Effect of Early Antibiotic Treatment on the Antibody Response to Cytoplasmic Proteins of *Brucella melitensis* in Mice

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To test whether antibiotic therapy hampers the antibody response to *Brucella* antigens, 30 BALB/c mice were infected with *Brucella melitensis* H38 and randomized for treatment with doxycycline administered intraperitoneally for 42 days starting at 7 or 28 days postinfection (p.i.) (groups DOX7 and DOX28, respectively) or for no treatment (control group). Antibodies to smooth lipopolysaccharide (LPS) reached peak levels (mean optical density [OD] = 2.618) between days 56 and 70 p.i. in the control group, and similar peak levels (mean OD = 2.486) were observed in the DOX28 group, but significantly lower peak levels (mean OD = 0.821) were observed at 28 days p.i. in the DOX7 group. The antibody response against cytoplasmic proteins depleted of LPS (CPs) reached maximal levels (mean OD = 2.402) between days 56 and 70 p.i. in the control group, but no response was detected in the DOX7 group. Anti-CP antibodies were detected in only three animals from the DOX28 group, at levels significantly lower than those in the control group (mean maximal OD = 0.791). The pattern of antibody response to an 18-kDa cytoplasmic protein of *Brucella* spp. was similar to that against the CP antigen. This study shows that early antibiotic treatment affects the antibody response of mice to cytoplasmic proteins of *Brucella* and, to a lesser extent, to LPS.

Human infection by *Brucella* spp. still constitutes an important health problem in many developing countries and in some developed areas of the world. Classical serological tests rely on the detection of antibodies to the bacterial smooth lipopolysaccharide (S-LPS), which may render false-positive results because of cross-reactivity with other gram-negative bacteria. In order to improve the specificity of the diagnosis, recent investigations have focused on the antibody response to *Brucella* proteins (1, 4, 5, 11). Members of our group have previously shown that the immunoglobulin G (IgG) response to cytoplasmic proteins depleted of LPS (CPs, formerly called LPS-free CYT) of *Brucella*, measured by enzyme-linked immunosorbent assay (ELISA), allows differentiation of active from inactive human brucellosis and shows good correlation with the clinical progression of the disease (3, 8). Similar results were obtained by measuring antibodies to an 18-kDa cytoplasmic protein of *Brucella* (9). Recent studies performed by members of our group have shown that IgM and IgG antibodies to CP and to the 18-kDa protein can be measured by the antibody response to S-LPS and cytoplasmic proteins of *Brucella* in mice randomized for antibiotic treatment at different times after experimental infection with *B. melitensis*.

Thirty female BALB/c mice were inoculated intraperitoneally with $8.55 \times 10^7$ CFU of the highly virulent strain *B. melitensis* H38. At 7 days postinfection (p.i.), three animals were killed by cervical dislocation, and their spleens were removed, homogenized, diluted serially, and plated onto tryptic soy agar to determine the number of viable *B. melitensis* cells. The remaining animals were randomly assigned to receive doxycycline (100 mg/kg of body weight/day, intraperitoneally) for 42 days starting at 7 days p.i. (group DOX7, *n* = 7) or at 28 days p.i. (group DOX28, *n* = 7) or to receive no antibiotic treatment (control group, *n* = 13). The procedure for bacterial counting was repeated (three animals each time) on days 21, 28, 42, and 70 p.i. for the control group and on days 14 and 42 of antibiotic therapy for group DOX7 (21 and 49 days p.i., respectively) and group DOX28 (42 and 70 days p.i., respectively). Animals in the control group were bled at weekly intervals from day 7 p.i. to day 70 p.i. Animals in groups DOX7 and DOX28 were bled weekly between days 14 and 42 of the antibiotic treatment. Each time, blood was obtained from all animals still not sacrificed.

Serum reactivity to the CP antigen of *Brucella* was assayed as described previously (8). Briefly, polystyrene plates (Maxisorp; Nunc, Roskilde, Denmark) were sensitized with 0.5 µg of CP per well and blocked with 3% skim milk in phosphate-buffered saline (PBS). The plates were washed with PBS-0.05% Tween 20, and the sera under study were added (diluted 1:100 in PBS-0.05% Tween 20 containing 1% skim milk). After incubation, the plates were washed and incubated with a horseradish peroxidase-conjugated antibody to mouse immunoglobulins (Axell, Westbury, N.Y.). The reaction was developed with o-phenylenediamine (2 µg/µl) and 0.03% H$_2$O$_2$ in 0.1 M citrate-phosphate buffer (pH 5.0) and was stopped with 4 N H$_2$SO$_4$. The resulting color was read at 492 nm in a Σ 960
ELISA microplate reader (Metertech Inc., Taipei, Taiwan). All sera from a given group were assayed in the same run. Sera obtained before the infection served as negative controls in the assays. A pool of sera obtained from untreated mice at 42 days p.i. was used as a positive control and to correct the results for interassay variation.

