_3HeB/FeJ spleen cells (103). Studies have documented also that the C_3H/HeJ mice are not deficient in B cells, but have a normal distribution and number of both T and B cells (32).

Experiments by Glode et al. (32) illustrate that the addition of normal C_3H/HeN macrophages or spleen cells to C_3H/HeJ spleen cell cultures fails to restore normal LPS responsiveness to the C_3H/HeJ cells. Conversely, neither macrophages nor spleen cells from C_3H/HeJ animals suppress the in vitro mitogenic response of

normal C₃H/HeN spleen cell cultures to LPS. In vivo, reconstitution of C₃H/HeJ animals following B-cell depletion by sublethal irradiation with spleen cells from normal C₃H/HeN mice results in acquired responsiveness by the C₃H/HeJ animals. The authors conclude, therefore, that unresponsiveness is a defect involving only the B cell and cannot be attributed to either the presence of suppressor cells or the absence of helper cells.

Statement of Thesis

The involvement of the thymus in the response to B. abortus has remained unclear despite numerous attempts to define the role of the thymus-derived lymphocyte. Some reports claim that BA is a thymus-independent antigen, while others claim that BA is a unique antigen, eliciting a thymus-independent primary response but a thymus-dependent secondary response. The main areas of investigation have involved the use of immunologically impaired (nude and ATxBM) mice or the transfer of primary and secondary responses to irradiated recipients to examine the requirement for T cells. While investigators have briefly examined the response of nude mice to BA, there are no data available on the kinetics of the nude response to various doses of BA over an extended period of time.

Furthermore, the paucity of data about the immunologic

properties of the Brucella lipopolysaccharides left this area open to investigation. An elucidation of these properties might indeed bear on the relatedness of the BA-LPS to the endotoxins extracted from the Enterobacteriaceae. In this area the C₃H/HeJ mouse would prove useful, as either a response or the lack of one might reveal certain similarities or differences between the various endotoxins.

The specific questions to which I directed my research are as follows:

- 1) Do normal and nude mice respond to primary and secondary exposures to B. abortus cells?
- 2) Are the kinetics of the response to BA cells by normal and nude mice similar over an extended period of time?
- 3) Do nude and normal mice synthesize similar amounts and class (es) of antibody in response to various doses of BA cells?
- 4) Are BA cells mitogenic?
- 5) Is BA-LPS immunogenic in both nude and normal mice? What, then, is the optimal immunizing dose?
- 6) What class (es) of antibody is elicited by immunization with BA-LPS?
- 7) What are the kinetics of the primary response to various doses of BA-LPS in normal and nude mice?
- 8) Does BA-LPS elicit a secondary response?

- 9) Is BA-LPS a mitogen?
- 10) Do C₃H/HeJ mice respond to BA cells and BA-LPS as immunogens and as mitogens?

MATERIALS AND METHODS

Animals

Mice used in these experiments were of three genetic backgrounds: Balb/c, nude (nu/nu) mice, eleventh and twelfth generations produced by successive cross-intercrossing onto a Balb/c background, and C3H/HeJ mice. The Balb/c and nude mice were derived from stock maintained in our laboratory; C3H/HeJ mice were purchased from Jackson Laboratories (Bar Harbor, ME). All received sterilized Purina Lab Chow 5010 and acidified chlorinated water ad libitum. Experimental animals ranged in age from six to sixteen weeks; those used in each experiment varied in age by less than two weeks.

Antigens and In Vivo Immunizations

Brucella abortus Antigen

Brucella abortus 0119-3 (BA) was obtained from Edward Sheehan (Veterinary Research Laboratory, Montana State University, Bozeman, MT.). This antigen suspension, prepared by the USDA as their tube agglutination test antigen, is a 4% (by volume) suspension of killed, phenolized Brucella abortus cells. Cell counts performed using a Petroff-Hauser counting chamber indicated that this stock preparation contained 10^{10} organisms per ml. Prior to immunization of animals, the cells were washed twice and then resuspended in

sterile phosphate buffered saline (PBS) to 4%. From this stock of washed cells, subsequent dilutions were made in sterile PBS. Mice received 0.25 ml of the appropriate antigen dilution intravenously (i.v.) via the lateral tail vein. Cell doses used are stated in the Results section.

Brucella abortus 0119-3 Lipopolysaccharide

Lipopolysaccharide from Brucella abortus 0119-3 (BA-LPS), extracted from cells according to the modification by Redfearn (76) of the phenol-water extraction procedure of Westphal (106), was generously supplied by Dr. Lois Jones (University of Wisconsin--Madison, Madison, WI.). BA-LPS solutions used for immunization were prepared in sterile PBS; mice received 0.25 ml i.v. of the appropriate dilution. Doses varied with the question under investigation and are given fully in the Results section.

Determination of Anti-Brucella Agglutinin Titers

Tube Agglutination Assay

For most experiments, the serum antibody response to BA and BA-LPS was quantitated using the tube agglutination test of Spink et al. (90). The indicator antigen used in this system is the USDA-prepared Brucella abortus 0119-3 tube agglutination antigen; the stock USDA antigen is diluted

1:100 in 0.5% phenolized physiological saline before use in the assay.

2-mercaptoethanol (2-ME) has been shown by Adler (2) to inhibit the agglutinating ability of IgM, due to destruction of the pentameric structure (27), otherwise maintained by disulfide bonds (43). To determine what portion of the total antibody response consisted of 2-ME resistant (2-ME-R) antibody, samples of sera were also treated with 2-ME prior to titration with Brucella antigen. The 2-ME concentration was selected by incorporating 2-ME over a dose range of 0.1 to 0.01 M in tube agglutination assays of anti-sheep erythrocyte serum samples. It was observed that over this dose range, no difference appeared in the 2-ME-R antibody titers, and 0.1 M 2-ME was arbitrarily used in most assays. Routinely, 0.1 ml serum was diluted 1:10 in 0.8 ml physiological saline plus 0.1 ml of a 1.0 M 2-ME stock solution, and incubated at 37°C for one hour before subsequent dilutions were made (22). Alternatively, 0.5 ml of 0.2 M 2-ME in physiological saline was added to 0.1 ml serum diluted 1:5; this mixture was then incubated one hour at 37°C before further dilution. (2-ME was purchased from Sigma Chemical Co., St. Louis, MO., #M6250.)

Microtiter Agglutination Assay

The antibody titers shown in Table II were determined using the microtiter system described by Claflin et al. (18) and modified by Jacobson et al. (37). Plastic U-bottom microtiter plates, 50 ul diluting loops, and 50 ul pipette droppers were obtained from Cooke Engineering (Alexandria, VA.). The indicator antigen used in this system is the Brucellin Ring Test Antigen prepared by the USDA and kindly supplied by Edward Sheehan. This antigen is a 4% (by volume) cell suspension of killed BA organisms coupled to a violet indicator dye. This stock suspension was diluted 1:10 in sterile PBS for use in the microtiter assay.

Stimulation of Cell Cultures by Mitogens

Mitogen assays were carried out according to the method of Spellman (89). Mitogens used were Concanavalin A (Con A) (Miles Laboratories, Inc., Kankakee, IL.); Escherichia coli 0113 lipopolysaccharide (LPS) extracted by the phenol-water method of Westphal (106), kindly supplied by Dr. J.A. Rudbach (University of Montana, Missoula, MT.); Brucella abortus 0119-3 (USDA tube agglutination test antigen), donated by Edward Sheehan; Brucella abortus 0119-3 lipopolysaccharide, a gift from Dr. Lois Jones.

All mitogen preparations were made in PBS; sterilization

procedures varied with the mitogen. Con A was filter-sterilized by passage through a 0.22 μ millipore filter using a Swinnex filter assembly and syringe; E. coli 0113 LPS was boiled for two-and-one-half hours; BA 0119-3 cells were diluted in sterile PBS; BA-LPS was heated for forty-five minutes at 65°C.

A spectrum of doses of each mitogen was used, as indicated in the Results section.

RESULTS

A series of experiments was performed to characterize the magnitude and kinetics of the antibody response by Balb/c, C3H/HeJ, and congenitally athymic nude mice to Brucella abortus cells. The data for these experiments are presented in Tables I through IV and Figure 1. All individual results may be found in the Appendix to Tables and Figures.

Determination of Optimal Dose of B. abortus Bacterin

In the literature concerning Brucella abortus, various doses of cells have been used for immunizations. Whether or not these doses elicited optimal humoral responses in mice was not indicated (22, 69, 100). Therefore, four-month-old male and female Balb/c mice were immunized with various doses of BA. They were bled on day 7 (1^0 = primary response) and on day 15 (R^0 = residual primary response); reimmunization on day 16 with a dose identical to that received on day 0 was followed on day 23 (2^0 = secondary response) by bleeding. Total serum antibody titers as well as 2-ME-R titers were determined.

Results of this experiment are shown in Table I. Geometric mean titers were essentially identical at all three bleeding times for mice receiving either 7.5×10^7 or 2.5×10^8 organisms for both first and second injections. Titers appeared somewhat lower in those animals receiving 7.5×10^8 organisms. All animals

TABLE I

Primary and Secondary Serum Antibody Responses of
Balb/c Mice to Various i.v. Doses of BA Cells

<u>Dose</u> ^a	<u>Serum Antibody Titers</u> ^b					
	<u>1^o Response</u>		<u>R^o Response</u>		<u>2^o Response</u>	
	(Day 7)		(Day 15)		(Day 23)	
	<u>Total</u>	<u>2-ME-R</u>	<u>Total</u>	<u>2-ME-R</u>	<u>Total</u>	<u>2-ME-R</u>
7.5 X 10 ⁷	95	< 10	80	< 10	538	134
2.5 X 10 ⁸	95	< 10	80	< 10	538	113
7.5 X 10 ⁸	48	< 10	80	< 10	640	80
2.5 X 10 ⁹	All mice died.					

^a The dose given in column was used for both first (day 0) and second (day 16) immunizations.

^b Titers represent the geometric mean of the individual titers of four mice.

receiving 2.5×10^9 cells died within four days following the first injection. Possibly this can be attributed to toxicity of the endotoxin present in the cell walls; Leong et al. (49) have demonstrated that BA-LPS extracted by the hot phenol-water method (76) is at least as toxic for mice as an equal quantity of E. coli LPS extracted by the Westphal procedure (106). For future experiments, then, an immunizing dose of 2.5×10^8 organisms was selected; this dose eliminated concern over mortality among experimental animals while eliciting responses similar to those using 7.5×10^8 BA cells. A dose of 2.5×10^8 cells correlates closely with that used by other investigators (37, 66, 96, 100).

Kinetics of the Balb/c Response to B. abortus Cells

Previous investigators (22, 37) have routinely bled BA-immunized mice at 7 day intervals; however, there is a scarcity of published data which supports or disputes this practice. Therefore, eight-to-nine-week-old Balb/c male and female mice were immunized with 2.5×10^8 BA cells, and groups of four mice were bled and sacrificed on days 2, 4, 6, 8, 10, 12, and 14 following immunization. Because limited volumes of sera were available, total antibody titers were determined using the microtiter technique, which has been utilized by other investigators (22, 30, 101). The data, presented in Table II, indicate that in Balb/c

TABLE II

Serum Antibody Titers to BA Cells at Short
Intervals Following Immunization

<u>Day of Immunization</u> ^a	<u>Total Serum Antibody Titers</u> ^b (Day 0)
-14	761
-12	538
-10	320
- 8	269
- 6	226
- 4	48
- 2	< 2
---	< 2

^aMice were immunized on various days prior to bleeding on day 0. A dose of 2.5×10^6 BA cells in 0.25 ml PBS was used for immunization.

