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Increased Sensitivity of *Corynebacterium parvum*-Treated Mice to Toxic Effects of Indomethacin and Lipopolysaccharide

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Female BALB/c and C3H/HeJ mice develop increased sensitivity to the toxic effects of indomethacin after injection of nonviable *Corynebacterium parvum*. The increased sensitivity developed within 4 days of intraperitoneal injection of the organisms and started to resolve 14 days after injection. The development of increased sensitivity was dependent on the quantity of organisms injected and the concentration of indomethacin utilized. The effect was not observed when *C. parvum*-treated animals were injected with aspirin. *C. parvum*-treated BALB/c mice also developed increased sensitivity to *E. coli* lipopolysaccharide (LPS). Although increased sensitivity to LPS and indomethacin paralleled each other in BALB/c mice, the experiments with the LPS-resistant C3H/HeJ mice indicated that the two phenomena could be separated. The pyridine extract residue of *C. parvum* was as effective as *C. parvum* whole cells in inducing indomethacin and LPS sensitivity. Therefore, activation of the reticuloendothelial system is probably a critical element in the induction of sensitivity to these agents.

Exposure of experimental animals to bacteria or bacterial products can lead to alterations in several components of the immune system (reviewed in reference 27). Lipopolysaccharide (LPS) is known to activate macrophages (2, 28; reviewed in references 17, 19, and 27) and B-lymphocytes (1; reviewed in references 19, 20, 26, and 27). Nonviable suspensions of mycobacterium are included in Freund complete adjuvant to enhance immune responsiveness (9; reviewed in references 16 and 27). Treatment of animals with killed *Corynebacterium parvum* leads to activation of the reticuloendothelial system (RES) and enhanced tumor immunity (13, 14; reviewed in references 12 and 24). Tuttle and Cantrell (24) have reported that these two activities of *C. parvum* can be separated by pyridine extraction of the nonviable organisms. In some of these instances, treatment with one bacterium can lead to enhanced sensitivity to another bacterium or bacterial product. Suter et al. (23) have reported that treatment of Swiss Webster mice with BCG leads to a lowering of the 50% lethal dose for *Escherichia coli* LPS from 357 to 7 μg. Peavy et al. (21) reported that BCG treatment of C57B1/6 mice (an LPS-sensitive strain) resulted in an enhanced sensitivity to LPS from a galactoseless mutant of *Salmonella typhimurium* but that similar treatment of C3H/HeJ mice (an LPS-resistant strain) did not lead to altered sensitivity to LPS. In contrast, Vogel et al. (25) reported that BCG treatment of C3H/HeJ mice leads to a lowering of the 50% lethal dose for *E. coli* LPS from >8 mg to 282 μg. Similar experiments with *C. parvum* have revealed that treated mice exhibit enhanced sensitivity to other bacteria (29) as well as bacterial products such as LPS (3, 7, 29). Several authors (discussed in references 3, 21, and 29) have concluded that the macrophage may play a central role in these altered responses to LPS.

Antiinflammatory drugs such as indomethacin and aspirin apparently exert their effect via inhibition of prostaglandin synthesis (8). Indomethacin, a nonsteroidal antiinflammatory drug, has been shown to be effective in altering the response of mice to LPS (11). Although drugs such as indomethacin are potent inhibitors of prostaglandin synthesis at low doses, at higher doses this drug can modify other enzyme systems (8; discussed in reference 15) and lead to toxic consequences. Interestingly, Boorman et al. (4) have reported that daily injections of high, but nontoxic, doses of indomethacin can lead to alterations in the immune system of the mouse. Injection of mice with 4 mg/kg for 6 days leads to splenomegaly, increased hematopoietic activity, and activation of macrophage functions (4). Animals pretreated with indomethacin exhibited enhanced resistance to *Listeria monocytogenes*, and macrophages derived from such animals exhibited enhanced phagocytosis (4). Thus, indomethacin can suppress inflammatory responses as well as enhance the activity of cells involved in such responses.

During the course of our studies on the effect of antiinflammatory agents on host responses to bacteria and bacterial products, it was discovered that *C. parvum*-treated mice develop increased sensitivity to concentrations of indomethacin that are not toxic to untreated mice. Some of the characteristics of this increased sensitivity parallel the increased sensitivity of these mice to LPS. These results may therefore provide a new avenue of approach to investigate the mechanisms by which LPS exerts its biological effects on the RES.

