Differential Interleukin-10 and Gamma Interferon mRNA Expression in Lungs of Cilium-Associated Respiratory Bacillus-Infected Mice

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Received 20 October 2000/Returned for modification 13 December 2000/Accepted 5 March 2001

The cilium-associated respiratory (CAR) bacillus is a gram-negative, extracellular bacterium that causes persistent respiratory tract infections in rodents. We have previously demonstrated that BALB/c mice are more susceptible to CAR bacillus-induced disease than resistant C57BL/6 mice, with elevations in pulmonary gamma interferon (IFN-γ) and interleukin (IL)-4. IL-10 is a type 2 cytokine that can increase host susceptibility to bacterial diseases through its anti-inflammatory effects, including suppression of macrophage function. The purpose of this study was to further describe the cytokine profiles associated with histologic lesions in CAR bacillus-infected mice and to assess the effects of cytokine depletion on the pathogenesis of disease. Six-week-old female BALB/c and C57BL/6 mice and mice with targeted mutations in IFN-γ and IL-4 were inoculated intratracheally with 10⁶ CAR bacillus organisms, and samples were collected at 6 to 7 weeks postinoculation. Lung samples were collected for histopathologic examination and analysis of cytokine mRNA. IFN-γ, IL-10, and IL-4 mRNA levels in the lungs of infected mice were semiquantitatively measured using a reverse transcriptase-mediated PCR assay and compared to those in uninfected control animals of each strain. BALB/c mice infected with CAR bacillus had a median lung lesion score of 6 and IL-10 and IL-4 mRNA levels were significantly elevated. The majority of C57BL/6 mice were resistant to disease characterized by lung lesions scores of 2 or less and a dominant IFN-γ mRNA cytokine profile. A few C57BL/6 mice with lesions scores 5 or greater had elevations in all three cytokines and were susceptible to disease. C57BL/6 IFN-γ knockout mice had increased disease with elevations in IL-10 and IL-4 mRNA, while BALB/c IL-4 knockout mice infected with CAR bacillus had a mild decrease in lesion severity and an attenuated IL-10 mRNA expression compared to wild-type BALB/c mice. These data indicate that IL-10 and IL-4 predominate in CAR bacillus-induced histologic lesions in mice, while IFN-γ may play a role in resistance to disease.

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To test our hypothesis and to further characterize the cytokine profiles associated with CAR bacillus-induced disease, mRNA levels of IL-4, IL-10, and IFN-γ in lung homogenates were evaluated in BALB/c and C57BL/6 (B6) mice. A predominant IL-10 mRNA expression was associated with severe lesions in BALB/c, whereas disease was less severe with a predominant IFN-γ mRNA expression in B6 mice. To assess whether IFN-γ or IL-4 contributed to the pathogenesis of CAR bacillus-induced disease, B6 mice with targeted mutations in IFN-γ and BALB/c mice with targeted mutations in IL-4 were inoculated with CAR bacillus. Lesions were more severe in B6 mice with an IFN-γ mutation, suggesting that IFN-γ may have a protective role against CAR bacillus disease. BALB/c mice with IL-4 deficiency had attenuated IL-10 responses and abrogated disease. These results suggest there is a dysregulation of the cytokine network in response to CAR bacillus-induced disease, with the type 2 cytokines IL-4 and IL-10 predominating during disease.

MATERIALS AND METHODS

CAR bacillus culture. A mouse isolate of CAR bacillus (provided by Tom Spencer, National Institutes of Health) was maintained in cell culture on murine 3T3 fibroblasts in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum (34). Prior to inoculation, flask containing CAR bacillus were scraped, and cellular debris was removed by centrifugation at 900 × g for 10 min. The bacteria were pelleted by centrifugation at 20,000 × g for 10 min and resuspended in 1 ml of phosphate-buffered saline (PBS; 15 mM NaH2PO4, 108 mM Na2HPO4, 1.4 M NaCl [pH 7.4]). Bacterial concentration was determined using a hemocytometer, and bacterial suspensions were diluted with PBS to achieve a final inoculum concentration of 108 CAR bacillus organisms per 30 μl of PBS (14). Prior to inoculation, the CAR bacillus inoculum was checked for cell viability by dual staining (13). Briefly, 2 × 106 CAR bacillus organisms were stained with 2 μg of fluorescein diacetate (5 mg/ml in acetone) and 0.6 μg of propidium iodide (20 μg/ml in PBS) at room temperature for 3 min and then placed on ice for 15 min. When viewed with a fluorescence microscope, viable cells appeared green and dead cells appeared red. Greater than 95% of the inoculum was viable prior to inoculation.