^bTiters were determined using the microtiter assay; the given titer represents the geometric mean for four mice.

mice, the total antibody titer increases steadily throughout this 0-14 day period; a peak response followed by subsidence of the antibody titer does not occur. Limited availability of animals precluded repetition of this study at bacterin doses other than 2.5×10^8 BA cells.

This pattern of steadily increasing antibody titer becomes more apparent in the data which follows. However, it is not evident in Table I. A possible explanation for this is the difference in the ages of the mice used in the experiments. The data shown in Table I was collected from animals four months old. For all other experiments investigating the humoral response to BA, the animals were six-to-nine-weeks-old.

Responses of Balb/c, C3H/HeJ, and Nude Mice to High, Intermediate, and Low Doses of BA

Tingle and Shuster (101) investigated the response of normal CBA/J and of ATxBM CBA/J mice to BA over an eight $\log_{10}$ dose range. I was interested in determining if the response of nude mice would be similar to the responses of the ATxBM animals. As control groups, normal Balb/c and C3H/HeJ mice were included in the experiment.

Three doses of BA were used; 7.5×10^8 , 7.5×10^6 , and 2.5×10^4 cells were given i.v. on day 0 in a volume of 0.25 ml

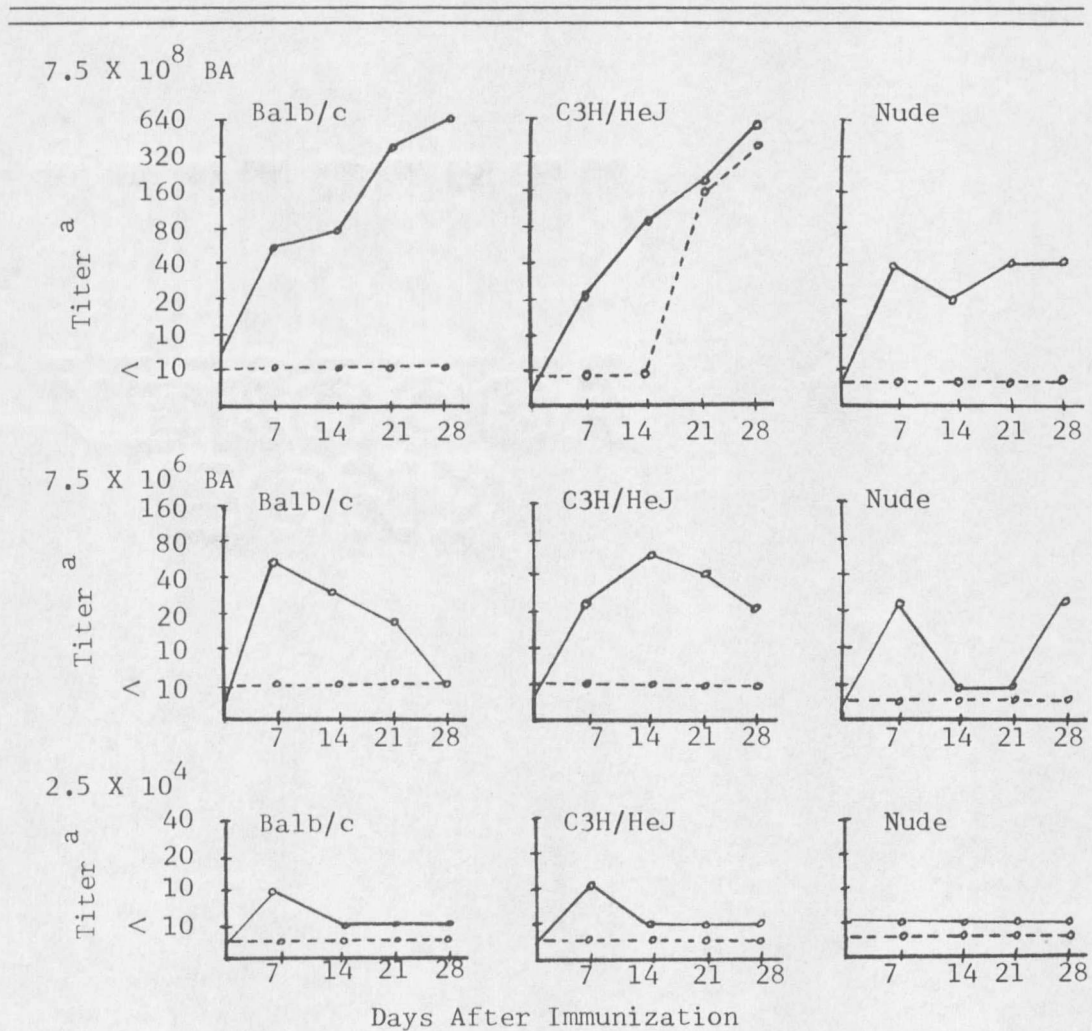
PBS. Following this single administration of antigen, mice were bled on days 7, 14, 21, and 28, and total antibody and 2-ME-R antibody titers were determined. The relative abilities of these mice to produce a humoral response to a single antigenic stimulation over a wide dose range are shown in Figure 1.

The most striking difference is that which appears in response to the high dose of 7.5×10^8 BA. Both Balb/c and C3H/HeJ animals respond to the single dose of immunogen with antibody synthesis which steadily increases to a titer of approximately 500. Both groups show a significant synthesis of 2-ME-R antibody, which first appears following day 14 and day 21 in C3H/HeJ and Balb/c mice, respectively. This pattern is conspicuously absent from the nude response. By day 7 a titer of 40 is reached, and the antibody titer plateaus from this point through the remaining 21 days of the experiment. The total antibody response appears to be IgM, as determined by treatment of sera with 2-ME; at no point is 2-ME-R antibody detectable.

In mice receiving 7.5×10^6 BA, a different trend is seen. Balb/c mice produce antibody which peaks at day 7, and decreases slowly over the 8-28 day period, until on day 28 no residual antibody can be detected. The C3H/HeJ response peaks on day 14, and subsides to one-half by day 28. In neither group is a 2-ME-R component detectable. The response of the nude group shows a

FIGURE 1

Dose-Response of Balb/c, C3H/HeJ, and Nude Mice
Receiving a Single Injection of Killed BA Cells



^a Total antibody titers (—) represent the geometric mean of the individual titers of four mice. 2-ME-R antibody titers (---) represent the titer of a pool of sera of the mice in each group.

cyclic phenomenon: the antibody titer peaks at day 7, declines below the sensitivity of the assay on days 14 and 21, and reappears again on day 28. A 2-ME-R response by nude mice is not detected at any point.

At a low dose of 2.5×10^4 BA, Balb/c and C3H/HeJ mice both produce antibody in quantities sufficient to be detected at day 7. The antibody appears to be totally 2-ME-sensitive. By day 14, however, the responses have subsided, and on days 14, 21, and 28, no antibody is detectable. No response by nude mice is found between days 7 and 28.

Despite the strain differences, these observations illustrate trends similar to those reported by Tingle and Shuster (101). It is apparent that the responses by normal and nude mice to BA differ both in the timing and magnitude of the antibody response as well as in the class of antibody synthesized.

Primary and Secondary Responses of Balb/c and Nude Mice to BA

The responses of Balb/c and nude mice were determined following primary and secondary immunizations with 2.5×10^8 BA. Ten six-week-old Balb/c and ten six-week-old nude mice were immunized on day 0, then bled on days 7 and 16. On day 17, representative mice from each group were reimmunized, and on day 23 all were bled. Total serum antibody titers and 2-ME-R titers are shown in Table III.

TABLE III
Primary and Secondary Responses of Balb/c
and Nude Mice to Killed BA Cells

	<u>Serum Antibody Titers</u> ^a						
	<u>Day 7</u>		<u>Day 16</u>		<u>Second Injection</u>	<u>Day 23</u>	
	<u>Total</u>	<u>2-ME-R</u>	<u>Total</u>	<u>2-ME-R</u>		<u>Total</u>	<u>2-ME-R</u>
Balb/c ^b	26	< 10	269	< 10	yes (5) ^c	>5120	1940
					no (3)	806	403
Nude	49	< 10	37	< 10	yes (6)	905	61
					no (4)	48	< 10

^a Titers represent the geometric mean of the individual titers of each group of mice.

^b A dose of 2.5×10^8 BA cells in 0.25 ml PBS was administered i.v. for all injections.

^c Number represents the number of mice per group; two Balb/c mice died following day 16 bleeding.

Several trends are apparent. As indicated in the previous experiment, the antibody response of Balb/c mice to BA at day 7 is quite small, but by day 16 has increased significantly. All antibody detected within this period is sensitive to treatment with 2-ME, indicating that only IgM has been synthesized in detectable amounts. By day 23, however, in mice not receiving a second injection, the antibody titer is much higher than that seen on day 16, and a considerable portion of this response is resistant to 2-ME reduction. A second immunization on day 17 results in a very high antibody titer, with a substantial 2-ME-R (IgG) component.

The response of nude mice to BA is quite different from that seen in normal Balb/c mice. On day 7 the total serum antibody response by nude mice is equal to or slightly greater than that of the Balb/c animals. Further, no increase nor decrease in this IgM titer occurs between days 7 and 23. A 2-ME-R titer is not detectable within this period. These results are in conflict with those reported by Crewther and Warner (22), who found significant 2-ME-R titers by day 16 in nude mice.

If the nude mice are reimmunized on day 17, heightened antibody synthesis occurs. However, the peak nude secondary response measures much less than the normal Balb/c response. Following a second injection, the nudes do synthesize a small but definitely

present 2-ME-R antibody, which is very small by comparison with the Balb/c 2-ME-R antibody titer.

Thus it would appear that in Balb/c and nude mice receiving 2.5×10^8 BA, the antibody responses are equivalent only at day 7 following immunization, and are widely divergent at days 16 and 23. These results substantiate and extend those reported in Figure 1.

Primary and Secondary Responses of C3H/HeJ Mice to BA

Spellman (89) demonstrated that C3H/HeJ mice do make a primary response to E. coli 0113 bacterin comparable to that made by normal Balb/c mice. In vivo, however, no secondary response could be elicited in the C3H/HeJ animals; rather, a second injection of E. coli bacterin elicited a second primary response; i.e., a response similar in magnitude and antibody class to a response following initial exposure. Therefore, the ability of C3H/HeJ mice to make both a primary and a secondary response to BA bacterin was investigated.

A dose of 2.5×10^8 BA cells was used for all immunizations. Sera for determination of the primary response was obtained on day 7 following immunization; residual primary and secondary response sera samples were obtained on day 23. All samples were assayed simultaneously using the tube agglutination test. Results

appear in Table IV. The primary, residual primary, and secondary responses of Balb/c and C3H/HeJ animals appear similar. Clearly the C3H/HeJ animals do make a secondary response when reimmunized with BA cells.

