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Investigation of Role of Nitric Oxide in Protection from *Bordetella pertussis* Respiratory Challenge

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*B. pertussis* is a human pathogen which possesses tropism for the respiratory system, causing an acute and sometimes persistent disease. Although pertussis vaccines have been in use for mass vaccination in most countries for many years and have led to a major decrease in the incidence of pertussis, the mechanism by which they induce protection against pertussis in children is still unclear. Recent evidence indicates that *B. pertussis* is a facultatively intracellular organism and that clearance involves activated macrophages (4, 15, 20, 21). The mechanism whereby macrophage activation results in the killing of facultatively intracellular pathogens is still incompletely determined. However, it has become increasingly apparent in recent years that NO and reactive nitrogen intermediates (nitrite and peroxynitrite) are potentially important mediators of the immune system (1). Production of NO by activated murine macrophages has been implicated as an antimicrobial effector mechanism against several pathogens (2, 5, 9). We have previously reported that macrophage activation produced by vaccination with WCV is associated with induction of NO synthesis by macrophages in response to in vitro stimulation with *B. pertussis* antigens. To determine whether NO production is an effector of protection or simply a marker of activation, the susceptibility of inducible nitric oxide synthase (type II, iNOS) knockout mice to infection with *B. pertussis* was examined. We showed that iNOS knockout mice were more susceptible to *B. pertussis* respiratory challenge than wild-type mice. iNOS-deficient mice also developed a less effective protective response than wild-type mice after the same immunization with WCV. This suggests that NO plays an important role in effecting protection against *B. pertussis* challenge.

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RESULTS

The time course of *B. pertussis* infection in wild-type and mutant mice was investigated by aerosol challenge on naive mice, and CFU counts in their lungs were taken at indicated time points (Fig. 1). Replication of the organisms resulted in similar increases in the numbers of bacteria recoverable from the two groups of mice, approximately 2.5 to 3.0 log units by day 7 to 10. However, bacterial counts subsequently declined more rapidly in wild-type mice than in iNOS knockout mice. Thus, by day 18, bacterial counts in iNOS-deficient mice were at least 0.5 log unit higher than those in wild-type mice, and this difference (*P* < 0.05) was maintained until the end of the experiment.

To assess the ability of murine peritoneal macrophages to generate NO in response to in vitro stimulation with HKC, mice were immunized with WCV or ACV. The peak time for production of NO in the macrophage cultures was approximately 10 to 15 days postimmunization (data not shown). Therefore, in this study all macrophages were isolated from mice on day 15 post immunization.

Peak NO concentrations in macrophage cultures from the wild type group were approximately threefold higher than those from the mutant group. Furthermore, macrophages from mice lacking iNOS produced lower NO concentrations in their cultures after in vitro restimulation with HKC than cells recovered from wild-type mice (Fig. 2a). This corresponded closely to the resistance to aerosol challenge observed in vivo in the two types of mice (Fig. 2b), where the CFU count per lung was approximately 2 log units lower in wild-type than in mutant mice.

Macrophages from mice immunized with ACV were also assayed for NO production with and without the addition of HKC. Unlike those from mice immunized with WCV, macrophages from mice immunized with ACV did not produce NO concentrations higher than those for the control group before the stimulation in vitro (baseline) (Fig. 3a). However, these macrophages were able to produce NO in response to *B. pertussis* HKC stimulation, although the levels were lower than those for the WCV-immunized group. NO concentrations in cultures from the wild-type group were more than threefold higher than those in cultures from the homozygous group. This also closely paralleled the pattern of in vivo protection against aerosol challenge, where CFU counts in the lungs of the mutant group were approximately 1.5 log units higher (*P* < 0.05) than those in the lungs of the wild-type group (Fig. 3b).

To investigate the relationship of the NO produced by activated macrophages to host defense against *B. pertussis* challenge, different immunization doses were used. Figure 4a shows that the synthesis of NO by macrophages in response to HKC restimulation was immunization dose dependent, and again, macrophages from the wild-type group had higher NO production than those from the mutant group. It was noted in the protection study that when mice were immunized with WCV at 1.0 IU/dose, similar reductions (of approximately 2.5 to 3.0 log units) in lung CFU counts occurred in both wild-type and mutant groups in comparison with the control group (Fig. 4b). This was in spite of the fact that cells from wild-type mice produced higher NO levels in their cultures than those from the mutant group. Reduction of the immunization dose from 1.0 to 0.25 IU resulted in different protection profiles for these two groups of mice, which corresponded with the NO concentrations achieved in their macrophage cultures. That is, the mutant mice showed a lower level of protection than the wild-type mice.