Mycoplasma screen. To ensure that the CAR bacillus inoculum was free of Mycoplasma, PCR assays were performed on the cell cultures prior to inoculation. Mammalian cell pellets and bacterial cell pellets from CAR bacillus cultures were resuspended in 200 μl of PBS, and DNA was isolated using a Qiaamp tissue kit (Qiagen, Santa Clarita, Calif.) according to the manufacturer’s instructions. Mycoplasma PCR assays, using primers known to amplify all species of Mycoplasma, were performed on isolated DNA as previously described (42). All inoculated mice were also screened for antibodies to Mycoplasma pulmonis by enzyme-linked immunosorbent assay (Research Animal Diagnostic and Investigative Laboratory, University of Missouri—Columbia). Select mice inoculated with CAR bacillus were further assessed for exposure to M. pulmonis by culturing lung swabs on selective media and by PCR-based testing of paraffin-embedded lung sections.

Mice. Six-week-old female mice were obtained free of pathogens including the respiratory pathogens CAR bacillus, M. pulmonis, Sendai virus, and pneumonia virus of mice. Mice were housed in microisolator cages in accordance with the Guide for the Care and Use of Laboratory Animals (23). BALB/c and B6 mice were obtained from the Frederick Cancer Research and Development Facility (Bethesda, Md.). BALB/c and/or C57BL/6 mice with targeted mutations in the IL-4 gene (BALB/c IL-4 knockout [KO] mice) and C57BL/6-fipm1172T mice with targeted mutations in the IFN-γ gene (B6 IFN-KO mice) were obtained from The Jackson Laboratory (Bar Harbor, Maine).

Experimental model. Mice were anesthetized with isoflurane, and a ventral incision was made to expose the trachea. Mice were given 30 μl of bacterial inoculum injected directly into the trachea. Control mice were sham inoculated with 30 μl of PBS. The skin incision was closed with surgical adhesive (Nexaband, Veterinary Products Laboratories, Phoenix, Ariz.). Mice were euthanized by CO2 asphyxiation, and samples were collected at 6 and 7 weeks postinoculation, a time at which susceptible mice develop significant disease (14). Samples were evaluated from five to seven inoculated wild-type mice of each strain, four to five inoculated mice of each mutant strain, and three to four sham-inoculated control mice from each strain.

A portion of the left lung lobe at the level of the bronchi was fixed in Omnifix (Ancon Genetics, Inc., Melville, N.Y.) and processed for histologic evaluation. Samples of lung were embedded in paraffin, 5-μm sections were stained with hematoxylin and eosin, and lesion severity was evaluated. Lesions were scored on a scale of 1 (no significant lesions) to 7 (severe bronchopneumonia with peri-bronchiolar lymphoid follicles and inflammatory cell infiltrates) on the basis of criteria previously described (14).

The remaining lung sample was processed for mRNA evaluation. The lung sample was placed in 2.0 ml of guanidine thiocyanate and phenol tissue lysis buffer (Ultraspec RNA isolation system; Biotex Laboratories, Inc., Houston, Tex.), homogenized in a glass-Teflon tissue homogenizer (Con-Torque power unit; Eberbach Corp., Ann Arbor, Mich.), and stored at −70°C until assayed.

Colonization status was determined by PCR and Southern blot analysis as previously described (14). Briefly, PCR was performed on DNA isolated from 50-μm sections of paraffin-embedded lung tissue. Southern blot analysis of PCR products was performed to confirm the colonization status of mice, using digoxigenin-labeled probes synthesized from DNA from a purified culture of CAR bacillus (DIG nonradioactive nucleic acid labeling system and detection system; Boehringer Mannheim Corp., Indianapolis, Ind.).

Competitive RT-PCR. Competitive reverse transcriptase (RT)-mediated PCR (RT-PCR) assays were used to assess type 1 (IFN-γ) and type 2 (IL-4 and IL-10) cytokine responses. Total RNA from homogenized lung sections was isolated using the Ultraspec RNA isolation system (Biotex) according to the manufacturer’s instructions and quantified (GeneQuant RNA/DNA calculator; Pharmacia Biotech, Piscataway, N.J.). Using oligo(dT) primers and a modified Moloney murine leukemia virus RT (Superscript; Life Technologies, Rockville, Md.), 2.5 μg of total RNA was reverse transcribed in duplicate. PCR mixtures contained 200 μM each deoxynucleoside triphosphate, 0.4 μM each primer, 3 mM MgCl2, and 1.25 U of AmpliTaq Gold (Perkin Elmer, Foster City, Calif.). The cDNA equivalent of 50 ng of total RNA was coamplified by PCR with serial dilutions of a competitor DNA plasmid (pCFH) that shared cytokine-specific primer binding sites (graciously provided by Argyrios N. Theofilopoulos, The Scripps Research Institute) (31). Samples were heated at 95°C for 9 min for initial denaturation and activation of the polymerase followed by 45 PCR cycles consisting of a 30-s denaturation at 94°C, 15-s annealing at 55°C (IFN-γ and hypoxanthine phosphoribosyltransferase [HPRT]) or 60°C (IL-4 and IL-10), and 45-s extension at 72°C. A final 10-min extension at 72°C was performed.