In summary, these experiments investigating the antibody responses to Brucella abortus 0119-3 bacterin by Balb/c and nude mice indicate the following: a) On day 7, normal Balb/c and nude responses to 2.5×10^8 or 7.5×10^8 BA are similar. The responses are widely divergent following this. b) The dose of BA determines the amount and class of antibody synthesized. c) Normal Balb/c mice given a high dose of BA respond during the first 28 days following immunization with a steadily increasing antibody titer. At this dose 2-ME-sensitive (IgM) antibody is first synthesized, with 2-ME-R (IgG) antibody appearing between days 16 and 23. d) Following a high dose of antigen, the nude response peaks prior to day 14 and plateaus through day 28. No 2-ME-R antibody is apparent. e) Responses by nude and normal mice to similar doses of BA differ in the timing and magnitude of the response as well as in the class of antibody synthesized. f) 2.5×10^4 BA cells approaches the minimal dose to which normal Balb/c animals will respond. Nude mice do not respond detectable to this dose. g) Nude mice do make a secondary response to 2.5×10^8 BA. This response consists of both IgM and IgG, but is markedly deficient

TABLE IV
Primary and Secondary Responses of Balb/c
and C3H/HeJ Mice to Killed BA Cells

	<u>Serum Antibody Titers</u> ^a				
	<u>Day 7 Response</u>		<u>Second Injection</u>	<u>Day 23 Response</u>	
	<u>Total</u>	<u>2-ME-R</u>		<u>Total</u>	<u>2-ME-R</u>
Balb/c ^b	25	< 10	yes (4) ^c	2560	640
			no (4)	134	20
C3H/HeJ	25	< 10	yes (4)	2560	640
			no (4)	160	40

^a Total titers represent the geometric mean of the individual titers within each group. 2-ME-R titers were determined on a pool of sera from the mice within each group.

^b A dose of 2.5×10^8 BA cells in 0.25 ml PBS was administered i.v. for all injections.

^c (Number) represents the number of mice per group.

when compared to the secondary response by normal Balb/c animals.

Those experiments involving C3H/HeJ mice can be briefly summarized: a) At all doses of BA cells examined, the C3H/HeJ primary response is similar to that of Balb/c mice, both in the magnitude of the antibody response and in the class of antibody synthesized. b) C3H/HeJ mice make a secondary response to Brucella abortus bacterin; this response is virtually identical to that made by Balb/c mice.

Responses of Balb/c, C3H/HeJ, and Nude Mice to BA-LPS

Several experiments were conducted to determine the antibody responses of Balb/c, C3H/HeJ, and nude mice to Brucella abortus 0119-3 lipopolysaccharide. The results of these experiments are shown in Tables V through VII and Figure 2. The data for individual animals may be found in the Appendix to Tables and Figures.

Determination of Optimal Dose of BA-LPS

Leong et al. (49) have demonstrated that their phenol phase preparation of BA-LPS was antigenic in rabbits, but no data were available regarding its immunogenicity in mice. Therefore, Balb/c mice received a single i.v. injection of phenol phase-extracted BA-LPS 0119-3 in 0.25 ml PBS. Groups were composed of four mice; each group received 100, 50, 10, or 1 μ g BA-LPS. Animals were bled on days 7 and 16, and total and 2-ME-R antibody titers were determined by agglutination assays using BA 0119-3 cells as indicator antigen. This titrating procedure has been used previously by Leong et al. (49). On day 17 all mice were reimmunized i.v. with 1 μ g BA-LPS in 0.25 ml PBS. This dose was selected because on day 7 following primary immunization, it had elicited a suboptimal antibody response. On day 23 the animals were bled, and secondary response titers were determined.

Results of this experiment are shown in Table V. Peak day 7

TABLE V
Primary and Secondary Responses of Balb/c
Mice to Various Doses of BA-LPS

<u>Dose</u> ^b	<u>Serum Antibody Titers</u> ^a					
	<u>1^o Response</u>		<u>R^o Response</u>		<u>2^o Response</u> ^c	
	(Day 7)		(Day 1)		(Day 23)	
	<u>Total</u>	<u>2-ME-R</u>	<u>Total</u>	<u>2-ME-R</u>	<u>Total</u>	<u>2-ME-R</u>
100 µg	24	< 10	28	< 10	269	113
50 µg	24	< 10	14	< 10	269	160
10 µg	40	< 10	< 10	< 10	269	113
1 µg	20	< 10	12	< 10	269	135

^a Titers represent the geometric mean of the individual titers of four mice.

^b Primary immunizing dose of BA-LPS was administered in 0.25 ml PBS via the lateral tail vein.

^c On day 17, all mice received a second injection of 1 µg BA-LPS in 0.25 ml PBS.

responses occurred in animals given 10 μ g BA-LPS, a peak which only slightly exceeds the day 7 titers reached in mice receiving 100, 50, or 1 μ g BA-LPS. By day 16, the responses to the three lower doses were less than those seen on day 7; in mice receiving 100 μ g BA-LPS, the day 16 titer appears comparable to the day 7 titers. From these data an optimal dose of 10 μ g BA-LPS was chosen for eliciting a primary response.

A second immunization on day 17 results in secondary titers which are very similar in the four groups of mice. Neither total nor 2-ME-R antibody titers reflect the differences in the quantities of BA-LPS administered as the priming dose on day 0.

Kinetics of the Primary Antibody Response to BA-LPS

Two experiments were performed to examine the kinetics of the primary antibody response to BA-LPS. The first experiment, which examined the antibody response at short intervals over the period day 0 to day 23, was designed to answer two questions. a) Because both the magnitude and the duration of the response to E. coli endotoxin differ between normal and C3H/HeJ mice (87), it was of interest to determine if a similar discrepancy might appear in this system. b) Cyclic antibody production is a phenomenon associated with the response to some bacterial lipopolysaccharides (63). Whether or not BA-LPS also elicits a cyclic response remains unanswered.

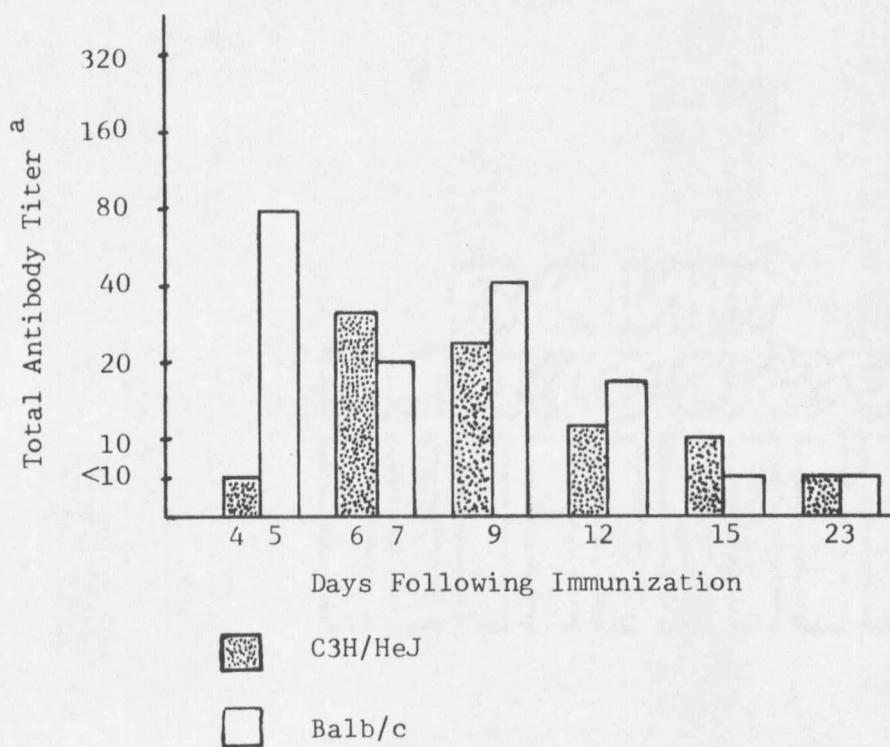
On day 0, mice received 10 μ g BA-LPS i.v., in a volume of 0.25 ml PBS. Slightly different intervals were initially selected for determination of antibody titers of the two strains because it has been shown that the C3H/HeJ response to E. coli LPS peaks and subsides more slowly than does the Balb/c response (87). Therefore, groups of four C3H/HeJ animals were bled on days 4, 6, 9, 12, 15, and 23, while Balb/c mice were bled on days 5, 7, 9, 12, 15, and 23.

The results of this experiment are shown in Figure 2. By day 5 the Balb/c response to BA-LPS reaches a high antibody titer which subsides (day 7), rises again (day 9), and then declines during days 12-23. Whether the slight rise on day 9 truly reflects a biological phenomenon of cyclic antibody production is doubtful, because a titer determined using the tube agglutination test is usually considered to be within one dilution of being precise.

The C3H/HeJ response differs from this pattern; by day 4 no response is detectable, and the peak response at day 6 is lower than the Balb/c peak on day 5. Data presented in Figure 2 do not address the possibility that the C3H/HeJ day 5 response may be comparable to that by Balb/c. However, Table VI contains data for the day 5 anti-Brucella antibody titer of both C3H/HeJ and Balb/c mice, and the day 5 Balb/c response is shown to be higher than that of C3H/HeJ animals. Following day 6, the C3H/HeJ response diminishes slowly, until at day 23 the residual primary response is comparable to that

FIGURE 2

Serum Antibody Responses of Balb/c and C3H/HeJ Mice on
Various Days After Immunization with B. abortus LPS



^a The titer shown is the geometric mean of the individual titers of four mice. 2-ME-R antibody was assessed for each group; throughout the experiment no 2-ME-R antibody was detected using the tube agglutination assay.

of the Balb/c mice. Cyclic antibody production is not readily apparent from these data. However, the possibility that it does occur between days 9 and 23 and should not be ruled out; a slightly altered bleeding schedule may have made the answer more apparent.

The second kinetics experiment conducted was much broader in scope. The antibody titers were determined over a long period of time following a single i.v. injection of BA-LPS. In addition to Balb/c and C3H/HeJ mice, nude mice were included in this experiment in the hope of determining whether or not the response to BA-LPS, like that to E. coli LPS (3, 56, 65, 78), could be considered to be independent of T-cell cooperation.

Mice were immunized i.v. on day 0, receiving BA-LPS injections of either 50 μg (high dose), 10 or 1 μg (intermediate doses), and 0.01 μg (low dose), solubilized in 0.25 ml PBS. Due to limited availability of nude mice, only Balb/c and C3H/HeJ mice were immunized with a dose of 10 μg BA-LPS. Animals were bled on days 5, 11, 17, and 30 following administration of the antigen. The data from this experiment appear in Table VI. At doses of 50 μg , 10 μg , and 1 μg BA-LPS, the Balb/c responses appear to be slightly but consistently higher than those of either C3H/HeJ or nude mice. In none of these three groups is 2-ME-R antibody demonstrable between days 0 and 17. A hint of cycling antibody is present: In all

TABLE VI

Serum Antibody Responses of Balb/c, C3H/HeJ, and Nude Mice
to Various Doses of B. abortus 0119-3 LPS

<u>Dose</u>	<u>Animals</u>	<u>Serum Antibody Titers</u> ^a		
		<u>Day 5</u>	<u>Day 11</u>	<u>Day 17</u>
50 μ g	Balb/c	48	28	40
	C3H/HeJ	17	12	34
	Nude	34	10	14
10 μ g	Balb/c	57	34	40
	C3H/HeJ	28	12	34
1 μ g	Balb/c	25	20	28
	C3H/HeJ	20	< 10	10
	Nude	24	14	10
10 ⁻² μ g	Balb/c	< 10	< 10	< 10
	C3H/HeJ	< 10	< 10	< 10
	Nude	< 10	< 10	< 10

^a Titers represent the geometric mean of the total serum antibody titers of four mice. 2-ME-R titers were determined on a pool of the sera of the mice in each group; all 2-ME-R titers were less than 10 at the three assay periods.

three groups the day 11 titers are slightly lower than those recorded for either day 5 or day 17. The differences in geometric mean titers is not large, but the trend becomes more apparent when individual titers are examined. Due to animal death in some groups, day 30 titers are not presented in Table VI. However, the serum antibody titers of surviving animals were determined. Day 30 titers, which are noted in the Appendix to Tables and Figures, were diminished from day 17, and no 2-ME-R antibody was detected.

