Secretion of Functional Monocyte Chemotactic Protein 3 by Recombinant Mycobacterium bovis BCG Attenuates Vaccine Virulence and Maintains Protective Efficacy against M. tuberculosis Infection

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Received 7 June 2006/Returned for modification 23 July 2006/Accepted 17 October 2006

A strain of Mycobacterium bovis BCG that secretes high levels of functional murine monocyte chemotactic protein 3 (BCGMCP-3) was developed. Mice vaccinated with BCGMCP-3 displayed increased lymphocyte migration in vivo and augmented antigen-specific T-cell responses compared to mice vaccinated with BCG alone. The level of protection afforded by BCGMCP-3 was equivalent to that with control BCG; however, immunodeficient mice infected with BCGMCP-3 survived significantly longer than mice infected with the control BCG strain. Therefore, BCGMCP-3 may be a safer alternative than conventional BCG for vaccination of immunocompromised individuals.

Tuberculosis (TB) remains a leading cause of worldwide mortality alongside human immunodeficiency virus and malaria. Recent figures suggest that each year there are approximately 8 million new cases and 2 million deaths (17). The current vaccine, Mycobacterium bovis bacillus Calmette-Guérin (BCG), is only partially effective against lung TB, the contagious form of the disease, with vaccine efficacy ranging from 0 to 80% (median of 50%) (7). Consequently, although BCG is considered a safe vaccine and has been administered since 1921 to an estimated 3 billion people, it has proved insufficient to control the spread of M. tuberculosis. Partially effective against lung TB, the contagious form of the disease, BCG is amenable to genetic manipulation, and overexpression of protective antigens in BCG can improve the efficacy of the vaccine in animal models of TB (9, 20, 23). An alternative approach is to express immunostimulatory molecules in BCG. Murine cytokines, including interleukin-2, interleukin-18, gamma interferon (IFN-γ), and granulocyte-macrophage colony-stimulating factor, can be secreted by BCG in a functional form; however, the ability of these vaccines to protect against TB has not been tested (13, 16, 18). The monocyte chemotactic proteins (MCP) are a subset of the CC or β-chemokines consisting of four members, MCP-1, -2, -3, and -4 (with MCP-5 in the mouse only), which are induced by viral and bacterial infections in many cell types (26, 31). MCP-3 is a pleiotropic chemokine that binds the receptors CCR1, CCR2, and CCR3 (15). These receptors are expressed on mononuclear phagocytes, T cells, B cells, natural killer cells, basophils, eosinophils, neutrophils, and particularly immature dendritic cells (1, 3, 11, 26, 32). MCP-3 is highly expressed by macrophages exposed to mycobacterial components or M. tuberculosis-infected mice (27, 30). Mice lacking CCR2 were acutely susceptible to M. tuberculosis infection and exhibited a significant reduction in early macrophage recruitment and a later defect in dendritic cell and T-cell migration (22). These results clearly demonstrate that chemokines, such as MCP-3, are vital in the response to M. tuberculosis infection, as they are required for the selective migration of the cells essential for the generation of a protective immune response.

In this report, we demonstrate that murine MCP-3 (mMCP-3) can be expressed and secreted by BCG in an active form. Secretion of MCP-3 markedly improved the immunogenicity of BCG after vaccination of mice. BCGMCP-3 was less virulent than BCG alone in immunodeficient animals; however, both strains displayed similar levels of protective efficacy against M. tuberculosis infection in immunocompetent mice.