Quantitation of cDNA. Target amplicons and competitor amplicons were electrophoretically separated on a 2% agarose gel (Nusieve; FMC Bioproducts, Rockland, Maine) in Tris-borate-EDTA buffer containing 0.01% ethidium bromide (10 μg/ml). The gel was placed on an UV light box, and a digital image was captured (Electrophoresis Documentation and Analysis System version 2.0; Eastman Kodak Co., Rochester, N.Y.). The bands were densitometrically analyzed (Kodak Scientific Imaging System version 2.0; Eastman Kodak Co., New Haven, Conn.) for each competitor dilution to determine the number of molecule equivalents of cytokine-specific mRNA in the sample as previously described (31). Cytokine mRNA estimates were normalized by dividing by the number of molecules of cytokine mRNA by the number of mRNA molecules of the housekeeping HPRT gene. RT-PCR evaluations of cytokines and HPRT were performed in triplicate for each animal, unless two consecutive tests yielded no detectable cytokine. The median value of the three runs was used to estimate mRNA levels for individual mice.

Statistical analysis. Data was evaluated using SigmaStat Statistical Software (SPSS Marketing, San Rafael, Calif.). Histologic scores are reported as median values and were analyzed using a one-way analysis of variance on ranks test followed by a pairwise comparison using Dunn’s method. The average (± standard error of the mean [SEM]) cytokine mRNA levels for each group of mice are reported. Differences in cytokine mRNA levels among mouse strains were analyzed using Student’s t test when data were normally distributed. If the normality test failed, a Mann-Whitney rank sum test was performed.

RESULTS

Predominant IL-10 in BALB/c mice with CAR bacillus infection. BALB/c mice were more susceptible than B6 mice to CAR bacillus-induced disease, as indicated by the more severe histologic lesion scores (Table 1). Cytokines in lung homogenates were evaluated by competitive RT-PCR to characterize
the immune response associated with lung lesions in wild-type mice (Fig. 1). Type 1 responses were assessed by the presence of IFN-γ, and type 2 responses were assessed by the presence of IL-4 and IL-10. Compared to controls, BALB/c mice infected with CAR bacillus had a 25-fold elevation in IL-10 mRNA (P < 0.02) and a 7-fold elevation in IL-4 mRNA (P < 0.03) but no significant elevation in IFN-γ mRNA. These data suggest that a type 2 response with predominant IL-10 mRNA expression may contribute to the pathogenesis of CAR bacillus infection.

**Resistant and susceptible B6 mice.** Histologic lesions of CAR bacillus-infected B6 mice revealed resistant and susceptible mice (Table 1). Of the seven B6 mice tested, five were resistant (as determined by lesion scores of 2 or less) and two were susceptible (as determined by lesion scores of 5 or greater). The cytokine mRNA levels were evaluated based on these differences (Fig. 2). In resistant B6 mice, IFN-γ mRNA expression was elevated twofold, with no detectable elevations in IL-4 or IL-10 mRNA levels. Susceptible B6 mice had elevations in all three cytokines. The predominant cytokine expressed was IL-10, the level of which was 22-fold higher than in control mice. The IL-4 mRNA level was 13-fold higher than in controls, while there was only a 6-fold increase in IFN-γ mRNA. These data suggest that type 1 cytokines have a protective role in CAR bacillus-induced disease. Furthermore, disease in susceptible B6 mice accompanied by a type 2 cytokine response was similar to that seen in susceptible BALB/c mice, supporting the suggestion that type 2 cytokines contribute to disease pathogenesis.

**Lesions are more severe in B6 IFN KO mice.** To further assess the protective role of IFN-γ in diseases, B6 IFN KO mice were infected with CAR bacillus. At 7 weeks postinoculation, lung samples were collected for histology and lung homogenates were assayed for IL-4 and IL-10 mRNA (Fig. 3). Lesions were more severe in B6 IFN KO mice (median lesion score of 4 [P < 0.001], versus 1 for resistant B6 mice). Associated with the increased disease severity, B6 IFN KO mice had a fourfold elevation in IL-4 mRNA (P < 0.05) and a 16-fold elevation in IL-10 mRNA (P < 0.01) compared to resistant B6 mice. These findings support the hypothesis that IFN-γ plays a protective role in CAR bacillus-induced disease and that when IL-10 predominates, disease is more severe.