Of the twelve mice immunized with 0.01 μ g BA-LPS, anti-Brucella antibody was detected in the serum of only one mouse (a nude). This serum was agglutinin-positive at a dilution of 1:20, and the antibody proved to be 2-ME-sensitive. Therefore, little difference in the primary responses of Balb/c, C3H/HeJ, and nude mice over a wide range of doses of BA-LPS could be detected. Based on these data, it would appear that the C3H/HeJ response to BA-LPS is similar to that of Balb/c mice; this observation is in contrast to the aberrant C3H/HeJ response to E. coli LPS. Furthermore, over the dose range examined, BA-LPS appears to be a T-independent antigen, because no significant difference can be demonstrated between the responses of the normal Balb/c and C3H/HeJ mice, and the functionally athymic nude mice.

Secondary Responses of Balb/c and C3H/HeJ Mice to BA-LPS

The secondary responses of Balb/c and C3H/HeJ mice to BA-LPS were examined; limited availability of nude mice prevented their inclusion in the experiment. Because previous experiments (89) had shown that C3H/HeJ mice fail to mount a secondary response to E. coli LPS under any conditions of immunization tested, it seemed worthwhile to investigate the ability of the C3H/HeJ mice to make a secondary response to BA-LPS. A dose of 10 µg BA-LPS administered i.v. was used for all immunizations. Ten Balb/c and ten C3H/HeJ mice were immunized on day 0, and all were bled on day 5. On day 18, five Balb/c and five C3H/HeJ mice were reimmunized; 5 days later, on day 23, all twenty mice were bled. Total antibody and 2-ME-R antibody titers were determined. Data for this experiment, presented in Table VII, show that the primary, residual primary, and secondary responses of Balb/c and C3H/HeJ mice are essentially identical. Mice of both strains clearly make a secondary response to BA-LPS. This is in direct opposition to the studies investigating the response of the C3H/HeJ mouse to E. coli LPS.

These experiments investigating the antibody response to Brucella abortus 0119-3 lipopolysaccharide by Balb/c and nude mice may be summarized as follows: a) Antibody responses to BA-LPS can be detected in the sera of both nude and normal animals within

TABLE VII

Primary and Secondary Responses of Balb/c and C3H/HeJ Mice to BA-LPS

	<u>Serum Antibody Titers</u> ^a					
	<u>1^o Response</u> ^b		<u>R^o Response</u>		<u>2^o Response</u>	
	(Day 5)		(Day 23)		(Day 23)	
	<u>Total</u>	<u>2-ME-R</u>	<u>Total</u>	<u>2-ME-R</u>	<u>Total</u>	<u>2-ME-R</u>
Balb/c	28	< 10	13	< 10	278	160
C3H/HeJ	25	< 10	15	< 10	278	160

^a Total antibody titers represent the geometric mean of the individual titers of 10 mice for the 1^o response and 5 mice for both the R^o and the 2^o responses. 2-ME-R titers represent the titer of a pool of sera from these mice.

^b All mice were immunized on day 0 with 10 µg BA-LPS given i.v. in a volume of 0.25 ml PBS. All mice were bled on day 5. On day 18 5 mice in each group were reimmunized with 10 µg BA-LPS. All mice were bled on day 23.
 1^o = day 5 response; R^o = day 23 response following a single day 0 injection;
 2^o = day 23 response following immunizations on both days 0 and 18.

5 days following immunization with 100, 50, 10, or 1 μ g BA-LPS.

b) The total primary response to BA-LPS appears to be IgM; 2-ME-R antibody (IgG) does not become detectable during the first 30 days following a primary immunization. c) Little difference is apparent in the timing or magnitude of the antibody response or in the class of antibody synthesized by Balb/c and nude mice following a single injection of BA-LPS. This suggests the response to BA-LPS may be thymus-independent. d) A second injection of BA-LPS within 16 days following primary immunization stimulates an increased antibody response, a large component of which is 2-ME-R. e) This secondary response antibody titer appears comparable in Balb/c animals receiving various priming doses of BA-LPS; neither the total nor the 2-ME-R antibody titer reflects the quantity administered in the first injection.

The experiments involving C3H/HeJ animals allow the following conclusions: a) C3H/HeJ mice make a primary response to BA-LPS over a dose range of 50 μ g to 1 μ g. This response is similar in magnitude to that made by Balb/c mice. b) The C3H/HeJ primary response, like that of Balb/c mice, appears to consist entirely of IgM; no 2-ME-R antibody is demonstrable. c) C3H/HeJ animals make a secondary response to BA-LPS which is comparable to a Balb/c secondary response, both in the magnitude and the class of antibody synthesized.

Mitogenic Responses of Spleen and Thymus Cell Cultures to BA and BA-LPS

Studies with mitogens were carried out on cultures of spleen cells derived from Balb/c, nude, and C3H/HeJ mice. Two control mitogens were included in all assays; Con A, a known stimulator of DNA synthesis in T cells of spleen and thymus (38, 39), and E. coli 0113 LPS, a powerful mitogen for B cells, including those B cells from spleens of nude mice (4). In all experiments with spleen cells (see Figure 3a) or thymus cells (see Figure 4a) from Balb/c or C3H/HeJ mice, the Con A responses were within normal stimulation indices reported by other investigators (4). The nude spleen cell cultures showed no stimulation by Con A. The mitogenic responses to E. coli LPS (see Figures 3b and 4b) were also normal in the Balb/c and nude cultures. It has previously been reported that this LPS preparation is weakly or non-mitogenic for C3H/HeJ spleen cells (88), and this was also confirmed.

The primary emphasis of the mitogenic studies was a) to determine if B. abortus 0119-3 cells and B. abortus 0119-3 lipopolysaccharide were also mitogenic, and b) to determine if the aberrant C3H/HeJ mitogenic response to E. coli LPS would also limit their response to BA-LPS. The failure of C3H/HeJ spleen cells to respond mitogenically to E. coli LPS has been attributed to a

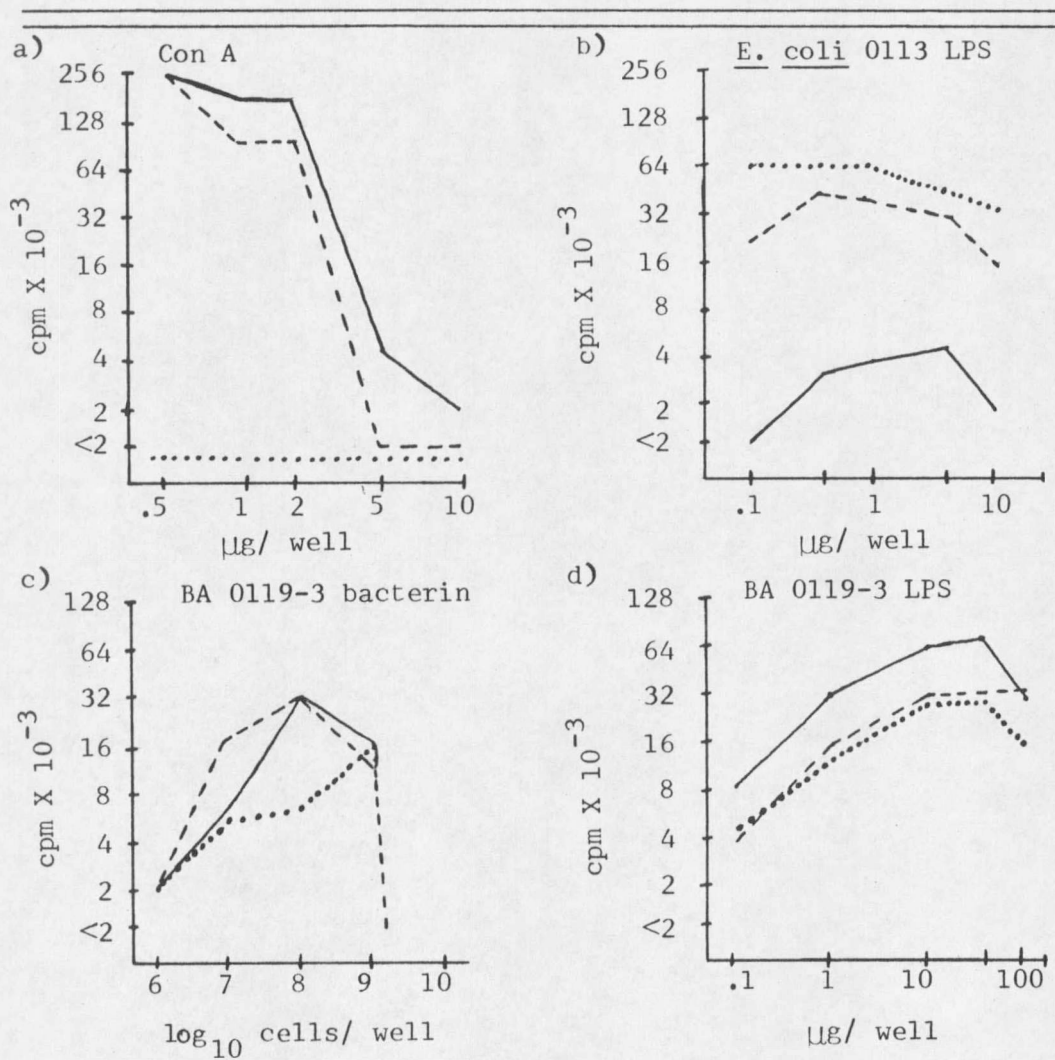
genetic defect which does not allow recognition of the Lipid A moiety of the endotoxin. B. abortus and E. coli lipopolysaccharides share the same Lipid A structure.

A preliminary assay (data not shown) indicated that both BA and BA-LPS were indeed mitogenic for Balb/c spleen cell cultures. However, it was unknown whether or not C3H/HeJ spleen cell cultures would respond to the endotoxin. Shown in Figure 3c and d are the data of a typical experiment investigating the effect of BA and BA-LPS upon spleen cells of Balb/c, nude, and C3H/HeJ mice.

BA cells were tested at final concentrations of 1×10^6 , 1×10^7 , 1×10^8 , 1×10^9 , and 2×10^9 cells per microtiter well (5×10^7 , 5×10^8 , 5×10^9 , 5×10^{10} , and 1×10^{11} cells per ml of mitogen preparation). The final concentrations of BA-LPS were 0.1, 1.0, 10, 50, and 100 μg per microtiter well (5.0, 50, 500, 2500, and 5000 μg per ml of mitogen preparation). Peak responses to BA occurred at 1×10^8 cells per well, and at this dose the Balb/c response was quite similar to that of the C3H/HeJ cultures. The nude response was maximal at 1×10^9 BA cells per well, but the response is considerably less than the peak for the Balb/c and the C3H/HeJ cultures. The peak response to BA-LPS by Balb/c, C3H/HeJ, and nude cultures occurs at a dose of 50 μg per well. At all doses of BA-LPS, the C3H/HeJ response is consistently higher than that of either Balb/c or nude cultures; all three curves, however,

FIGURE 3

Mitogenic Responses of Balb/c, C3H/HeJ, and Nude Spleen Cells
to Con A, *E. coli* LPS, BA, and BA-LPS



CPM ^3H Scintillation (Background Subtracted)

Balb/c ----	Background	2,000
C3H/HeJ ———	Background	4,085
Nude	Background	2,604

are very nearly superimposable, thus crediting the idea that BA-LPS is a mitogen only for B cells.

