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Ig Heavy Chain Complex-Linked Genes Influence the Immune Response in a Murine Cryptococcal Infection

Julie A. Lovchik,* Julie A. Wilder,* Gary B. Huffnagle,† Roy Riblet,‡ C. Richard Lyons,§ and Mary F. Lipscomb*‡

A murine pulmonary infection with Cryptococcus neoformans (Cne) has been used to determine mechanisms regulating effective T cell-mediated immunity in the lungs. In BALB/c and C.B-17 mice, following intratracheal deposition of Cne, the fungus initially grows rapidly and is then progressively cleared from the lungs. Cne clearance in C.B-17 mice requires CD4 and CD8 T cells, IFN-γ, and NO. Clearance in congenic BALB/c mice proceeds more slowly than in C.B-17 mice, even though the only genetic difference between these strains is at the Ig H chain-containing region of chromosome 12. Examination of the pulmonary immune response in the two strains revealed that both cleared lung Cne by T cell-dependent mechanisms and generated equivalent levels of NO. Furthermore, both strains recruited equal numbers of macrophages, lymphocytes, and neutrophils to the lungs, although BALB/c mice recruited higher numbers of eosinophils. Notably, leukocytes isolated from BALB/c lungs during infection secreted lower levels of IFN-γ and higher levels of the Th2 cytokines IL-4 and IL-5 as compared with lung leukocytes from C.B-17 mice. Furthermore, serum levels of IgM, IgG1, IgG2a, and IgG3 anti-Cne Abs generated during infection were significantly greater in BALB/c mice than C.B-17 mice. These data suggest that although both BALB/c and C.B-17 mice clear pulmonary cryptococcosis through T cell-mediated mechanisms, Ig H chain-linked genes in BALB/c mice are associated with a decreased effectiveness of the host response, which we suggest might influence the balance in Th1/Th2 T cell subset development or increase anti-Cne Abs, or both.


Cryptococcus neoformans (Cne) is an important opportunistic fungal pathogen that is acquired via the respiratory tract. It causes several clinical syndromes, but most commonly presents as meningitis. Cryptococcosis is a major cause of morbidity and mortality among patients with AIDS as well as in patients receiving immunosuppressive therapy (1, 2). In addition to patients with known immunodeficiencies, some immunocompetent individuals also develop cryptococcosis (2). The establishment of Cne infections in immunocompetent individuals could be due to increased virulence or dose of the organism. Alternatively, susceptibility in certain immunocompetent individuals may reflect genetically determined differences in the ability to generate a protective immune response against the organism.

We have established a murine pulmonary Cne infection model in which the organism is inoculated into the trachea to dissect mechanisms involved in a protective pulmonary immune response (3). Pulmonary clearance of a moderately virulent Cne strain, 52D, varies among immunologically intact mouse strains, providing an opportunity to identify genes that play a role in regulating pulmonary immunity against this fungal pathogen. C.B-17 mice clear 52D, and clearance depends on the development of a Th1-type response to Cne and production of IFN-γ (4–6). IFN-γ is required for the activation of recruited macrophages to produce NO. Thus, if anti-IFN-γ Ab is administered after the initial development of an immune response and before full activation of recruited macrophages, or if the NO pathway is inhibited by treatment with the arginine analogue, N⁴-monomethyl-L-arginine, lung clearance is abrogated (6).

BALB/c mice also clear pulmonary cryptococcal infections with Cne 52D (3). However, preliminary studies indicated that at early time points in the infection, BALB/c mice consistently had more Cne in their lungs than C.B-17 mice, although both mouse strains reduced their lung burdens to similar low numbers late in the infection. The two mouse strains are genetically identical except at the telomeric region of chromosome 12, which, in addition to other genes, encodes the Ig H chain locus (IgH) (7).