MATERIALS AND METHODS

Animals. Female BALB/c mice (8 to 10 weeks of age) were obtained from Charles River Canada, and female C3H/HeJ mice (10 to 12 weeks of age) were from Jackson Laboratories. Animals were maintained 2 to 4 weeks after shipment before being used in experiments.

Reagents. Indomethacin was obtained from Merck Sharp & Dohme and Sigma Chemical Co. Aspirin was purchased from Sigma. Indomethacin was dissolved in 95% ethanol to yield a stock solution of 10 mg/ml. Aspirin was dissolved in 95% ethanol to yield a stock solution of 50 mg/ml. Immediately before use, the test materials were diluted in phosphate-buffered saline (PBS) (pH 7.4) such that the appropriate dose could be administered by intraperitoneal injection of 0.25 ml. In some experiments, control animals were injected with 0.25 ml of the appropriate dilution of 95% ethanol in PBS. LPS (*E. coli* 055:B5 Westphal; lot no. 718368) was pur-

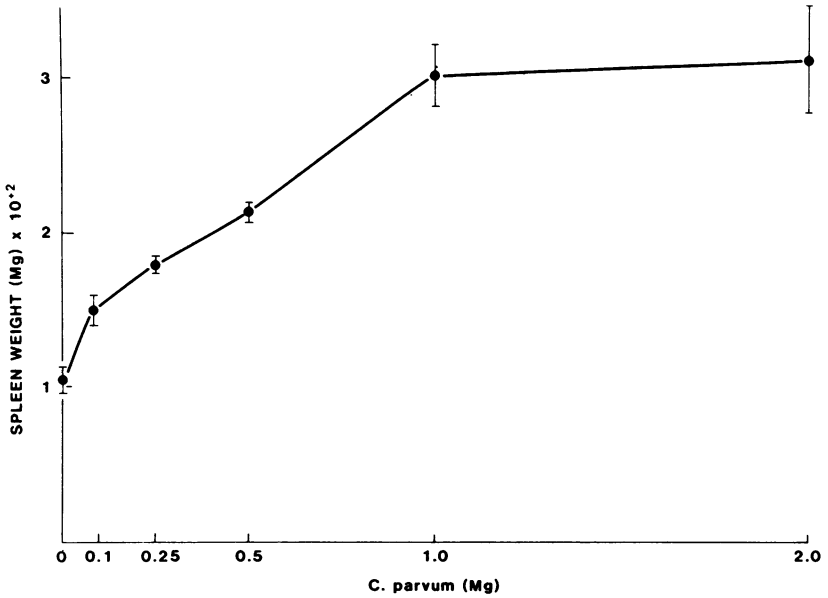

FIG. 1. Induction of splenomegaly by *C. parvum*. Groups (five to eight animals per group) of BALB/c female mice were injected with the indicated quantities of *C. parvum* suspended in PBS. Seven days postinjection, the animals were sacrificed, and the spleens were removed. The indicated values represent the mean ± the standard error of the mean weight of the spleens in milligrams. A repeat of this experiment yielded nearly identical results.

chased from Difco Laboratories and dissolved in PBS. *C. parvum* (*Propionibacterium acnes* 482) was obtained from RIBI Immunochem Research, Inc., Hamilton, Mont., as whole cells (lot no. 551 and 552), the pyridine extract residue (PER) of *C. parvum* (lot no. 450), or the pyridine extract (PE) of *C. parvum* (lot no. 405). Suspensions of *C. parvum* or *C. parvum* fractions were prepared in PBS. Samples of the suspension were injected via the intraperitoneal route.

RESULTS

Injection of increasing quantities of *C. parvum* into the peritoneal cavity of BALB/c mice led to definite splenomegaly by day 7 postinjection (Fig. 1). Splenomegaly could be detected 7 days after injection of 0.1 mg of *C. parvum*. Injection of 1.0 mg of *C. parvum* led to increases in spleen weight that were 309% of the weight of the spleens