Serum reactivity against the 18-kDa cytoplasmic protein of Brucella was measured by indirect ELISA, using a recombinant protein prepared in our laboratory. Blocking of plates, testing of sera, addition of the conjugates, and development of the reaction were performed as described above.

Serum reactivity against S-LPS was measured by indirect ELISA. Plates were sensitized with 5 μg of Brucella S-LPS per well, prepared by proteinase K digestion of the cytoplasmic fraction of Brucella abortus (originally containing 10 mg of S-LPS per ml), prepared as described previously (8). Blocking of plates, testing of sera, addition of the conjugates, and development of the reaction were performed as described above.

Effectiveness of antibiotic therapy. Prior to randomization, at 7 days p.i., infected mice had high bacterial counts (mean ± standard deviation, 5.14 ± 0.72 CFU/spleen). In the control group, the splenic bacterial count remained high, with only minor changes, until the end of the experiment (5.50 ± 0.43 CFU/spleen at 70 days p.i.). A progressive splenomegaly was observed in untreated mice, for which the spleen weights were 0.191 ± 0.016 g, 0.358 ± 0.108 g, and 0.724 ± 0.186 g at 7, 21, and 28 days p.i., respectively. In contrast to the control group, no bacteria were found in the spleens of animals from groups DOX7 and DOX28 on days 14 and 42 of antibiotic therapy, confirming the effectiveness of the treatment. On day 14 of therapy (21 days p.i.), the mean spleen weight of the DOX7 group was lower than that of the control group (0.221 ± 0.035 g versus 0.358 ± 0.108 g). In the DOX7 group, the spleen weight on day 42 of therapy (49 days p.i.) was also lower than that observed at comparable times (42 days p.i.) for the control group (0.154 ± 0.026 g versus 0.410 ± 0.100 g). Similarly, the mean spleen weight of the DOX28 group was lower than that of the control group, both on day 14 of therapy (42 days p.i., 0.107 ± 0.028 g versus 0.410 ± 0.100 g) and on day 42 of therapy (70 days p.i., 0.188 ± 0.049 g versus 0.528 ± 0.051 g).

Antibody response to Brucella antigens. Figure 1 shows the antibody response to S-LPS in four mice of each group, which were monitored until the end of the antibiotic treatment (groups DOX7 and DOX28) or until 70 days p.i. (control group). In the control group, anti-S-LPS antibody levels showed a steady increase starting at 21 days p.i., reaching a peak (mean optical density [OD] = 2.618) between days 56 and 70 p.i. In group DOX7, in contrast, a much lower increase in the levels of anti-S-LPS antibodies was observed in three animals, whose response reached a plateau at 28 days p.i. (mean peak OD = 0.821). The remaining mouse did not show an anti-S-LPS response. Three animals in group DOX28 developed a significant anti-S-LPS response (mean peak OD = 2.486), similar to that observed in the control group. In these mice, however, the maximum antibody response was reached between days 28 and 42 p.i., earlier than in the control group. Although the fourth animal in group DOX28 developed a lower anti-S-LPS response (peak OD = 1.054), its antibody levels did not increase significantly after day 28 p.i. At 49 days p.i., the mean ODs were 1.449 in the control group, 0.616 in the DOX7 group, and 2.109 in the DOX28 group (excluding the poor responder).

The antibody response to CP developed by the same animals is shown in Fig. 2. Three of the four mice in the control group showed a steady increase in the level of anti-CP antibodies starting on day 28 p.i., while a significant increase was not observed in the fourth animal until day 49 p.i. The peak response (mean OD = 2.402) was reached between days 56 and 70 p.i. In contrast, no anti-CP response was detected until the end of the follow-up in the four mice from group DOX7. Three animals from group DOX28 developed a low anti-CP response, but no anti-CP antibodies were detected in the fourth animal during the whole follow-up. The maximum response of the three responders (mean OD = 0.791) was reached between days 28 and 49 p.i. At 49 days p.i., the mean

FIG. 1. Antibody response to Brucella S-LPS in control (A), DOX7 (B), and DOX28 (C) groups. Serum reactivity was measured by ELISA. Each symbol represents results for an individual mouse.
ODs of anti-CP antibodies were 1.266 in the control group, 0.048 in the DOX7 group, and 0.671 in the DOX28 group (responders only).