The goal of the current study was to determine whether the genetic differences encoded in the unshared regions of chromosome 12 in BALB/c and C.B-17 mice influenced the T cell immune response generated against Cne or, alternatively, whether Ab levels might differ and perhaps suggest a role for Ab in lung clearance. The results demonstrated that although both strains required T cells to initiate cryptococcal lung clearance and produced similar amounts of NO at the same rate, they differed in lymphocyte cytokine secretion profiles and in serum anti-Cne Ab levels during infection. Therefore, an IgH-linked gene (or genes) on chromosome 12 influenced the rate of lung clearance, T cell subset development, and serum anti-Cne Ab levels during a pulmonary cryptococcal infection.
Materials and Methods

Reagents

HyClone RPMI 1640 media, HBSS, and Dulbecco’s PBS (HyClone, Logan, UT) were used throughout these studies. Media was certified as endotoxin free by vendor, supplemented with 100 U/ml penicillin/ streptomycin and 2 mM l-glutamine (Life Technologies, Grand Island, NY), 1 mM sodium pyruvate and 1 mM nonessential amino acids (Cellgro-Fisher Scientific, Pittsburgh, PA), and 5–10% FBS (Life Technologies) as noted below. FBS was heat-inactivated at 56°C for use in assays. Anti-CD4 (GK1.5) and anti-CD8 (YTS 169.4) mAbs, were produced as ascites in SCID mice and purified on a protein A-Sepharose affinity chromatography column (BioRad Laboratories, Rockville, NY) as previously described (8).

Cytokine secretion cultures

Lung leukocytes were prepared as described above and cultured in RPMI 1640 with 10% FBS supplemented with 2 μg/ml amphotericin B (Sigma) to block Cne growth in culture, 2.5 × 10^-3 M 2-ME (Eastman Kodak, Rochester, NY), 1 μg/ml indomethacin (Sigma), and 250 U/ml casein (Worthington Biochem, Freehold, NJ). Amphotericin B was previously found to have no effect on the secretion of cytokines in 24 h cultures, but any effect in the 48 h cultures should be uniform for both C.B-17 and BALB/c cell cultures. Indomethacin and catalase are consistently used in lung cell cultures to avoid the effects of PG and oxygen radicals on sup- pressor lymphocytes. Again, the use of similar agents in cultures of cells from both strains compared should allow for valid comparisons. Cells were cultured in duplicate at 5 × 10^5 cells/ml in 24-well tissue culture plates and either cultured in medium alone or stimu- lated with 5 μg/ml Con A (Sigma). In some experiments, 1 μg/ml of anti-IL-4 receptor Ab (Genzyme, Cambridge, MA) was added to cultures to optimize assessment of secreted IL-4. LALN cells were cultured to above except at 5 × 10^4 cells/ml in duplicate in 96-well plates and cultured in either media alone or with added Con A. Supernatants were collected at 48 h for cytokine analysis.

Cytokine ELISAs

Cytokines were analyzed using a two-site sandwich ELISA. Capture mAbs for IL-4 (11B11), IL-5 (TRFK5), and IFN-γ (R46A2) were obtained from PharMingen (San Diego, CA) and bound to ELISA plates diluted in 0.1 M Na2HPO4 solution (pH 9.0). Nonspecific binding was blocked with a 1% BSA/PBS solution. Biotinylated detection mAbs included: anti-IL-4 (BVD6-24G2), anti-IL-5 (TRFK4), and anti-IFN-γ (XMG1.2; PharMingen). Streptavidin-HRP (1 mg/ml) in blocking buffer was added to detect bound cytokines in the assays and developed using an ABTS (azo-bis- 3-ethylbenzthiazoline-6-sulfonic acid) solution, and the OD at 405 nm was determined. Cytokines were quantified by comparison to standard curves using recombinant IL-4, IL-5, and IFN-γ (PharMingen). An internal standard was included to monitor reproducibility of ELISAs using recombinant IL-4, IL-5, and IFN-γ obtained from Genzyme. Detection limits for each cytokine were assigned as the lowest concentration in the linear portion of the standard curve: IL-4 (0.25 ng/ml), IL-5 (1 ng/ml), and IFN-γ (1.56 ng/ml).