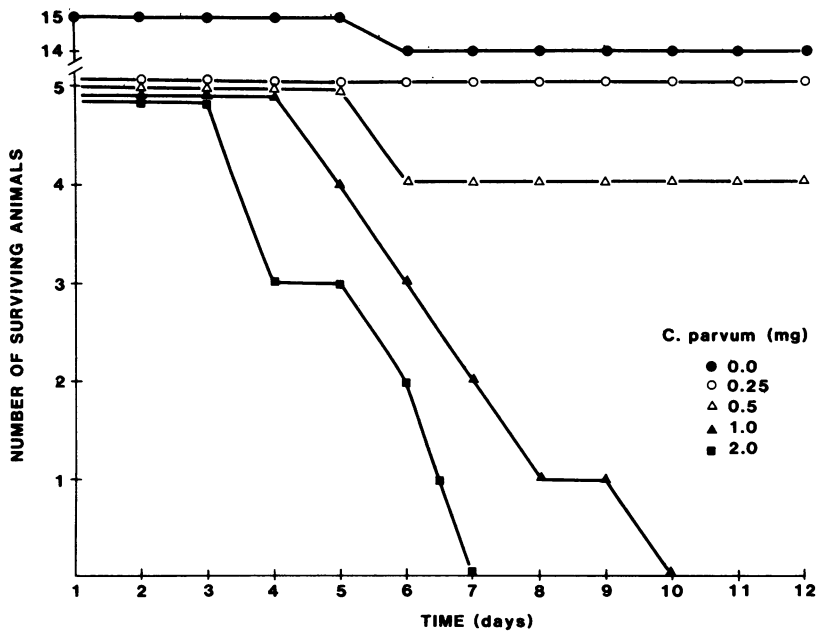

FIG. 2. Sensitivity of *C. parvum*-treated mice to indomethacin. Groups of BALB/c mice were injected with the indicated quantities of *C. parvum* whole cells. Seven days postinjection, the animals received daily intraperitoneal injections of 100 μg of indomethacin. Injections of indomethacin were performed between 8 and 10 a.m. Deaths were recorded at 8 a.m. and at 8 p.m. A repeat of this experiment yielded nearly identical results.

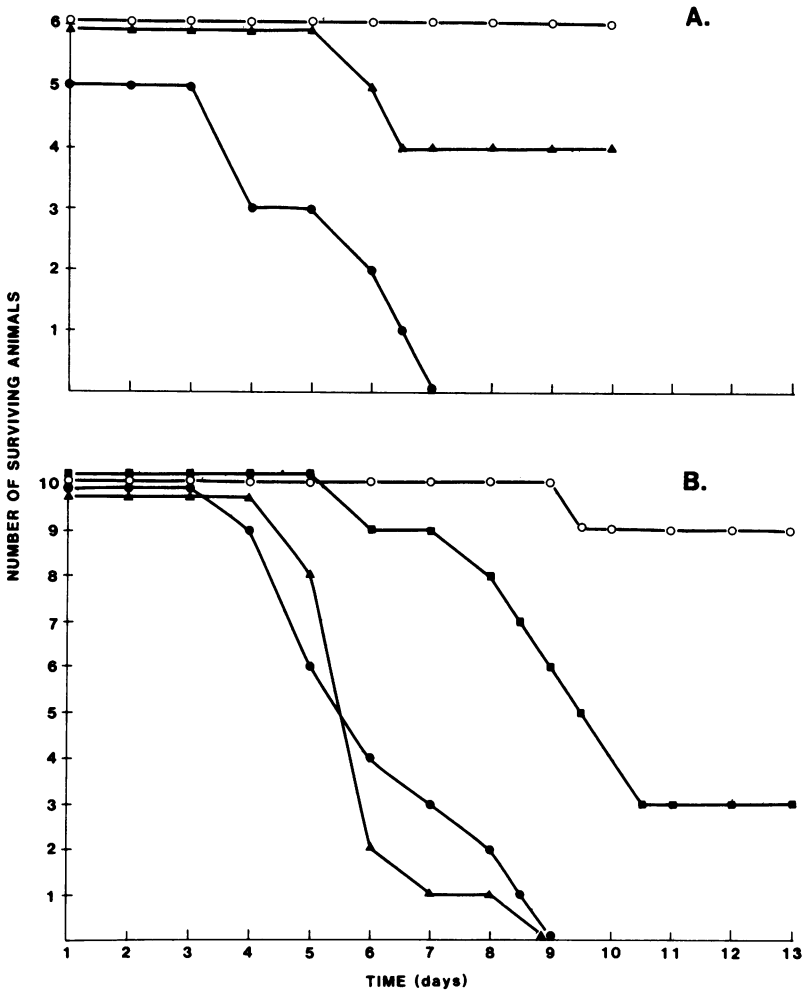

FIG. 3. Kinetics of the induction and resolution of sensitivity to indomethacin in *C. parvum*-treated BALB/c mice. (A) BALB/c mice were injected with saline (○) or 2.5 mg of *C. parvum* (●, ▲). Beginning on days 7 (●) and 14 (▲) posttreatment, the animals were injected daily with 100 μg of indomethacin for 10 days. (B) BALB/c mice were injected with saline (○) or 2.5 mg of *C. parvum* (●, ▲, ■). On days 1 (■), 4 (▲), and 7 (●) posttreatment, daily injections of 75.0 μg of indomethacin were initiated (day 1). The experiment was stopped after 13 injections of indomethacin. A repeat of these experiments revealed similar results.

from the controls. Increasing the quantity of *C. parvum* injected to 2.0 mg per animal led to only a slight increase in splenomegaly (318% of control values). Induction of splenomegaly by *C. parvum* has been attributed to its ability to activate the RES (reviewed in reference 24).