**Attenuated type 2 cytokines alter lesion severity in CAR bacillus-infected mice.** To examine the role of attenuated type 2 cytokines in the pathogenesis of CAR bacillus-induced disease, histologic lesion scores and IFN-γ and IL-10 mRNA levels were assessed in BALB/c IL-4 KO mice (Fig. 4). Infected BALB/c IL-4 KO mice had a fivefold decrease in IL-10 mRNA compared to wild-type BALB/c mice (P < 0.05). There was a corresponding decrease in median lesion score; however, this decrease was not significant (P = 0.11). These data indicate that IL-4 deficiency in BALB/c mice results in decreased IL-10, with slightly abrogated disease. Although IL-10 was decreased,
it remained the dominant cytokine during disease, which may be the reason there was not a more profound change in disease severity.

DISCUSSION

We previously demonstrated that BALB/c mice are more susceptible to CAR bacillus-induced disease than B6 mice (14). In those studies, 6 to 7 weeks postinoculation, more than 80% of the BALB/c mice infected (14 of 17) developed a bronchopneumonia with a lesion score greater than 3 with heavy bacterial colonization. In contrast, more than 60% of the B6 mice (11 of 17) had a lesions score of 2 or less with minimal bacterial colonization. The present study corroborates these findings and further identifies differential cytokine expression associated with disease.

Mice that developed bronchopneumonia in response to CAR bacillus infection had an associated IL-10 and IL-4 cytokine response. Conversely, IFN-γ was the predominant cytokine expressed in resistant mice. When IL-4 was eliminated from susceptible BALB/c mice, IL-10 mRNA expression decreased, as did lesion severity, albeit not significantly. When IFN-γ was eliminated from resistant B6 mice, disease progression was more severe and IL-10 mRNA expression increased. These findings support the hypothesis that type 2 cytokines play a role in the pathogenesis of CAR bacillus disease, while IFN-γ appears to contribute to resistance to CAR bacillus disease.

CAR bacillus-induced disease was more severe in mice when the IL-10 response dominated, suggesting that this cytokine either contributes to disease pathogenesis or is elevated in response to the disease state. What role IL-10 plays in CAR bacillus-induced disease pathogenesis is unclear. IL-10 is a cross-regulatory cytokine originally found in Th2 cells that acts synergistically with IL-4 to inhibit IFN-γ production by Th1 cells (21) and influences macrophage and B-cell function (18). The production of proinflammatory cytokines (TNF-α, IL-1, IL-6, and IL-12) by macrophages is inhibited in the presence of IL-10. The result is a suppressed macrophage with ineffective microbicidal activity (4). In contrast to its effects on macrophages and T cells, IL-10 enhances B-cell activity, with increased production of immunoglobulins M and A (12, 28).

It is possible that CAR bacillus initiates an anti-inflammatory response with IL-10 production that decreases bactericidal effector cells allowing persistent colonization (44). An alternative explanation is that IL-10 is produced in response to an overexuberant immune response to prevent host cell damage. For example, the down-regulation of proinflammatory cytokines, particularly TNF-α, by IL-10 is one mechanism used to regulate the host immune response (35). We have previously demonstrated persistent elevations of TNF-α during CAR bacillus disease (14), and others have associated overexuberant TNF-α responses with acute respiratory distress syndrome (38). It is conceivable that instead of having a detrimental effect on the host immune response, IL-10 responses may be a means of limiting the severity of disease caused by CAR bacillus-induced TNF-α. Further studies are necessary to clarify the role of IL-10 in CAR bacillus disease pathogenesis.

In contrast to susceptible mice, the majority of B6 mice were resistant to disease. However, resistance to CAR bacillus disease is not an all-or-none phenomenon, as demonstrated in our previous study (14). In our studies, the majority of B6 mice were resistant to disease. However, 3 of 17 mice developed histologic lesions with a score greater than 3 and were heavily colonized with CAR bacillus (14). In this study, two B6 mice developed disease. Interestingly, disease in these two mice, like that of BALB/c mice, was accompanied by a predominant IL-10 response; further suggesting that this cytokine either contributes to disease progression or is produced, with ineffective results, in response to disease.

