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

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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 athy-mic 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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Date November 11, 1976

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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.

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 desribed 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), adjuvanticity (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 preformed 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 μg) 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

C_3H/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 C_3H/HeN strain, were derived from the C_3H/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.

Sultzer was the first to report the resistance of the C_3H/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 X CWB) X 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 C_3H/HeN spleen cell cultures to LPS. In vivo, reconstitution of C_3H/HeJ animals following B-cell depletion by sublethal irradiation with spleen cells from normal C_3H/HeN mice results in acquired responsiveness by the C_3H/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 supressor 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 C_3H/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 C_3H/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^{o} = primary response) and on day 15 (R^{o} = residual primary response); reimmunization on day 16 with a dose identical to that received on day 0 was followed on day 23 (2^{o} = 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

Dose [a]	Serum Antibody Titers [b]					
	1^{o} Response (Day 7)		R^{o} Response (Day 15)		2^{o} Response (Day 23)	
	Total	2-ME-R	Total	2-ME-R	Total	2-ME-R
7.5×10^{7}	95	< 10	80	< 10	538	134
2.5×10^{8}	95	< 10	80	< 10	538	113
7.5×10^{8}	48	< 10	80	< 10	640	80
2.5×10^{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 X 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 X 10^8 organisms was selected; this dose eliminated concern over mortality among experimental animals while eliciting responses similar to those using 7.5 X 10^8 BA cells. A dose of 2.5 X 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 X 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

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

[a]Mice were immunized on various days prior to bleeding on day 0. A dose of 2.5×10^8 BA cells in 0.25 ml PBS was used for immunization.

[b]Titers 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 subsidance 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