Expression of murine MCP-3 by BCG augments lymphocyte migration in vivo. In order to construct BCG secreting MCP-3, the gene encoding mMCP-3 was amplified by PCR from the MCP-3/MSP4/5 vector (25) and cloned into pRBD4, a derivative of the pMV261 expression vector (18). This resulted in pJEX57, in which the chemokine is fused to the M. bovis BCG Ag85B secretory signal sequence. Plasmid pMV261 was used as the control vector (29). Both pJEX57 and pMV261 were transformed into M. bovis BCG Pasteur strain 1173P2 as previously described (21). BCG strains were grown, as described previously (28), and mycobacterial cell lysates were prepared by sonication of cultures harvested at mid-log phase. The expression of mMCP-3 was verified by immunoblotting using the 12CA5 monoclonal antibody specific for the hemagglutinin epitope tag present at the N terminus of the mature protein. The size of the 14.3-kDa protein correlated with the predicted mass of the fusion of uncleaved Ag85B signal peptide and recombinant MCP-3 (Fig. 1A). Supernatants from 10-day cultures were collected and secreted mMCP-3 detected using an MCP-3-specific capture enzyme-linked immunosorbent assay (PeproTech, Rocky Hill, NJ). MCP-3 was readily detected in supernatants of BCGMCP-3 cultures but not in BCG containing the control vector (BCGpG) (Fig. 1B).

We next determined if MCP-3 was secreted by BCG in a...
A protein of approximately 14 kDa was detected only in BCGMCP-3. A cross-reactive protein of 42 kDa was detected in both strains. An mMCP-3-specific enzyme-linked immunosorbent assay was used to detect secretion of the mature protein in 10-day-culture supernatants. (C) C57BL/6 mice (n = 3) were infected intraperitoneally with 1 × 10^6 CFU BCGMCP-3 or BCGCt. Four days after intraperitoneal infection with rBCG, the numbers of CD4^+ (black columns), CD8^+ (gray columns), and CD11b^- (white columns) leukocytes in peritoneal fluid were analyzed by flow cytometry. The results are expressed as the means of triplicates ± standard errors of the means, and significance of differences was determined by analysis of variance (*, P < 0.05; **, P < 0.005). The data are representative of one of two individual experiments. (D) C57BL/6 mice (n = 3) were infected s.c. with 5 × 10^5 CFU of BCGMCP-3 or BCGCt. Five weeks postimmunization, the mean number of IFN-γ-secreting cells from cultured draining lymph node (LN) cells restimulated with 10 μg/ml BCG lysate was measured by enzyme-linked immunospot assay. The results are expressed as means of triplicates ± standard errors of the means (*, P < 0.05; **, P < 0.001). The data are representative of one of three individual experiments.

form that could influence cellular movement in vivo. Six- to 8-week-old female C57BL/6 mice (Animal Research Centre, Perth, Australia) were infected intraperitoneally with 1 × 10^6 CFU of BCGMCP-3 or BCGCt. Four days postinfection, the cellular infiltrates into the peritoneal cavity were analyzed by flow cytometry with anti-CD11b–fluorescein isothiocyanate, anti-CD4–allophycocyanin, and anti-CD8–peridinin chlorophyll protein (BD Pharmingen, San Diego, CA). As shown in Fig. 1C, there was an increased number of CD4^+ and CD8^+ T cells in the peritoneal exudate of BCG-vaccinated mice compared to that of uninfected animals. Vaccination with BCGMCP-3 resulted in a significantly greater number of CD4^+ and CD8^+ T cells than vaccination with BCG alone (Fig. 1C). A decrease in CD11b^- cells was also observed after vaccination with the recombinant BCG (rBCG) strains, and this was most apparent after vaccination with BCGMCP-3. Although this decrease was not statistically significant, the results suggest the secretion of MCP-3 by BCG may also influence migration of cells out of the peritoneal fluid. The results indicate that BCG-derived mMCP-3 is able to influence cellular migration in vivo, implying that the chemokine is efficiently processed in a functionally active form by BCGMCP-3.