A panel of BALB/c mice was injected with the same quantities of *C. parvum* used in the experiment described above, and after 7 days they were injected daily with 100 μg of indomethacin or diluent. This concentration of indomethacin was not toxic for untreated age-and sex-matched BALB/c mice (Fig. 2). This concentration of indomethacin was also not toxic to animals injected with 0.25 mg of *C. parvum* and only marginally toxic to animals injected with 0.5 mg of *C. parvum* (1 of 5 dead) (Fig. 2). In contrast, all of the animals pretreated with 1.0 or 2.0 mg of *C. parvum* were dead after nine and six injections, respectively, of indomethacin (Fig. 2). None of the animals pretreated with 1.0 or 2.0 mg of *C. parvum* and then injected with the diluent died after 10 injections (data not shown).

Induction and resistance to indomethacin toxicity. To determine whether the sensitivity to indomethacin described above was a temporary or permanent change in the RES, BALB/c mice were injected with 2.5 mg of *C. parvum*, and then sensitivity to indomethacin was assessed 7 and 14 days postinjection. The sensitivity of treated mice to indomethacin had declined by day 14 postinjection of *C. parvum* (Fig. 3A). Therefore, it would appear that *C. parvum* induces a temporary alteration in sensitivity to this drug.

Conversely, it was also important to ascertain the kinetics of the induction of the increased sensitivity to indomethacin. A panel of BALB/c mice was injected with 2.5 mg of *C. parvum* whole cells on day 7, 4, or 1 before initiation of daily injections of 75 μg of indomethacin. Animals injected with *C. parvum* 4 or 7 days before the start of indomethacin treatment were equally sensitive to the toxic effects of this drug (Fig. 3B). Animals injected with *C. parvum* 1 day before indomethacin treatment were less sensitive to the drug than the above-described groups of this panel, but they were still more sensitive than the controls (Fig. 3B). Of 10 animals in the experimental groups, 3 survived the indomethacin treatment, and the number of injections of the drug required to observe the toxic effects was greater than that required to observe the toxic effects in the groups injected

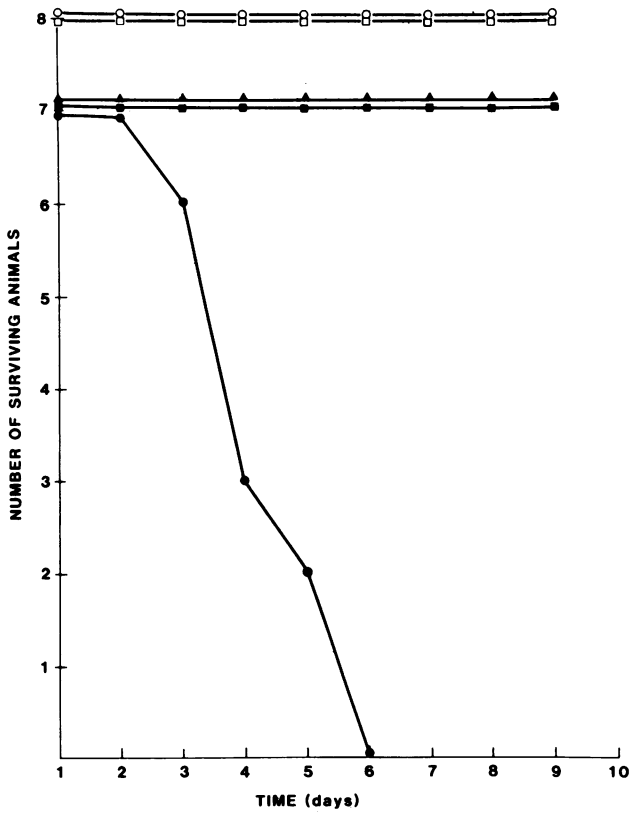

FIG. 4. Specificity and dose dependency of the increased sensitivity of *C. parvum*-treated BALB/c mice to antiinflammatory drugs. BALB/c mice were injected with either PBS (○, □) or 2.5 mg of *C. parvum* whole cells (●, ▲, ■). Seven days later, groups of mice were started on daily injections of 75 (○, ●) or 37.5 (▲) μg of indomethacin or 1 mg of aspirin (□, ■). The experiment was terminated after nine injections. A repeat of these experiments revealed similar results.

with *C. parvum* 4 or 7 days before initiation of the drug injections (Fig. 3B).