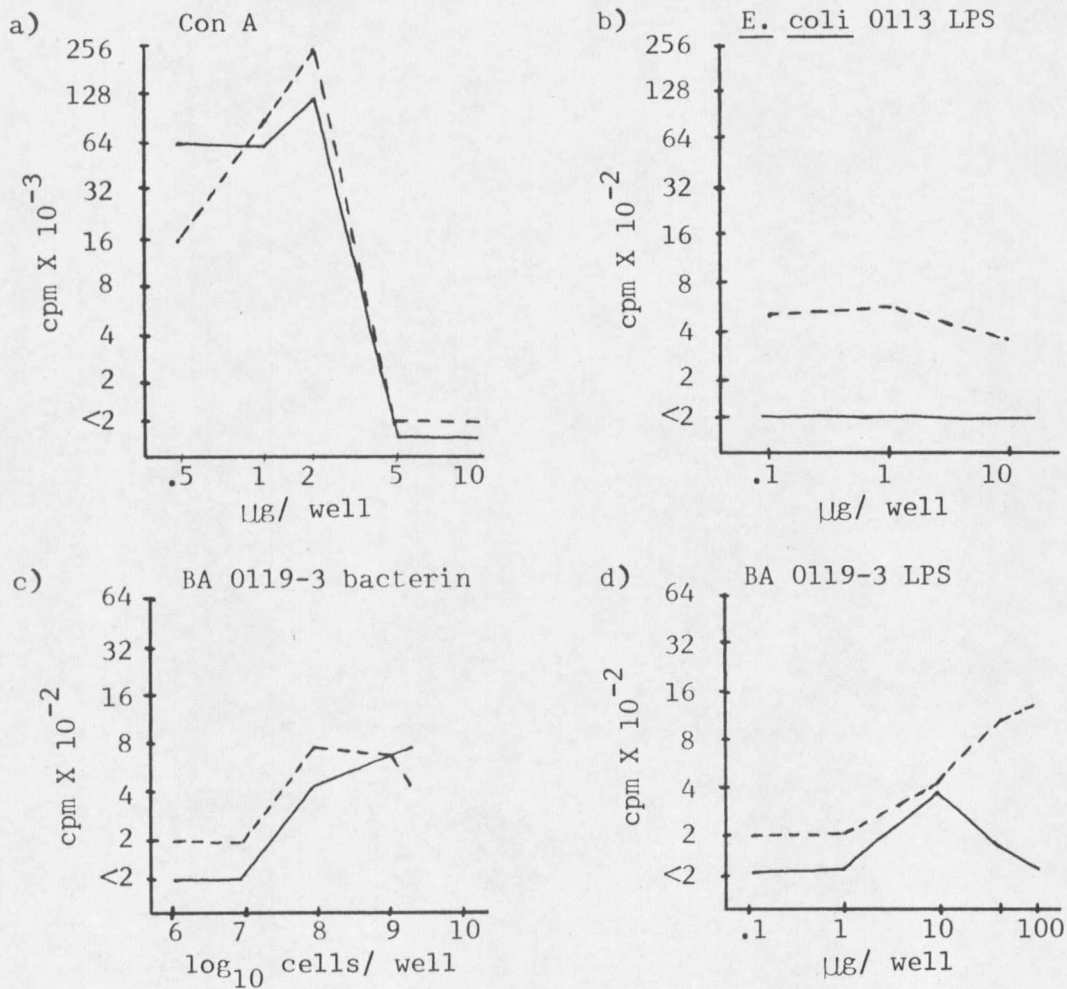
Kruger and Gershon (46) have reported that B. abortus is a stimulator of DNA synthesis in thymocytes in vivo. Therefore, mitogen assays were also performed on cultures of thymus cells derived from Balb/c and C3H/HeJ mice, to determine if the in vivo observations were reflected in an in vitro system. Data are shown in Figure 4.

BA and BA-LPS were used at the same doses indicated for the spleen cell assays. Mitogenic responses to both BA and BA-LPS are very low, the maximal stimulation being four-fold over background levels. The slight stimulation is probably attributable to the presence of B cells trafficking through the thymus or to contamination of the thymus cell preparation with parathymic lymph node cells, rather than to a direct mitogenic effect of BA or BA-LPS on thymocytes.

These mitogenic assays determined that B. abortus 0119-3 and B. abortus 0119-3 LPS are indeed mitogenic for spleen cells of normal Balb/c animals and elicit comparable responses from the C3H/HeJ strain, a low responder to the lipopolysaccharide of E. coli.

FIGURE 4

Mitogenic Responses of Balb/c and C3H/HeJ Thymus Cells
to Con A, *E. coli* LPS, BA, and BA-LPS



CPM ^3H Scintillation (Background Subtracted)

Balb/c	-----	Background	480
C3H/HeJ	—————	Background	374

DISCUSSION

The experiments presented in this paper examined the antigenicity and mitogenicity of Brucella abortus 0119-3 bacterin and Brucella abortus 0119-3 purified endotoxin in Balb/c, C3H/HeJ, and congenitally athymic "nude" mice. The results can be summarized as follows:

- 1) A primary immunization of the mice with increasing doses of BA reveals that the antibody titers and the class of antibody synthesized are dose-dependent in Balb/c and C3H/HeJ mice. Specifically, I have found that "low" (2.5×10^4 BA) and "intermediate" (7.5×10^6 BA) doses elicit only IgM antibodies in Balb/c and C3H/HeJ animals. The low dose response peaks on day 7 and cannot be detected by day 14. The intermediate dose also elicits a peak response by day 7-14, but diminishes more slowly and is not detectable by day 28. High doses of BA cells (7.5×10^8) in either Balb/c or C3H/HeJ mice yield a much different kinetic and antibody class profile. With this amount of antigen, the antibody response is evident by day 7 and steadily continues to increase at least to day 28. Further, this high BA dose is required to elicit the appearance of IgG antibody. The IgG component of the humoral response is evident by day 21 in C3H/HeJ and by day 28 in Balb/c mice. In both strains, IgG constitutes a major portion of the antibody

detected on day 28.

Nude mice do not respond to the low dose of BA. The response of nude mice to the intermediate dose of BA cells is curious in that the antibody titer peaks on day 7, decreases below detectable levels on days 14 and 21, and again increases on day 28. Injection of 7.5×10^8 BA cells into nude mice results in a peak antibody titer on day 7, and this level is maintained at least to day 28. The nude mice produce 2-ME-sensitive antibodies and never exhibit 2-ME-R antibodies. The cyclic and plateau responses by nude mice to the intermediate and high doses of BA, respectively, will be discussed again.

- 2) Secondary responses to BA by Balb/c, C3H/HeJ, and nude mice were also studied. The animals were primed on day 0 with 2.5×10^8 BA cells and boosted on day 17 with 2.5×10^8 BA. The humoral response was examined on days 7 and 23. I have found that all three mice strains were able to generate 2-ME-R antibodies, with some 2-ME-sensitive component still detectable. The Balb/c and C3H/HeJ mice responded identically, and both strains demonstrated 2-ME-R titers many fold greater than the 2-ME-R response of nude mice.

- 3) Primary antibody responses were determined in Balb/c, C3H/HeJ, and nude mice to immunizations with BA-LPS. Secondary antibody responses were determined in Balb/c and C3H/HeJ mice. Primary injections of the BA-LPS, 1-100 μg per mouse, indicated that the optimum immunogenic dosage range was quite broad. A single injection of 50, 10, or 1 μg BA-LPS elicits only a 2-ME-sensitive response which is evident by days 4 or 5, and persists to at least day 17 in C3H/HeJ mice and to day 30 in Balb/c and nude mice. All three mice strains appeared to yield approximately equal and parallel responses. This data on the primary antibody response strongly suggests that BA-LPS may be a thymus-independent antigen. 2-ME-R antibodies could be elicited to BA-LPS only when the animals had received two immunizations. A 2-ME-R component was found in Balb/c and C3H/HeJ mice which were primed on day 0 and boosted on day 18 with 10 μg of the endotoxin. Unfortunately, insufficient numbers of nude mice precluded an examination of their secondary responses to BA-LPS.
- 4) Lastly, mitogenic studies were performed with BA bacterin and BA-LPS. E. coli 0113 LPS and Con A were also employed as positive controls on the assays.
- a) Spleen cells: Con A responses were as expected. Both

the C3H/HeJ and Balb/c cells gave strong mitogenic responses to this mitogen, and, of course, the nude mice cells did not respond. E. coli 0113 LPS could elicit positive mitogenic responses in the Balb/c and nude cells. E. coli 0113 LPS did not stimulate the C3H/HeJ spleen cells. The aberrant C3H/HeJ response to endotoxin has been well characterized.

The mitogenicity of BA and BA-LPS was studied for two interrelated reasons. Since many investigators have concluded that a mitogenic event is a prerequisite for antibody synthesis, it was of interest to determine if these two antigenic materials were also mitogenic. Secondly, because the C3H/HeJ mouse strain is well known for its lack of responsiveness to many of the biological properties of LPS from E. coli or Salmonella spp., I wanted to determine if these two antigens, which elicited strong antibody responses in the C3H/HeJ mouse were also mitogenic for this particular strain. The results were clear-cut: both BA and BA-LPS were mitogenic for spleen cells from Balb/c, C3H/HeJ, and nude mice.

b) Thymus cells: The responses of thymocytes from Balb/c and C3H/HeJ mice to Con A, E. coli LPS, BA, and BA-LPS paralleled the responses seen in the spleen cell cultures, with the exception that only marginal stimulations were found with E. coli LPS, BA, and BA-LPS. Possible sources of B cell

contamination of the thymocyte preparations are discussed in subsequent paragraphs.

My studies indicate that BA and BA-LPS are antigenic and mitogenic substances capable of eliciting 2-ME-sensitive antibodies, and capable of subsequently eliciting 2-ME-R antibodies under appropriate immunization protocols. However, there do exist some reports which are at variance with my findings. Therefore, I will now discuss my findings in the light of these reports and point out differences in techniques or experimental protocols which may account for the conflicting reports on the BA antigens. Special attention will also be given to interpretation of the results of these mitogen studies.

BA as a Thymus-Independent Antigen

Several studies have been performed using killed Brucella abortus cells as an antigen. The data from these studies have not been clear-cut and leave some question as to whether or not BA is a thymus-independent antigen. There are basically two systems which have been used in the experiments involving BA:

- 1) cell transfer experiments investigating the ability of anti-theta treated immune spleen cell populations to transfer a secondary response to BA to sublethally irradiated recipients (66, 96, 100).

- 2) experiments comparing the response to BA of immunologically impaired animals with the responses of normal animals (22, 26, 37, 69, 82, 101).

The data accumulated using these two systems can be briefly summarized as follows. The cell transfer experiments tend to support the hypothesis that BA is a thymus-independent antigen. Treatment of immune spleen cell populations with anti-theta serum plus complement prior to transfer into irradiated recipients causes little or no diminution of the anti-BA titer when compared with the responses transferred by normal mouse serum-treated spleen cells.

The second type of experiments permit an opposing view. The primary (22, 37) and secondary (37) titers of nude mice are significantly depressed compared to those of normal animals, but can be partially restored if the animals are reconstituted with thymocytes prior to immunization. ATxBM animals also produce lower titers of antibody to BA than do normal mice (101). Differences are apparent in the magnitude of the antibody response and in the class of antibody synthesized.