Immunization with BCGMCP-3 enhances antigen-specific immune responses. We next assessed if the secretion of MCP-3 by BCG could influence antimycobacterial immunity compared to the parental BCG strain. Female C57BL/6 mice were infected via subcutaneous (s.c.) injection at the base of the tail with 5 × 10^5 CFU of BCGMCP-3 or BCGCt. Five weeks postimmunization, single-cell suspensions from draining lymph nodes were resuspended in RPMI medium containing 10% (vol/vol) fetal calf serum, 10 mM HEPES buffer, 10 mM sodium bicarbonate, 2 mM l-glutamine, and 50 μM 2-mercaptoethanol. Antigen-specific IFN-γ-secreting cells restimulated with BCG lysate (10 μg/ml) were measured by enzyme-linked immunospot assay. The results indicate that BCGMCP-3 is able to influence cellular migration in vivo, implying that the chemokine is efficiently processed in a functionally active form by BCGMCP-3.
Therefore, the secretion of functional MCP-3 by BCG can influence the development of Th1-like immunity.

Expression of MCP-3 by BCG protects against aerosol M. tuberculosis challenge and influences BCG virulence in immunodeficient mice. After verifying that MCP-3 expressed by rBCG was secreted and functional, we investigated if the recombinant strain could enhance the protective efficacy against M. tuberculosis challenge. Five and 12 weeks postvaccination with 5 × 10^8 CFU of BCG<sub>MCP-3</sub> and BCG<sub>Ct</sub>, mice were exposed to M. tuberculosis H37Rv (ATCC 27294) via the aerosol route (12). Four weeks following M. tuberculosis challenge, the bacterial loads in the lungs and spleens of infected mice were determined by plating on Middlebrook 7H11 agar supplemented with 10% oleic acid-albumin-dextrose-catalase enrichment. Both BCG<sub>MCP-3</sub> and BCG<sub>Ct</sub> afforded a significant reduction in bacterial growth in the lungs (Fig. 2A and B) and spleens (data not shown) compared to levels for nonimmunized mice. There was no significant difference in protective efficacy afforded by the two strains. In order to assess the influence of MCP-3 secretion on in vivo growth of the recombinant vaccine, mice were infected with 1 × 10^6 CFU of BCG<sub>MCP-3</sub> or BCG<sub>Ct</sub> via intravenous (i.v.) injection and bacterial loads determined. BCG<sub>MCP-3</sub> was cleared more rapidly than BCG<sub>Ct</sub>, particularly in the lungs of infected mice (Fig. 2C and D). To further assess the safety of BCG<sub>MCP-3</sub>, RAG-1<sup>−/−</sup> mice were infected i.v. with 1 × 10^6 CFU of BCG<sub>MCP-3</sub> or BCG<sub>Ct</sub>. BCG-infected mice were monitored daily and culled if displaying signs of ill health, including reduced activity, ruffling of fur, and weight loss (exceeding 15% of age-matched controls). Survival was calculated on a Kaplan-Meier nonparametric survival plot, and significance was assessed by log rank Mantel-Cox test. RAG-1<sup>−/−</sup> mice infected with BCG<sub>Ct</sub> succumbed to infection at approximately 100 days (Fig. 2E). Mice infected with BCG<sub>MCP-3</sub> survived significantly longer than those infected with the control BCG strain. Together, these data indicate that secretion of MCP-3 by BCG reduced the virulence of the vaccine while maintaining protective efficacy.