Specificity of the indomethacin effect on *C. parvum*-treated BALB/c mice. In previous experiments, animals were injected with 75 to 100 μg of indomethacin per day. To determine whether the toxic effects observed were specific for this empirically determined dose of a nonsteroidal antiinflammatory drug, a panel of BALB/c females was injected with PBS or 2.5 mg of *C. parvum* whole cells. Seven days later, groups of animals were injected daily with 75 or 37.5 μg of indomethacin or 1 mg of aspirin. All of the animals treated with 75 μg of indomethacin were dead after 6 days (Fig. 4). However, all of the *C. parvum*-treated animals receiving 37.5 μg of indomethacin or 1 mg of aspirin per day were still alive after 9 days. Therefore, the observed toxicity of indomethacin was restricted to high doses of the drug and was not observed with very high doses of the other antiinflammatory drug tested, aspirin (Fig. 4).

Ability of fractions of *C. parvum* to induce indomethacin sensitivity. *C. parvum* treatment of mice results in the alteration of several host systems (reviewed in references 12 and 24). Tuttle and Cantrell (24) have reported that the induction of antitumor immunity and activation of the RES by *C. parvum* can be separated by extraction of *C. parvum* whole cells with pyridine. Treatment of mice with material extracted with pyridine does not lead to splenomegaly but does lead to enhanced tumor immunity. In contrast, treatment of mice with the residue remaining after pyridine extraction leads to splenomegaly and activation of the RES (24). To ascertain whether the sensitivity to indomethacin of mice treated with *C. parvum* whole cells was due to alterations in antitumor immunity or activation of the RES, a panel of BALB/c mice was injected with PBS, 2.5 mg of *C. parvum* PE, or 2.5 mg of *C. parvum* PER. Seven days later, five animals from each group were sacrificed, and the spleens were removed and weighed. The values obtained for the controls, *C. parvum* PE-treated, and *C. parvum* PER-treated animals were 117 ± 9, 132 ± 20, and 337 ± 28 mg, respectively. Therefore, only *C. parvum* PER induced splenomegaly.

The remainder of the animals in each group were injected daily with 75 μg of indomethacin. All of the animals treated with *C. parvum* PER were dead after 8 days, whereas only 1 of 11 animals treated with *C. parvum* PE died over a 10-day period (Fig. 5). Therefore, development of sensitivity to indomethacin was associated with activation of a host system by *C. parvum* PER and not with those systems altered by *C. parvum* PE.

Sensitivity of *C. parvum*-treated BALB/c mice to LPS. Mice injected intravenously with *C. parvum* have been previously reported to develop enhanced susceptibility to the toxic effect of LPS (3, 7, 21, 29). To determine whether development of increased sensitivity to indomethacin paralleled development of increased sensitivity to LPS, a panel of BALB/c mice was injected intraperitoneally with 2.5 mg of *C. parvum* whole cells. Seven days later, untreated mice and