The question of the thymus-dependence or independence of BA remains unanswered by these observations. The experiments involving T-lymphocyte deficient animals appear to indicate that thymic function is indeed necessary for a normal response to BA. Only

two experiments contradict this conclusion. Rouse and Warner (82) immunized chickens with 20×10^9 BA cells and found similar responses by normal chickens and those treated with anti-lymphocyte serum. Mosser et al. (69) studied the responses of normal and neonatally thymectomized irradiated mice given 5×10^9 killed BA, and found no significant difference between the anti-BA antibody responses of the two groups. Sinclair and Elliot (86) and Taylor and Wortis (99) have demonstrated that the need for T cell involvement may be partially overcome by a large enough antigen dose. The data from cell transfer experiments, however, speak against a T-cell requirement for a normal response to BA.

There exists a second explanation for the differences observed in the responses of normal and thymically-impaired animals. Cheers and Waller (15) recently published data suggesting that the macrophages of nude and ATxBM mice are more active phagocytically than those of normal mice; the authors suggest that this condition results from continual stimulation by the endotoxins of Gram-negative organisms. Whether or not this is the cause, or whether the condition is simply a compensatory mechanism developed in the absence of a fully functional immune system has not been determined.

Accelerated clearance of antigen by activated macrophages in nude mice equally well explains the difference in the antibody

responses of nude and normal mice. Through their reticuloendothelial system, the nudes may clear a larger portion of the Brucella bacterin more quickly, whereas in the normal animals this antigen may persist and be degraded slowly over a longer period of time. Slow degradation of the antigen would account for the steadily increasing titers seen in normal mice, while more rapid clearance could account for the plateau nude antibody responses. A similar picture of high and low responder strains appears in the work of Wiener and Bandieri (107) with keyhole limpet hemocyanin as antigen. These investigators demonstrated that peritoneal macrophages from low responder strains degrade the antigen more quickly than do the high responder macrophages. Rapid degradation of the antigen prevents continued stimulation of lymphocytes and thus results in lowered antibody titers.

My data indicate that the nude response to BA is severely restricted in comparison to the steadily increasing normal response, regardless of the dose of antigen used. Superficial examination of this data leads one to conclude that BA is a thymus-dependent antigen. However, BA may well be a thymus-independent antigen, as the cell transfer experiments suggest, RES clearance rate accounting for the apparent discrepancy between the responses of nude and normal animals. One valuable study which might answer

this question would be the monitoring of the clearance of radio-labelled BA cells by nude and normal mice. Without knowledge of clearance rates by these animals, it is impossible to determine which, if any, of the above hypotheses are correct.

2-ME-R Antibody Titers to BA

Other investigators have previously reported that mice receiving a single i.v. injection of BA shown significant 2-ME-resistant antibody titers by day 14 following the primary injection. Most of these determinations of 2-ME-R antibody were performed using the microtiter system, as described by Claflin et al. (18). For several reasons I elected to minimize the use of the microtiter system in my work.

- 1) The possibility of error is much greater in the microtiter system due to the small volumes involved.
- 2) Eight primary and secondary anti-Brucella serum samples were titrated using both the microtiter assay and the tube agglutination test. In the microtiter system, primary titers (both total and 2-ME-R) were consistently higher, while secondary total and 2-ME-R titers were consistently lower than in the agglutination test. As I was interested in observing secondary responses, I selected the tube agglutination technique, because it may be the more accurate and

sensitive assay.

That other investigators find a 2-ME-R antibody component by day 7 while I do not find this component may be due to the use of different techniques in detecting 2-ME-R antibody. That is, differences demonstrated by microtiter and tube agglutination assays may be due, at least in part, to the variations in the protocols involving the addition of the 2-ME to the serum samples. In the tube agglutination assay, serum is incubated with 2-ME for one hour prior to the addition of antigen. In the microtiter system, the antigen is suspended in PBS containing 2-ME. The 2-ME has no opportunity to reduce the antibody prior to addition of antigen, and formation of antigen-antibody complexes may be protective against antibody reduction by 2-ME. A second possibility exists. The antigen used in the microtiter assay is the Ring Test antigen, killed B. abortus 0119-3 coupled to a blue indicator dye. That the 2-ME also reduces this dye is apparent from the color change which occurs in a suspension of the Ring Test antigen prepared in PBS with 0.2 M 2-ME. Perhaps in the presence of the dye, the effective 2-ME concentration is lowered and some IgM escapes reduction.

I found an apparent lack of a 2-ME-R component in the anti-Brucella response, assayed by agglutination, prior to 14-21 days following primary antigen administration. This, however, does not

preclude the possibility that IgG synthesis has occurred prior to this time. Firstly, the sensitivity of the assay must be considered. Secondly, Adler (2), working in the anti-sheep red blood cell system, found a class of 7S lysins and agglutinins of γ_1 and γ_2 mobilities were indeed sensitive to 2-ME. These antibodies were encountered during the early phases of the 7S antibody formation to sheep red blood cells (days 5-10 following a primary immunization and day 3 following a secondary immunization). These 2-ME-sensitive 7S antibodies fully maintained their combining ability, although they did lose their agglutinating capacity. Therefore, some 2-ME-sensitive 7S anti-Brucella antibodies may be synthesized prior to the time at which 2-ME-R antibody is detected using the tube agglutination test.

Mitogenic Properties of BA and BA-LPS

The mitogen assays reported in the Results section clearly indicate that BA and BA-LPS are mitogenic for spleen cells from Balb/c and nude mice, and elicit comparable responses from mice of the C3H/HeJ strain, a low responder to the mitogenic effects of E. coli LPS. My data indicate little or no mitogenic effect upon thymocytes. However, the slight stimulation seen in thymus cell cultures may be due to a small number of B cells from parathymic lymph nodes inadvertently included in the thymus cell preparation.

The small stimulation may also be due to some recirculating B cells trafficking through the thymus. Whether or not BA and/or BA-LPS are mitogenic for thymocytes, as suggested by the in vivo data of Kruger and Gershon (46), cannot really be determined without demonstrated T cell homogeneity.

That the mitogenic effects of BA and BA-LPS are due to the same moiety cannot be stated with certainty, because moieties other than the endotoxin may be mitogenic. For example, Damais et al. (23) have demonstrated that the peptidoglycan of E. coli evokes blast transformation in B cells. It has further been reported that insoluble peptidoglycan preparations from Bacillus cereus (17) and B. megaterium (23), from Nocardia rubra and N. opaca (17), as well as a peptidoglycan-containing fraction of Listeria monocytogenes (20) all induce mitogenesis in mouse spleen cell cultures.

Nor can it be stated that the mitogenic stimulus provided by the BA-LPS lies in the Lipid A moiety which BA-LPS shares with E. coli LPS. The BA-LPS is much less highly purified than the E. coli LPS preparations; it contains significantly more protein and more lipid. The possibility cannot be excluded that the mitogenicity is due to some component other than the Lipid A.

In considering the fact that C3H/HeJ and Balb/c mice respond similarly to the immunogenic and mitogenic effects of both the BA

and the BA-LPS, one must take into account the recently published observations of Skidmore et al. (88). These authors examined the responsiveness of C3H/HeJ spleen cell cultures to mitogenic stimulation by E. coli LPS extracted by various methods. LPS using the TCA (14) or butanol (67) methods did elicit mitogenic responses in cultures of C3H/HeJ spleen cells; LPS extracted using the phenol-water method of McIntire et al. (57) or the phenol-chloroform-petroleum ether method of Galanos et al. (30) failed to elicit any response. LPS extracted by the phenol-water procedures of Westphal and Jann (105) or Morrison and Leive (67) were very weakly mitogenic for C3H/HeJ spleen cell cultures. Skidmore et al. contend that the structural integrity of the Lipid A moiety is critical in determining responsiveness or unresponsiveness in B cells of C3H/HeJ animals to lipopolysaccharide. However, more recent work by Morrison et al. (68) and by Sultzter and Goodman (94) presents evidence that these "positively mitogenic" lipopolysaccharide preparations are contaminated with a small polypeptide (molecular weight 10,000) which is, in itself, mitogenic for spleen cells of C3H/HeJ mice.

The B. abortus lipopolysaccharide used in the studies reported here was extracted using the Redfearn (76) and Leong (49) modifications of the Westphal hot phenol-water procedure. Although the modifications are slight, the BA and E. coli

endotoxins partition into the phenol and aqueous phases, respectively, due to their dissimilar biochemical compositions. Two possibilities emerge:

- 1) The BA-LPS is mitogenic for C3H/HeJ spleen cells due to the presence of some moiety other than Lipid A. A mitogenic polypeptide similar to that identified by Morrison et al. (68) and Sultzner and Goodman (94) in E. coli LPS preparations may be a contaminant of the BA-LPS preparation used.
- 2) If Lipid A is the mitogenic portion of the BA-LPS, it may be presented to the C3H/HeJ B cell in a form slightly different from the Lipid A of the Westphal-extracted E. coli LPS, due to the differences in the extraction procedures. In this form it may be capable of eliciting responses in spleen cell cultures of C3H/HeJ animals.

Several workers have speculated that Brucella cells and Brucella endotoxins are very different from members of the Enterobacteriaceae, in terms of the responses they evoke in experimental animals. The main conclusion expressed is that the "typical" response profile seen with enterobacterial cells and endotoxins is the result of previous exposure to these ubiquitous organisms; the response to BA and BA-LPS deviates significantly from this because the animals have had no previous natural

exposure to these antigens.

Leong et al. (49) reported that phenol-phase BA-LPS possesses, in common with the endotoxins of the Enterobacteriaceae, certain biological properties, including toxicity, lethality, and pyrogenicity. These investigators also performed hybridization experiments using the endotoxins extracted from Brucella abortus and from members of the Enterobacteriaceae. These experiments involved the admixture of various deoxycholate-generated fragments from the various endotoxins and detecting renatured hybrids by positive immunoprecipitin lines with monospecific antisera toward one of the endotoxins. The experiments demonstrated that hybrid formation did occur between BA-LPS and E. coli 0111 LPS and between BA-LPS and Salmonella enteritidis LPS. Although which moieties are identical was not determined, this has been interpreted to indicate some homology between BA-LPS and enterobacterial endotoxins.

The experiments reported here demonstrate the following differences in the response to BA and BA-LPS and the responses to the cells and endotoxins of the Enterobacteriaceae reported by other investigators.

E. coli cells and E. coli LPS have been shown to be thymus-independent antigens. Both primary and secondary responses to these antigens are characterized as IgM; an IgG response does not occur, except in hyper-immunized animals. Both the E. coli

bacterin and the purified LPS are mitogenic for B cells, but not for T cells. The C3H/HeJ strain of mice is refractory to many of the above mentioned properties. Their primary response to the LPS is aberrant, and they make a secondary response to neither the LPS nor the bacterin, in vivo. Furthermore, these mice are unresponsive to the mitogenic properties of the LPS, but do respond in mitogen assays to the bacterin at levels similar to Balb/c mice.

The response to BA and BA-LPS contrasts sharply with several of the above noted properties of E. coli bacterin and LPS. The class of antibody synthesized in response to immunization with bacterin is highly dependent on the dose of bacterin administered; high doses of antigen elicit a primary response consisting of both IgM and IgG. Secondary responses are characterized by a substantial 2-ME-R component, both in normal and nude mice. While the role of the T-lymphocyte in the response to BA has not been clearly defined, the data presented here suggest that BA-LPS may well be a thymus-independent antigen. Nude and normal mice respond similarly to a primary exposure to BA-LPS over a wide dose range. Primary immunization with BA-LPS does not trigger formation of IgG antibody, but a second exposure does elicit antibody of both IgM and the IgG classes. The primary and secondary responses to both BA and BA-LPS by C3H/HeJ animals are quite comparable to the Balb/c response in all aspects. Both BA bacterin

and purified BA-LPS are strongly mitogenic for spleen cells of Balb/c and nude mice, and are equally stimulatory for spleen cells of C3H/HeJ animals.