In this report we have shown that a murine chemokine expressed by BCG can enhance the safety profile of the BCG vaccine while still providing an equivalent level of protective efficacy against aerosol M. tuberculosis challenge. This is the first time that expression of a functional mammalian chemokine by a recombinant BCG strain has been reported. The mature protein secreted by BCG demonstrated functional activity, as both CD4<sup>+</sup> and CD8<sup>+</sup> T cells infiltrating the peritoneal cavities of mice were significantly increased upon infection with BCG<sub>MCP-3</sub> compared to levels upon infection with control BCG strain (Fig. 1C). The migration of both human and mouse CD4<sup>+</sup> and CD8<sup>+</sup> T cells in response to MCP-3 has been well documented, as both T-cell subsets express the chemokine receptors CCR1, -2, and -3 (8, 24). Fioretti et al. observed that human MCP-3-transfected tumors displayed increased CD4<sup>+</sup> and CD8<sup>+</sup> T-cell subsets in vivo compared to mock-transfected controls (8, 24). The increase in lymphocyte migration upon immunization with BCG<sub>MCP-3</sub> correlated with an enhanced antigen-specific IFN-γ response in vivo directed toward BCG antigens. Previous studies have demonstrated that DNA vaccines encoding human immunodeficiency virus and malarial antigens fused with MCP-3 enhance the antigen-specific immune response (4, 25). Similarly, fusion of MCP-3 to a tumor antigen enhanced the protective response in a T-cell-dependent manner upon tumor challenge (5). The ability of BCG-derived MCP-3 to influence T-cell migration (Fig. 1C) may directly increase the attraction of antigen-reactive T cells to the site of antigen presentation. Indeed, we observed enhanced stimulation of antigen-specific, IFN-γ-secreting T cells 14 days postvaccination with BCG<sub>MCP-3</sub> compared to results postvaccination with control BCG (data not shown), suggesting an early effect of MCP-3 on cell migration and resultant T-cell immunity.
The secretion of MCP-3 by BCG led to an enhancement in antigen-specific IFN-γ responses postvaccination; however, this did not translate into an increase in protective efficacy compared to results with the control BCG strain (Fig. 2). This suggests that IFN-γ release may not be an absolute correlate of protection against \textit{M. tuberculosis}, and such a finding has been demonstrated previously for anti-TB vaccine candidates (10, 12, 14). Despite this lack of improved protective efficacy, secretion of MCP-3 by BCG significantly prolonged the survival of RAG-1−/− mice infected with this strain compared to that of mice infected with the parental strain (Fig. 2E). This enhanced survival may be attributed to the attenuated phenotype of BCG-MCP-3 in vivo (Fig. 2C and D). Indeed, a murine melanoma tumor transfected with human MCP-3 displayed attenuated growth in nude mice, and this effect was attributed to an increase in intratumor macrophage infiltrate (6). As the BCG-MCP-3 vaccine strain showed an improved safety profile without any loss in protective efficacy, this vaccine may have applications for control of TB in human populations, especially in immunocompromised individuals.

We thank Michael O’Donnell, University of Iowa Hospitals and Clinics, Iowa City, IA, for providing the pRBD4 vector and Terry Spithill, Monash University, Victoria, Australia, for providing the mMCP-3 cDNA. This work was supported by the National Health and Medical Research Council of Australia. A. A. Ryan was supported by an Australian postgraduate award. The support of the NSW Health Department and the ARC Special Research Grant of Australia. A. A. Ryan was supported by an Australian postgraduate award. The support of the NSW Health Department and the ARC Special Research Grant of Australia. We thank the NSW Health Department for providing the University of Iowa Hospitals and Clinics, Iowa City, IA, for providing the pRBD4 vector and Terry Spithill, Monash University, Victoria, Australia, for providing the mMCP-3 cDNA. This work was supported by the National Health and Medical Research Council of Australia. A. A. Ryan was supported by an Australian postgraduate award. The support of the NSW Health Department and the ARC Special Research Grant of Australia. A. A. Ryan was supported by an Australian postgraduate award. The support of the NSW Health Department and the ARC Special Research Grant of Australia. We thank the NSW Health Department for providing the University of Iowa Hospitals and Clinics, Iowa City, IA, for providing the pRBD4 vector and Terry Spithill, Monash University, Victoria, Australia, for providing the mMCP-3 cDNA. This work was supported by the National Health and Medical Research Council of Australia. A. A. Ryan was supported by an Australian postgraduate award. The support of the NSW Health Department and the ARC Special Research Grant of Australia.

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Editor: J. L. Flynn