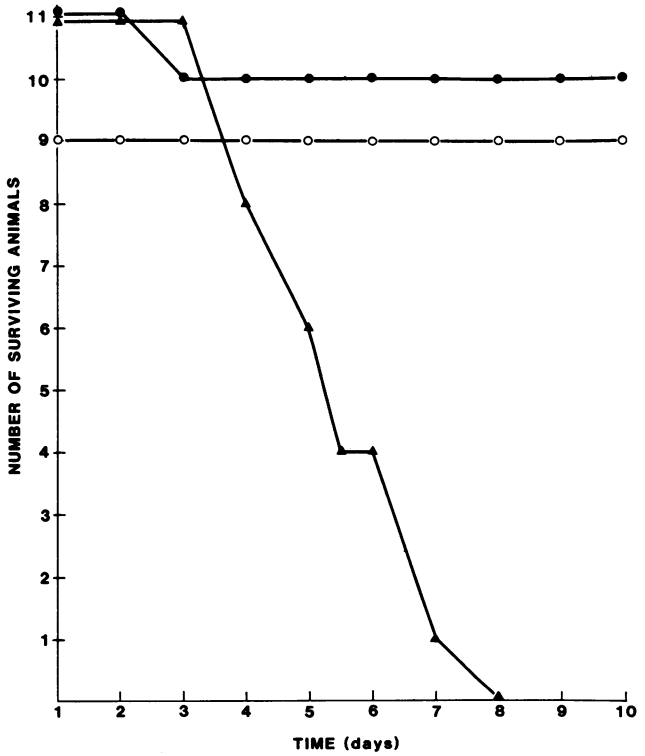

FIG. 5. Induction of increased sensitivity to indomethacin by fractions of *C. parvum*. Groups of BALB/c mice were injected with PBS (○), 2.5 mg of *C. parvum* PE (●), or 2.5 mg of *C. parvum* PER (▲). Seven days after treatment, daily injection of 75 μg of indomethacin was initiated (days 1 to 10). None of the *C. parvum* PER-treated animals injected with diluent died during the course of the experiment.

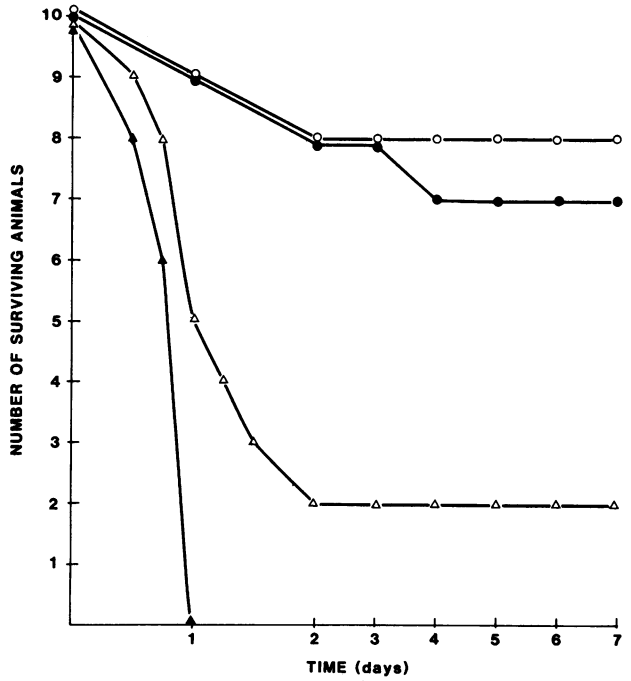

FIG. 6. Sensitivity of *C. parvum*-treated BALB/c mice to LPS. Groups of BALB/c mice were injected with either PBS (○, ●) or 2.5 mg of *C. parvum* (△, ▲). Seven days posttreatment, the animals were injected intraperitoneally with 150 (○, △) or 225 (●, ▲) μg of LPS.

C. parvum-treated animals were injected intraperitoneally with 150 or 225 μg of LPS. These doses of LPS were marginally toxic to the normal mice (Fig. 6). Of 10 animals in these groups, only 2 died by day 2. In contrast, all of the *C. parvum*-treated mice injected with 225 μg of LPS were dead within 24 h, and 8 of 10 mice injected with 150 μg of LPS were dead by 48 h postinjection (Fig. 6). Additional experiments have shown that 100 μg of LPS per mouse killed 0 of 10 mice in both control groups and in animals treated with 2.5 mg of *C. parvum* (data not shown). Further experiments with *C. parvum* PE- and *C. parvum* PER-treated animals have demonstrated that the enhanced sensitivity to LPS is associated with *C. parvum* PER rather than *C. parvum* PE. All of the *C. parvum* PER-treated animals (10 of 10) injected with 225 μg of LPS were dead after 24 h, whereas only 2 of 10 *C. parvum* PE-treated animals died within 5 days after LPS injection. Therefore, development of enhanced sensitivity of LPS paralleled the development of sensitivity to indomethacin.