Thus it appears that while BA and BA-LPS do share some features with the Enterobacteriaceae, the immunologic responses which the Brucella bacterin and lipopolysaccharide elicit in animals are distinctly different from those elicited by E. coli or Salmonella spp.

SUMMARY

Many conflicting reports appear in the literature about the ability of the Brucella to elicit the biologic and immunologic responses evoked by the Enterobacteriaceae. Many of the earlier reports either confirmed or denied the cytotoxic, pyrogenic, and antigenic properties of killed Brucella cells. Failure to elicit the above listed responses by some investigators led to the conclusion that either there was something very different about the Brucellae, or there was something very different about the mouse response to these organisms. More recently, attention has focused on the requirement for T cells in the antibody response to Brucella cells: Proponents of Brucella abortus as a thymus-independent antigen or as a thymus-dependent antigen can be cited.

The data on the properties ascribed to the Brucella lipopolysaccharides are extremely difficult to interpret because different investigators have used various fractions from phenol-water purifications as the endotoxin preparation. In 1960 Redfearn correctly reported that the BA-LPS partitioned into the phenol phase of the Westphal extraction procedure. However, most investigators had examined (or still do examine) the properties of only the aqueous phase material because the endotoxins of the Enterobacteriaceae have long been known to partition into the aqueous phase. Consequently, much of the literature incorrectly

characterizes the BA-LPS as lacking immunogenicity, pyrogenicity, lethality, and toxicity. Studies which have utilized the phenol phase endotoxin have shown that indeed the BA-LPS does possess the properties of antigenicity, pyrogenicity, lethality, and toxicity.

The conflicting reports on the immunogenic properties of BA as well as the lack of information about the immunologic properties of the purified phenol phase endotoxin left these areas open for my investigation. This report examines several parameters of the antibody and mitogenic response to BA and BA-LPS. Balb/c and nude mice were used to investigate the role of T lymphocytes in the response to these antigens. C3H/HeJ mice, shown to have an aberrant response to the endotoxins of E. coli and Salmonella spp., were included in these studies for the purpose of further delineating differences or similarities in the responses elicited by Brucella abortus and those elicited by E. coli and Salmonella spp.

It was determined that the antibody response to BA in normal mice is highly dose-dependent; both the magnitude of the antibody titer and the class of antibody synthesized vary with the dose administered. Only a high (7.5×10^8) dose of BA cells can elicit a detectable 2-ME-R response following a single injection of antigen. The magnitude of this response increases steadily during days 0-28. In nude mice the response to the same high dose

deviates significantly from the normal. By day 7 maximal response is attained, and this antibody titer is maintained through day 28. At no point is 2-ME-R antibody detected. At lower doses of antigen only 2-ME-sensitive antibody is detected, and the response peaks early and declines by day 28 in both nude and normal animals. Balb/c, C3H/HeJ, and nude mice all make a secondary response to BA cells. A large portion of this response is 2-ME-resistant.

BA-LPS is immunogenic over a dose range of 1 μg to 100 μg . Balb/c, C3H/HeJ, and nude mice respond similarly to the high, intermediate, and low doses examined. Following a single administration of 50 μg or 1 μg BA-LPS, responses are evident by day 5 and remain above detectable levels until at least day 17. During this period, only 2-ME-sensitive antibody is detectable. None of the mice respond to a dose of 10^{-2} μg BA-LPS. These data suggest that BA-LPS may be a thymus-independent antigen. Balb/c and C3H/HeJ mice make strong secondary responses to BA-LPS. This antibody response consists of both 2-ME-sensitive and 2-ME-R antibody. Secondary responses by nude mice were not examined.

Assays to determine possible mitogenic activity of BA and/or BA-LPS were performed using Con A and E. coli LPS as control mitogens. Cultures of spleen cells from Balb/c, C3H/HeJ, and nude

mice responded strongly to both BA and BA-LPS. Slight stimulation of cultures of Balb/c and C3H/HeJ thymus cells is probably due to low numbers of contaminating B cells, but the possibility that BA and BA-LPS are weakly mitogenic for T cells cannot be ruled out on the basis of the experiments reported here.

Thus it can be concluded that BA and BA-LPS possess the properties of immunogenicity and mitogenicity, features common to E. coli and Salmonella spp., and to their lipopolysaccharides. Differences lie in the class of antibody synthesized to both the bacterin and the endotoxin. Further, C3H/HeJ mice respond normally to Brucella abortus and its purified endotoxin. While no conclusion can be drawn about the role of the thymus in the response to Brucella bacterin, my data suggest that BA-LPS is a thymus-independent antigen.

APPENDIX

APPENDIX TO TABLES

Table I

7.5 X 10 ⁷ cells	1 ⁰	Total	80, 80, 160, 80;	$\bar{x} = 95^{**}$
		2-ME-R	<10, <10, <10, <10;	$\bar{x} = <10$
	R ⁰	Total	40, 80, 160, 80;	$\bar{x} = 80$
		2-ME-R	<10, <10, <10, <10;	$\bar{x} = <10$
	2 ⁰	Total	640, 640, 640, 320;	$\bar{x} = 538$
		2-ME-R	160, 160, 320, 40;	$\bar{x} = 134$
2.5 X 10 ⁸ cells	1 ⁰	Total	160, 40, 40, 320;	$\bar{x} = 95$
		2-ME-R	<10, <10, <10, <10;	$\bar{x} = <10$
	R ⁰	Total	160, 40, 80, 80;	$\bar{x} = 80$
		2-ME-R	<10, <10, <10, <10;	$\bar{x} = <10$
	2 ⁰	Total	1280, 80, 640, 1280;	$\bar{x} = 538$
		2-ME-R	160, 40, 160, 160;	$\bar{x} = 113$
7.5 X 10 ⁸ cells	1 ⁰	Total	40, 40, 40, 80;	$\bar{x} = 48$
		2-ME-R	<10, <10, <10, <10;	$\bar{x} = <10$
	R ⁰	Total	80, 160, 40, 80;	$\bar{x} = 80$
		2-ME-R	<10, <10, <10, <10;	$\bar{x} = <10$
	2 ⁰	Total	640, 1280, 320, 640;	$\bar{x} = 640$
		2-ME-R	160, 640, 10, 80;	$\bar{x} = 80$

Table II

Day -14	640, 640, 1280, 640;	$\bar{x} = 761$
-12	640, 320, 640, 640;	$\bar{x} = 538$
-10	320, 640, 320, 160;	$\bar{x} = 320$
- 8	320, 320, 160, 320;	$\bar{x} = 269$
- 6	320, 160, 320, 160;	$\bar{x} = 226$
- 4	40, 40, 80, 40;	$\bar{x} = 48$
- 2	<2, <2, <2, <2;	$\bar{x} = <2$

** $\bar{x}$ is used throughout this appendix to denote the geometric mean of the individual titers given.

Figure 1

7.5 X 10⁸ cells

Balb/c	Day 7	Total	40, 80, 80, 40;	$\bar{x} = 57$
		2-ME-R	<10	
	Day 14	Total	80, 160, 80, 160;	$\bar{x} = 113$
		2-ME-R	<10	
	Day 21	Total	320, 640, 160, 640;	$\bar{x} = 380$
		2-ME-R	<10	
	Day 28	Total	640, 640, 160, 640;	$\bar{x} = 452$
		2-ME-R	320	
C3H/HeJ	Day 7	Total	40, 40, 40, 10;	$\bar{x} = 28$
		2-ME-R	<10	
	Day 14	Total	160, 80, 80, 160;	$\bar{x} = 113$
		2-ME-R	<10	
	Day 21	Total	320, 320, 160, 320;	$\bar{x} = 269$
		2-ME-R	160	
	Day 28	Total	1280, 640, 320, 320;	$\bar{x} = 538$
		2-ME-R	320	
Nude	Day 7	Total	80, 40, 40, 20;	$\bar{x} = 40$
		2-ME-R	<10	
	Day 14	Total	20, 20, 20, 20;	$\bar{x} = 20$
		2-ME-R	<10	
	Day 21	Total	40, 40, 40, 40;	$\bar{x} = 40$
		2-ME-R	<10	
	Day 28	Total	40, 40, 40, 40;	$\bar{x} = 40$
		2-ME-R	<10	

7.5 X 10⁶ cells

Balb/c	Day 7	Total	80, 40, 80, 40;	$\bar{x} = 57$
		2-ME-R	<10	
	Day 14	Total	80, 40, 20, 20;	$\bar{x} = 34$
		2-ME-R	<10	
	Day 21	Total	80, 20, 10, <10;	$\bar{x} = 17$
		2-ME-R	<10	
	Day 28	Total	<10, <10, <10, <10;	$\bar{x} = <10$

Figure 1, continued.

		2-ME-R	< 10
C3H/HeJ	Day 7	Total 2-ME-R	40, 20, 20, 40; $\bar{x} = 28$ < 10
	Day 14	Total 2-ME-R	80, 80, 80, 40; $\bar{x} = 67$ < 10
	Day 21	Total 2-ME-R	80, 40, 40, 40; $\bar{x} = 48$ < 10
	Day 28	Total 2-ME-R	160, 20, < 10, 20; $\bar{x} = 23$ < 10
Nude	Day 7	Total 2-ME-R	20, 40, 40, 20; $\bar{x} = 28$ < 10
	Day 14	Total 2-ME-R	20, < 10, 10, < 10; $\bar{x} < 10$ < 10
	Day 21	Total 2-ME-R	< 10, < 10, < 10, < 10; $\bar{x} < 10$ < 10
	Day 28	Total 2-ME-R	20, 20, 20, 40; $\bar{x} = 24$ < 10
2.5 X 10 ⁴ cells			
Balb/c	Day 7	Total 2-ME-R	20, 10, 10, < 10; $\bar{x} = 10$ < 10
	Day 14	Total 2-ME-R	< 10, < 10, < 10, < 10; $\bar{x} < 10$ < 10
	Day 21	Total 2-ME-R	< 10, < 10, < 10, < 10; $\bar{x} < 10$ < 10
	Day 28	Total 2-ME-R	20, < 10, < 10, < 10; $\bar{x} < 10$ < 10
C3H/HeJ	Day 7	Total 2-ME-R	10, 10, < 10, 20; $\bar{x} = 10$ < 10
	Day 14	Total 2-ME-R	< 10, < 10, < 10, < 10; $\bar{x} < 10$ < 10
	Day 21	Total 2-ME-R	< 10, < 10, < 10, < 10; $\bar{x} < 10$ < 10

Figure 1, continued.

Nude	Day 28	Total	< 10, < 10, < 10, < 10; $\bar{x} < 10$
		2-ME-R	< 10
	Day 7	Total	< 10, < 10, < 10, < 10; $\bar{x} < 10$
		2-ME-R	< 10
	Day 14	Total	< 10, < 10, < 10, < 10; $\bar{x} < 10$
		2-ME-R	< 10
	Day 21	Total	< 10, < 10, < 10, < 10; $\bar{x} < 10$
		2-ME-R	< 10
	Day 28	Total	< 10, < 10, < 10, < 10; $\bar{x} < 10$
		2-ME-R	< 10

Table III

Balb/c	Day 7	Total	40, 40, 20, 40, 20, 40 ⁺ , 10, 10 ⁺ , 40, 40; $\bar{x} = 26$
		2-ME-R	< 10, < 10, < 10, < 10, < 10, < 10, < 10, < 10, < 10, < 10; $\bar{x} < 10$
	Day 16	Total	160, 640, 160, 320, 320, 320, 320, 160; $\bar{x} = 269$
		2-ME-R	< 10, < 10, < 10, < 10, < 10, < 10, < 10, < 10; $\bar{x} < 10$
	Day 23 yes	Total	>5120, >5120, 5120, 5120, >5120; $\bar{x} > 5120$
		2-ME-R	5120, 2560, 2560, 1280, 640; $\bar{x} = 1940$
	no	Total	1280, 640, 640; $\bar{x} = 806$
		2-ME-R	640, 320, 320; $\bar{x} = 403$
	Nude	Total	20, 80, 80, 160, 40, 40, 20, 80, 40, 40; $\bar{x} = 49$
		2-ME-R	< 10, < 10, < 10, < 10, < 10, < 10, < 10, < 10, < 10, < 10; $\bar{x} < 10$

⁺ denotes mice which died prior to a day 16 bleeding.