In the majority of B6 mice, no disease developed and the predominant cytokine expressed was IFN-γ, suggesting that this cytokine helps to protect against CAR bacillus-induced disease. Like IL-10, IFN-γ is an important regulator of macrophage activity; however, the effects of these two cytokines are often opposing. IFN-γ derived from T cells and NK cells potentiates the microbicidal effects of macrophages (2) and enhances innate immunity. For example, studies with M. pulmonis have demonstrated that there is an increase in the mRNA expression of TNF-α and IFN-γ within the first 48 h postinoculation in resistant B6 mice that are able to clear infection (3). This clearance is most likely the result of a robust innate immune response mediated by alveolar macrophages (11) whose activity is enhanced by IFN-γ (2). Susceptible mice do not have evidence of an early innate immune response (3). These mice develop severe lesions and have persistent elevations in cytokines that likely contribute to the pathogenesis of disease (26). It is conceivable that a similar situation occurs during CAR bacillus disease in resistant B6 mice; however, studies evaluating the acute phases of CAR bacillus disease are necessary to elucidate the role of innate immunity in conferring resistance to CAR bacillus disease.

In CAR bacillus-induced disease, when IFN-γ was eliminated (B6 IFN KO mice), there was an increase in lesion scores compared to wild-type resistant B6 mice, and this increased disease was accompanied by elevations in mRNA of the type 2 cytokines IL-4 and IL-10. Since mice deficient in IFN-γ have a reduced innate immunity that can affect the clearance of pathogens (2, 24), these results suggest that innate immune responses play an important role in conferring protection against CAR bacillus. These findings also support the hypothesis that IL-10 either contributes to disease progression or is produced in response to disease.

Because the production of other type 2 cytokines is reduced

FIG. 4. Lung homogenate cytokine mRNA levels (average plus SEM) and median lesion scores from BALB/c IL-4 KO mice infected with CAR bacillus. *, significant difference in cytokine levels (P < 0.05) between BALB/c IL-4 KO (n = 4) and BALB/c mice (n = 5).
in mice with targeted mutations in IL-4 (27, 39), the elimination of this cytokine from susceptible BALB/c mice was expected to result in a decrease in IL-10 expression, an increase in IFN-γ expression, and abrogation of disease. While there was an attenuation of IL-10 expression and disease, IFN-γ was not the predominant cytokine expressed. This is likely the result of redundancy in the cytokine network. For example, IL-13, another type 2 cytokine that has functions very similar to those of IL-4 (36), was present and active in IL-4 KO mice and could have resulted in down-regulation of IFN-γ.

Why all B6 mice were not resistant to disease is unknown. There are several factors that may cause disparity of the immune response resulting in differential disease susceptibility, including host factors, bacterial virulence factors, and dose of inoculum. It is unlikely that host or bacterial factors resulted in the differences in disease expression since these mice originated from the same source and were inoculated at the same time with the same inoculum. However, the dose of inoculum could alter the immune response and the cytokine expression associated with CAR bacillus disease. At high antigen concentrations, the immune response tends to skew toward a type 2 cytokine profile (5, 22). It is possible that the difference in disease expression in B6 mice is that some mice inadvertently received a higher dose of bacterial inoculum. CAR bacillus frequently clumps, and while every effort was made to reduce the degree of clumping in the inoculum, it is possible that some B6 mice were inadvertently given a higher dose, skewing the cytokine response to a predominant type 2 response, resulting in disease expression and bacterial colonization. Regardless of the mechanism responsible for causing some B6 mice to develop disease, disease associated with CAR bacillus in both B6 mice and BALB/c mice was associated with elevated IL-10 mRNA expression, which supports our hypothesis that type 2 cytokines predominate in CAR bacillus-induced disease.

In conclusion, increasing severity of disease caused by CAR bacillus was associated with increasing IL-10 mRNA levels. This increase in IL-10 could have two effects on the outcome of CAR bacillus disease. First, IL-10 may contribute to disease progression by causing down-regulation of protective cytokines such as IFN-γ, with concurrent inhibition of macrophages and their microbicidal capacity. The other possibility is that increases in IL-10 reflect an up-regulation of this cytokine in response to production of proinflammatory cytokines such as TNF-α induced by CAR bacillus. In either case, it is clear that IL-10 plays a prominent role in the pathogenesis of CAR bacillus disease, and future studies are needed to delineate this role.

ACKNOWLEDGMENTS

We thank Robert S. Livingston for assistance with RT-PCR assays and Beth Livingston, Peg Hogan, Kim Mullinas, Laurie Roesel, and the Research Animal Diagnostic and Investigative Laboratory support staff for their assistance.

This work was supported by Department of Health and Human Services grants RR 08624-01 and 5 T32 RR 07004-22 from the National Institutes of Health.

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Editor: J. D. Clements