Development of sensitivity to indomethacin in C3H/HeJ mice. From the experiments described above, it appeared that both LPS sensitivity and indomethacin sensitivity could be altered by treating BALB/c mice with *C. parvum* or *C. parvum* PER. To investigate whether the two sets of observations were more than casually associated, C3H/HeJ mice were treated with *C. parvum* and *C. parvum* PER. This strain of mouse is genetically resistant to LPS (discussed in references 10 and 22). These animals are resistant to the toxic effects of LPS in vivo, and lymphoid cells from these animals do not respond to LPS in vitro. However, agents such as glucan have been reported to alter the sensitivity of C3H/HeJ mice to LPS (18). To determine whether the *C. parvum* used in these studies could alter the sensitivity of C3H/HeJ mice to LPS, a panel of animals was injected intraperitoneally with 2.5 mg of *C. parvum*. Seven days later, five control mice and five treated mice were sacrificed, and the spleens were removed and weighed. Splenomegaly was evident in the treated group (260 ± 26 mg per spleen versus 115 ± 12 mg per spleen for the controls). The remaining animals were then injected intraperitoneally with either 200 (8 controls and 13 treated) or 300 μg of LPS (8 controls and 13 treated). No deaths occurred in any of the four groups over the next 7 days. The animals were sacrificed, and the spleens were removed and weighed. The average spleen weights for the controls plus 200 μg of LPS, controls plus 300 μg of LPS, treated plus 200 μg of LPS, and treated plus 300 μg of LPS were 114 ± 7, 106 ± 14, 462 ± 113, and 494 ± 120 mg, respectively. Therefore, *C. parvum* treatment of C3H/HeJ mice does not alter the sensitivity of these mice to doses of LPS in excess of those utilized to demonstrate lethality in *C. parvum*-treated BALB/c mice (Fig. 6). In this respect, these results with *C. parvum* are similar to those obtained by Peavy et al. (21) for BCG treatment of C3H/HeJ mice but differ from the results of Vogel et al. (25), who reported that BCG treatment of C3H/HeJ mice leads to enhanced sensitivity to LPS.

Therefore, a panel of C3H/HeJ mice was injected with PBS, 2.5 mg of *C. parvum* whole cells, or 2.5 mg of *C. parvum* PER. Seven days later, four animals from each group were sacrificed, and the spleens were removed and

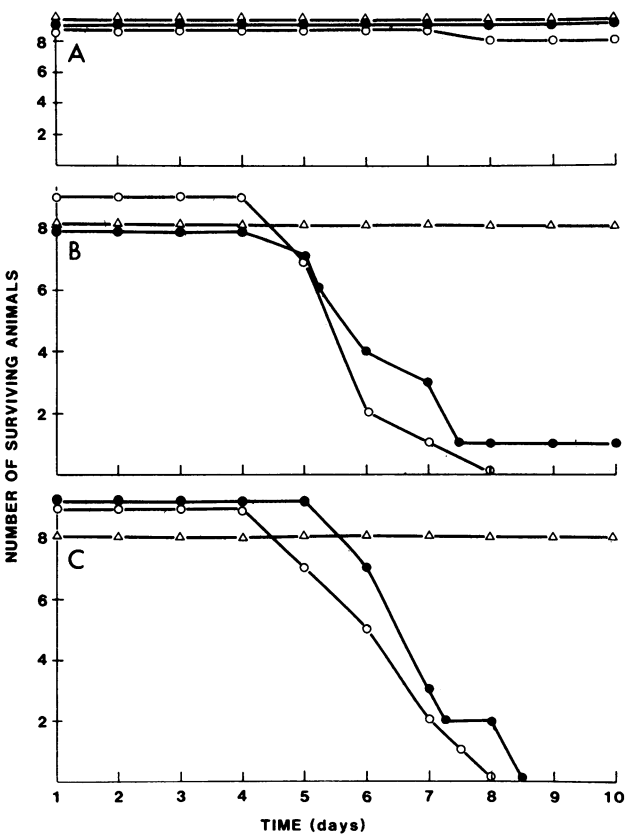

FIG. 7. Sensitivity of *C. parvum*-treated C3H/HeJ mice to indomethacin. Panels of C3H/HeJ female mice were injected with PBS (A), 2.5 mg of *C. parvum* whole cells (B), or 2.5 mg of *C. parvum* PER (C). Seven days posttreatment, groups of animals were injected daily with 75 (●) or 100 (○) μg of indomethacin or 1 mg of aspirin (△). This experiment was repeated three times with similar results.

weighed. The average weights of the spleens from the control group and the animals treated with *C. parvum* whole cells or *C. parvum* PER were 89 ± 5, 314 ± 35, and 273 ± 35 mg, respectively. Therefore, the treated animals developed splenomegaly. Each panel of mice was then separated into three groups which were injected daily with 75 or 100 μg of indomethacin or 1 mg of aspirin. Mice treated with *C. parvum* whole cells and *C. parvum* PER were very sensitive to indomethacin (Fig. 7). Aspirin treatment of these animals did not lead to any deaths over a 10-day injection schedule (Fig. 7). These results are very similar to those obtained previously with BALB/c mice (Fig. 2 and 5).