Table III, continued.

Day 16	Total	80, 40, 20, 160, 40, 80, 10, 40, 20, 40; $\bar{x} = 37$
	2-ME-R	< 10, < 10, < 10, < 10, < 10, < 10, < 10, < 10, < 10, < 10; $\bar{x} < 10$
Day 23 yes	Total	2560, 1280, 320, 1280, 640, 640; $\bar{x} = 905$.
	2-ME-R	--, 20, < 10, 320, 160, 160; $\bar{x} = 61$
no	Total	80, 10, 80, 80; $\bar{x} = 48$
	2-ME-R	< 10, < 10, < 10, < 10; $\bar{x} < 10$

Table IV

Balb/c	Day 7	Total	10, 40, 40, 20, 20, 20, 40, 40; $\bar{x} = 25$
	Day 23 yes	Total	2560, 2560, 2560, 2560; $\bar{x} = 2560$
	no	Total	80, 160, 160, 160; $\bar{x} = 134$
C3H/HeJ	Day 7	Total	20, 20, 40, 20, 20, 40, 20, 40; $\bar{x} = 25$
	Day 23 yes	Total	2560, 2560, 2560, 2560; $\bar{x} = 2560$
	no	Total	160, 160, 160, 160; $\bar{x} = 160$

Table V

100 μ g	Day 7	Total	40, 20, 20, 20; $\bar{x} = 24$
		2-ME-R	< 10, < 10, < 10, < 10; $\bar{x} < 10$
	Day 16	Total	80, 40, < 10, 40; $\bar{x} = 28$
		2-ME-R	20, < 10, < 10, < 10; $\bar{x} < 10$
	Day 23	Total	640, 160, 320, 160; $\bar{x} = 269$
		2-ME-R	320, 80, 80, 80; $\bar{x} = 113$

Table V, continued..

50 μg	Day 7	Total	10, 40, 40, 20; $\bar{x} = 24$
		2-ME-R	< 10, < 10, < 10, < 10; $\bar{x} < 10$
	Day 16	Total	20, 20, 20, < 10; $\bar{x} = 14$
		2-ME-R	< 10, < 10, < 10, < 10; $\bar{x} < 10$
	Day 23	Total	160, 320, 640, 160; $\bar{x} = 269$
		2-ME-R	160, 160, 160, 160; $\bar{x} = 160$
10 μg	Day 7	Total	20, 40, 40, 80; $\bar{x} = 40$
		2-ME-R	< 10, < 10, < 10, < 10; $\bar{x} < 10$
	Day 16	Total	< 10, < 10, < 10, 40; $\bar{x} < 10$
		2-ME-R	< 10, < 10, < 10, < 10; $\bar{x} < 10$
	Day 23	Total	160, 640, 160, 320; $\bar{x} = 269$
		2-ME-R	80, 320, 80, 80; $\bar{x} = 113$
1 μg	Day 7	Total	< 10, 10, 80, 40; $\bar{x} = 20$
		2-ME-R	< 10, < 10, < 10, < 10; $\bar{x} < 10$
	Day 16	Total	< 10, < 10, 40, 20; $\bar{x} = 12$
		2-ME-R	< 10, < 10, < 10, < 10; $\bar{x} < 10$
	Day 23	Total	320, 80, 640, 320; $\bar{x} = 269$
		2-ME-R	160, 80, 80, 320; $\bar{x} = 134$

Figure 2

Balb/c

Day 5	Total	20, 80, 320, 80; $\bar{x} = 80$
	2-ME-R	< 10
Day 7	Total	40, 10, 20, 20; $\bar{x} = 20$
	2-ME-R	< 10
Day 9	Total	20, 80, 40, 40; $\bar{x} = 40$
	2-ME-R	< 10
Day 12	Total	< 10, 20, 40, 20; $\bar{x} = 17$
	2-ME-R	< 10
Day 15	Total	10, < 10, 10, 10; $\bar{x} < 10$
	2-ME-R	< 10
Day 23	Total	10, < 10, 10, < 10; $\bar{x} < 10$
	2-ME-R	< 10

Figure 2, continued

C3H/HeJ

Day 4	Total 2-ME-R	<10, <10, <10, <10; <10	$\bar{x} = <10$
Day 6	Total 2-ME-R	10, 40, 40, 20; <10	$\bar{x} = 34$
Day 9	Total 2-ME-R	20, 40, 20, 20; <10	$\bar{x} = 24$
Day 12	Total 2-ME-R	40, <10, <10, 20; <10	$\bar{x} = 12$
Day 15	Total 2-ME-R	<10, 10, 10, 20; <10	$\bar{x} = 10$
Day 23	Total 2-ME-R	20, <10, 10, <10; <10	$\bar{x} = <10$

Table VI

50 μg

Balb/c	Day 5	Total	40, 80, 80, 40;	$\bar{x} = 48$
	Day 11	Total	20, 40, 40, 20;	$\bar{x} = 28$
	Day 17	Total	80, 20, 40, 40;	$\bar{x} = 40$
	Day 30	(2 mice) Total 2-ME-R	20, 40; < 10	$\bar{x} = 28$
C3H/HeJ	Day 5	Total	20, 10, 20, 20;	$\bar{x} = 17$
	Day 11	Total	20, 10, < 10, 20;	$\bar{x} = 12$
	Day 17	Total	40, 40, 20, 40;	$\bar{x} = 34$
	Day 30	(4 mice) Total $\bar{x}$ 2-ME-R	< 10, < 10, < 10, 40; < 10 < 10	

Table VI, continued.

Nude	Day 5	Total	20, 80, 40, 20; $\bar{x} = 34$
	Day 11	Total	20, 20, < 10, 10; $\bar{x} = 10$
	Day 17	Total	20, 20, < 10, 20; $\bar{x} = 14$
	Day 30	(3 mice) Total	20, 40, 40; $\bar{x} = 32$ 2-ME-R < 10
10 μ g			
Balb/c	Day 5	Total	40, 80, 80, 40; $\bar{x} = 57$
	Day 11	Total	20, 40, 80, 20; $\bar{x} = 34$
	Day 17	Total	20, 80, 40, 40; $\bar{x} = 40$
	Day 30	(4 mice) Total	< 10, 20, 40, 40; $\bar{x} = 20$ 2-ME-R < 10
C3H/HeJ	Day 5	Total	40, 20, 20, 40; $\bar{x} = 28$
	Day 11	Total	< 10, 20, 10, 20; $\bar{x} = 12$
	Day 17	Total	20, 40, 40, 40; $\bar{x} = 34$
	Day 30	(4 mice) Total	< 10, 20, < 10, < 10; $\bar{x} < 10$ 2-ME-R < 10
1 μ g			
Balb/c	Day 5	Total	40, --, 20, 20; $\bar{x} = 25$
	Day 11	Total	20, 40, 20, 10; $\bar{x} = 20$
	Day 17	Total	20, 40, 20, 40; $\bar{x} = 28$
	Day 30	(2 mice) Total	20, < 10; $\bar{x} = 10$ 2-ME-R < 10
C3H/HeJ	Day 5	Total	--, 20, 20, 20; $\bar{x} = 20$
	Day 11	Total	< 10, < 10, 20, < 10; $\bar{x} < 10$
	Day 17	Total	10, < 10, 20, 10; $\bar{x} = 10$
	Day 30	(4 mice) Total	10, < 10, < 10, < 10; $\bar{x} < 10$ 2-ME-R < 10

Table VI, continued.

Nude	Day 5	Total	20, 20, 20, 40; $\bar{x} = 24$
	Day 11	Total	10, < 10, 40, 20; $\bar{x} = 14$
	Day 17	Total	10, < 10, 20, 10; $\bar{x} = 10$
	Day 30	(3 mice) Total	< 10, 10, < 10; $\bar{x} < 10$
		2-ME-R	< 10
10^{-2} μ g Balb/c	Day 5	Total	< 10, < 10, < 10, < 10; $\bar{x} < 10$
	Day 11	Total	< 10, < 10, < 10, < 10; $\bar{x} < 10$
	Day 17	Total	< 10, < 10, < 10, < 10; $\bar{x} < 10$
	Day 30	(2 mice) Total	< 10, < 10; $\bar{x} < 10$
		2-ME-R	< 10
C3H/HeJ	Day 5	Total	< 10, < 10, < 10, < 10; $\bar{x} < 10$
	Day 11	Total	< 10, < 10, < 10, < 10; $\bar{x} < 10$
	Day 17	Total	< 10, < 10, < 10, < 10; $\bar{x} < 10$
	Day 30	(3 mice) Total	< 10, < 10, < 10; $\bar{x} < 10$
		2-ME-R	< 10
Nude	Day 5	Total	20, < 10, < 10, < 10; $\bar{x} < 10$
	Day 11	Total	< 10, < 10, < 10, < 10; $\bar{x} < 10$
	Day 17	Total	< 10, < 10, < 10, < 10; $\bar{x} < 10$
	Day 30	(4 mice) Total	< 20, < 20, < 10, < 10; $\bar{x} < 10$
		2-ME-R	< 10

Table VII

Balb/c	Day 5	Total	20, 40, 40, 20, 20, 40, 10, 40, 40, 40; $\bar{x} = 28$
	Day 23	Total	< 10, 40, 10, 20, 10; $\bar{x} = 13$
	R_0^{20}	Total	160, 320, 160, 640, 320; $\bar{x} = 278$

C3H/HeJ

Day 5 Total 40, 40, 20, 10, 40, 40, 10, 40,
20, 20; $\bar{x} = 24$

Day 23

R^0 Total < 10, 20, 40, 20; 10; $\bar{x} = 15$

2^0 Total 320, 320, 160, 640, 160; $\bar{x} = 278$

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ABBREVIATIONS

ATxBM	Adult thymectomized-lethally irradiated-bone marrow reconstituted mice
BA	<u>Brucella abortus</u>
BA-LPS	<u>Brucella abortus</u> lipopolysaccharide
B cell	Bursa-equivalent lymphocyte
Con A	Concanavalin A
i.v.	Intravenous
KDO	2-Keto-3-Deoxyoctonate
LPS	Lipopolysaccharide
2-ME	2-mercaptoethanol
2-ME-R	2-mercaptoethanol resistant antibody
PBS	Phosphate buffered saline
PEC	Peritoneal exudate cells
POL	Polymerized flagellin
PVP	Polyvinylpyrrolidone
R ⁰	Residual primary response
SIII	Pneumococcal polysaccharide type III
T cell	Thymus-derived lymphocyte
1 ⁰	Primary antibody response
2 ⁰	Secondary antibody response

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The immune and mitogenic response by Balb/c, C3H/HeJ, and nude mice to Brucella abortus bacterin and endotoxin

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