To investigate further the cellular and humoral immune responses induced by immunization of these two types of mice with WCV and ACV, pertussis-specific antibody production,
macrophage activation, and in vivo protection were assessed 4 weeks after immunization. Macrophages from both wild-type and mutant mice immunized with WCV produced approximately 5.5-fold-higher NO concentrations in their cultures after stimulation with HKC than macrophages from the ACV-immunized group ($P < 0.05$) (Fig. 5a). The NO concentration in macrophage cultures from the mutant group immunized with WCV was approximately half that for the wild-type group.

There was no difference ($P > 0.05$) in NO induction between the mutant group immunized with ACV and the control group.

Titers of antibody to PT, FHA, and PRN (69 kDa) were much higher in the ACV group than in the WCV group (Table 1). The ratio of IgG1 to IgG2a showed that mice immunized with WCV gave a response shifted towards Th1, whereas those immunized with ACV gave a response biased towards Th2. No difference in antibody production was found between wild-type
and mutant mice immunized with WCV. However, among mice immunized with ACV, lower levels of antibodies to FHA and PRN were observed in the mutant group than in the wild-type group. Aerosol challenge of mice at 4 weeks after immunization showed that mice immunized with WCV had developed better protection ($P < 0.05$) than mice immunized with ACV despite higher antibody responses developing in the latter group (Fig. 5b). Comparison of wild-type mice and mutant mice showed that the former group were better protected from the challenge than the latter group ($P < 0.05$) when immunized with WCV or ACV.

**DISCUSSION**

Infection by *B. pertussis*, usually manifesting as pertussis (whooping cough), is still an important cause of morbidity and mortality among children in many parts of the world (18). It is also being recognized increasingly as a significant agent of respiratory disease in adults (Editorial, Lancet **339**:526–527, 1992). The results of recent clinical trials have indicated that both established WCVs and the new generation of ACVs can stimulate protection in children (6, 7). We have previously reported that macrophage activation produced by vaccination...
with WCV is associated with induction of NO synthesis by macrophages in response to in vitro stimulation with *B. pertussis* antigens (20, 21). In the present study, iNOS knockout mice were used to further examine the role of NO in protection from *B. pertussis* respiratory challenge.

It has been reported previously that iNOS-deficient mice produced more gamma interferon (IFN-γ) and less interleukin 10 than similarly treated intact mice following infections and antigenic stimulation (10, 12, 13). In the present study, the results showed that elimination of *B. pertussis* from the lungs of infected iNOS knockout mice was slower than elimination from the lungs of wild-type mice after respiratory challenge. The iNOS-deficient mice also showed a lower level of protection than the wild-type mice following the same immunization with WCV or ACV in spite of producing a greater IFN-γ response to the bacterial antigens in vitro (data not shown). This provides a further indication that NO is an important effector molecule in protection against *B. pertussis* challenge.

Macrophages from wild-type and mutant mice immunized with the WCV all produced NO in response to in vitro stimulation with bacterial cells. This suggests that this type of vaccine is a very powerful inducer of NO synthase even in iNOS-deficient mice. That the latter still produced some NO in spite of not completely eliminating it (3, 8). In the present study, these mice produced substantially less NO than wild-type mice. However, NO may not be the only effector of protection. It was notable that when mice were immunized with higher doses of the vaccine, there was no difference in protection between wild-type and mutant mice, even though the wild-type mice produced more NO in their macrophage cultures. This may have been because the high vaccine doses stimulated an adequate NO response in the knockout mice and the greater amount produced by the wild-type mice added nothing further to protection, or it may suggest that at high vaccine doses another mechanism comes into play which is not dependent on the bactericidal action of NO.

Macrophages isolated from mice immunized with WCV produced larger amounts of NO than those from the control group without additional stimulation. Our previous studies showed that this NO production was increased by adding HKC but not by IFN-γ and that NO induced by HKC was only partially blocked by concentrations of anti-IFN-γ which completely blocked NO production in control cell cultures (20). Taken together, these results suggested that these macrophages had already been activated in vivo. It is noteworthy that macrophages from mice immunized with ACV did not produce NO in vitro in the absence of stimulant. However, NO production was significantly increased by the addition of HKC, and this was clearly associated with protection in vivo. These results suggested that there might be a difference in the degree of macrophage activation produced in vivo by immunization with these two different types of vaccines. Furthermore, mice immunized with ACV produced lower IFN-γ levels (data not shown) in culture than those immunized with WCV after stimulation in vitro. This may indicate that mice immunized with WCV developed a stronger Th1 type response than those that received ACV.

Although pertussis vaccination is used throughout the world and has made a major contribution to decreasing morbidity and mortality from pertussis, its precise mode of action is still unclear. There is, however, increasing recognition of the importance of cell-mediated immunity in protection against *B. pertussis*. The present study using iNOS knockout mice has provided direct evidence that the reactive nitrogen intermediates play an important role in the immune response induced by both WCV and ACV and that this is associated with protective immunity in vivo. This adds further weight to the hypothesis that activation of the killing mechanisms of macrophages helps to eliminate intracellular *B. pertussis* and hence to clear infection.

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