DISCUSSION

The results presented in this report demonstrate that injection of *C. parvum* into mice leads to enhanced sensitivity to both indomethacin and LPS. Since *C. parvum* PER was as effective in inducing this enhanced sensitivity as was *C. parvum* whole cells, it is likely that the basis for the enhanced sensitivity to these chemically very different agents is based on the activation of the host RES. Although the characterization of the induction of sensitivity to indomethacin and LPS revealed similarities, it is apparent from the C3H/HeJ experiments that the biochemical basis for the increased sensitivity to these agents is not identical. That is, resistance to LPS did not lead to resistance to indomethacin toxicity.

The mechanism by which indomethacin induces toxicity in the *C. parvum*-treated mice cannot be determined from the data. The finding that high levels (75 to 100 μg/day) of indomethacin, but not lower concentrations (37.5 μg/day), were toxic to the treated animals likely eliminates the possibility that the effect resides primarily in the ability of this drug to inhibit prostaglandin synthesis (8; discussed in reference 15). This conclusion is reinforced by the apparent lack of toxicity of very high doses of aspirin (1 mg/day = 50 mg/kg per day) in the treated animals (Fig. 4 and 7). Therefore, indomethacin toxicity is probably due to an effect on other metabolic systems. Doses of indomethacin comparable to those used in this study have been reported to lead to activation of the macrophage system in mice as well as other immune alterations (4). Animals treated with indomethacin for 6 days developed splenomegaly and some altered lymphocyte activities, but the clearance of [^{125}I]triolein by the RES was unaltered (4). In addition, several activities associated with activated macrophages (phagocytosis, clearance of *L. monocytogenes*) were detected after exposure to concentrations of indomethacin used in the present study (4). It should be noted that intraperitoneal injection of *C. parvum* into mice leads to the appearance of activated macrophages in the peritoneum (5, 6). Therefore, the central cell population in both *C. parvum*-treated animals and indomethacin-treated animals may be the macrophage. It is not known whether macrophages from *C. parvum*-treated animals are unique in their responsiveness in indomethacin. Chapes and Haskill (6) have cultured peritoneal macrophages from mice stimulated by intraperitoneal injection of *C. parvum* with indomethacin, but they did not report any unusual observation.

In contrast, macrophages from *C. parvum*-treated mice have been reported to exhibit enhanced sensitivity to the toxic effects of LPS (29). Yoshikai et al. (29) have reported that incubation of peritoneal macrophages from *C. parvum*-treated mice with concentrations of LPS which were nontoxic to macrophages from control mice leads to greater than 50% cell death by 6 h. They suggested that this effect may be at least partially responsible for the increased sensitivity of *C. parvum*-treated mice to the lethal effects of LPS. Ferluga et al. (7) have also reported that *C. parvum*-treated BALB/c mice were highly susceptible to the toxic effects of LPS. They provided evidence that extensive liver damage followed injection of LPS into such animals. However, in their study all injections were intravenous. Of interest, however, was their finding that a single injection of indomethacin (subcutaneously) immediately before injection of LPS prevented some of the liver damage. These authors did not report any adverse effect of indomethacin on the *C. parvum*-treated mice. This may not be unexpected since several injections of indomethacin were required to observe toxicity in the present study.

Goodrum et al. (11) have also reported that indomethacin antagonizes the toxic effects of LPS in mice. In their study, they found that treatment of mice with indomethacin 1 h before LPS injection leads to decreased release of a macrophage product, glucocorticoid-antagonizing factor, into the plasma. In addition, a single injection of indomethacin delayed the onset of LPS toxicity in lead acetate-treated mice (11).

From the reports described above as well as the present study, it would appear that indomethacin and LPS may exert influences on macrophages that are either similar or antagonistic, depending on the concentration used in the experiment and the state of the cells. It is obvious that our understanding of the interaction of indomethacin and LPS with macrophages (as well as other relevant cell types) must await additional investigation. However, it is plausible to speculate at this time that the continued presence of high concentrations of indomethacin causes the release of factors, such as glucocorticoid-antagonizing factor or interleukin-1, from macrophages, which in turn influence other essential biological systems. If such is the case, the results obtained in the present study with C3H/HeJ mice would indicate that either indomethacin bypasses the LPS-dependent steps in the process or there are multiple biochemical pathways to the same endpoint. In this context, the C3H/HeJ mouse may serve as a good model to investigate these possibilities.

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