The kinetics of the antibody response to the 18-kDa cytoplasmic protein were similar to those of the anti-CP response in all three groups (not shown). In the control group, the anti-18-kDa-protein response began to appear at 28 days p.i., but peak levels (mean OD = 2.670) were attained between days 56 and 70 p.i. No anti-18-kDa-protein response was detected in animals from the DOX7 group. Only two animals from the DOX28 group developed a significant response to the 18-kDa protein, with peak levels (mean OD = 2.410) at 70 days p.i.

The aim of the present study was to address whether early antibiotic treatment hampers the development of the antibody response to Brucella antigens in a murine model of brucellosis. The results obtained seem to support that hypothesis. The anti-CP antibody response of mice that received antibiotic therapy from day 28 p.i. was lower than that found in untreated animals. Moreover, mice whose treatment began at 7 days p.i. did not develop anti-CP antibodies. The antibody response to S-LPS was also affected, but only in those mice whose treatment began at 7 days p.i. These results suggest that the time that elapses between the infection and the onset of the antibiotic treatment is a determining factor in the strength of the antibody response to cytoplasmic proteins of Brucella spp. A possible explanation to these findings is that therapy at the initial stages of the disease results in a rapid clearance of the bacteria from infected tissues and reduces the chances of interaction between bacterial antigens and the immune system. Since the immunogenicity of Brucella cytoplasmic proteins is lower than that of S-LPS, this premature clearance would affect primarily the anti-CP response. Notwithstanding, the anti-LPS response could also be affected. As shown by Gazapo et al. (7), if the level of anti-LPS IgG antibodies is below the cutoff when antibiotic therapy is started, they are not detected unless a relapse occurs.

The effect of the antibiotic treatment on the murine antibody response to Brucella antigens was also examined by Domingo et al. (6), using a model similar to ours. In that study, BALB/c mice were infected with B. melitensis 16 M and were treated with doxycycline administered orally for 45 days starting at day 31 p.i., comparable to our DOX28 group. Compared to that of untreated mice, the anti-S-LPS response of treated mice appeared enhanced between days 43 and 76 p.i., which contrasts with our results. Although in two animals from our DOX28 group anti-S-LPS levels peaked earlier than in mice in the control group, these levels were attained before the treatment was begun. This early response was also observed in some animals from the control group that were sacrificed for bacterial cultures (not shown). The reasons for the discrepancy between the results of Domingo et al. and ours are not clear, but some experimental differences could account for it. First, the infecting dose used by these authors was significantly higher than ours (9.2 $\times$ 10$^5$ versus 8.55 $\times$ 10$^3$). Second, although the daily dose of doxycycline was the same in both studies, we administered it in a single dose instead of twice a day. Finally, while we administered the antibiotic intraperitoneally instead of orally, it has been shown that levels of doxycycline in plasma are very similar for both routes (14). Although Domingo et al. also observed lower levels of antibodies to cytosolic antigens of Brucella in treated mice, it must be stressed that, in contrast to the CP antigen used in our study, the cytosolic fraction they used was likely to contain significant amounts of S-LPS.

At present, we do not know whether antibiotics affect the cell-mediated immune response to Brucella, which is known to contribute to protection against this bacterium (10, 12). However, this is suggested by a study showing a higher rate of relapse among brucellosis patients treated early than among those treated later (2). Interestingly, a short duration of illness before therapy was also observed by Pellicer et al. (13) in patients suffering a relapse of brucellosis. Others have postulated that early antibiotic therapy could affect the development of

**FIG. 2.** Antibody response to Brucella CP antigen in control (A), DOX7 (B), and DOX28 (C) groups. Serum reactivity was measured by ELISA. Each symbol represents results for an individual mouse.
cell-mediated immunity to *Brucella* and, thus, reduce the diagnostic value of the delayed-type hypersensitivity reaction (15). Further studies are needed to assess the effect of early antibiotic therapy on the cellular response to *Brucella* and on the relapse rate for patients with this disease.

Members of our group have previously shown that the anti-protein antibody response is useful in differentiating active from inactive human brucellosis and that it correlates with the clinical outcome of patients (3, 8). This response, however, may be absent in patients diagnosed and treated within a few days of the onset of clinical disease. The frequency of such early therapy in a given setting would depend on, among other things, the presentation of the disease (which in turn depends on the virulence of the infecting species) and on the local facilities and guidelines for the management of brucellosis. In our studies, the lack of antibody response to *Brucella* proteins was observed in about 0.5% of more than 1,000 patients assayed for anti-CP antibodies (unpublished results).

In summary, this study shows that early antibiotic therapy affects the antibody response to cytoplasmic proteins of *Brucella* in mice. These results are consistent with the lack of antiprotein response in some humans receiving early treatment for brucellosis. At present these findings have primarily diagnostic importance, since the role of these antibodies in the protective immune response against *Brucella* has not yet been established.

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