Nitrite/nitrate assays

Urine nitrate was reduced to nitrite using nitrate reductase of Pseudomonas oleovorans (no. 8062; American Type Culture Collection) as previously described (9), and nitrite levels were then determined using the Griess assay (10).

Detection of anti-Cne-reactive Ab

A loopful of Cne strain 52D was removed from a Saubouraud dextrose agar slant (in stationary phase) and washed three times with saline. Then, 1 × 10^6 Cne were cultured with a 1/10 dilution of mouse sera from mice obtained from Harlan or our colony for 30 min on ice in a total volume of 50 μl staining buffer (1% FCS-PBS). Cne were washed three times with staining buffer before the addition of 50 μl FITC-labeled goat F(ab)2 anti-mouse IgG1, IgG2a, or IgG3 (Southern Biotechnology Associates, Birmingham, AL) at a final concentration of 10 μg/ml, or FITC-goat F(ab)2 anti-mouse IgM (Organon Teknika, Durham, NC) at a final dilution of 1/100. All secondary Abs were H chain specific. Cells were incubated on ice for 30 min, washed three times with staining buffer, and fixed with 0.5% paraformaldehyde. Cell-associated fluorescence was measured using a Becton Dickinson FACScan, and data was analyzed using PC-LYSIS software. To establish a standard curve, mAbs specific for Cne capsular poly- saccharide and representing each of the four isotypes to be quantified were using an ice-cold isotonic 0.14 M ammonium chloride solution (pH 7.4). Cells were counted on a hemocytometer and cell types were determined by performing differentials on Wright-Giemsa-stained cytospin preparations (300). Three slides were counted for cytokine secretion studies. Lung cell cultures were spun through a 30% Percoll/PBS solution (Pharmacia, Piscataway, NJ) to eliminate red cell ghosts and cellular debris from the lung cell preparation. Cells were incubated on 100 mm2 tissue culture plates for 2 h in 5% FBS/complete RPMI 1640 at 37°C in 5% CO2 to remove adherent cells. Nonadherent cells were collected, pelleted, resuspended in RPMI 1640 with 10% FBS, and counted on a hemocytometer with trypan blue to enumerate live cells. To prepare LALN cells for cytokine analysis, LALNs were removed from each mouse and disrupted between two sterile, frosted glass slides to obtain a single-cell suspension. The LALN cells were washed with HBSS and resuspended in RPMI 1640 with 10% FBS.
incubated with Cne at 1.0, 0.1, and 0.01 μg/ml and detected with the appropriate FITC-labeled secondary. Mean fluorescence intensity was plotted against concentration, and a curve was generated. The mean fluorescence intensity of each mouse serum was interpolated using the standard curve to determine the concentration. IgG1 anti-Cne (439) was a gift from Dr. Arturo Casadevall.

Statistical analysis

Differences in all measured variables between BALB/c and C.B-17 mice were analyzed using unpaired t tests employing Statview software (Abacus Concepts, Berkeley, CA) with the exception of differences in the levels of anti-cryptococcal Abs. Differences in Ab levels between strains were analyzed using ANOVA after rank transformation of the data (Excel; Microsoft, Redmond, WA).

Results

Cne lung clearance in BALB/c and C.B-17 mice

Previous studies demonstrated that after i.t. deposition, Cne proliferates in the lungs of resistant C.B-17 mice during the first week of infection. A decrease in lung CFU begins between day 7 and 14, and by day 90, organisms are often below the level of detection (<50 CFU) (6), suggesting that these mice may eventually completely clear the infection. Comparison of Cne clearance in BALB/c and C.B-17 mice revealed that from day 14 until day 56, BALB/c mice had significantly higher numbers of Cne as compared with C.B-17 mice (Fig. 1A). Thus, although at day 84 both mouse strains reached very low and similar CFU, pulmonary clearance progressed less efficiently in BALB/c mice.

Requirement for T cells in clearance of Cne by BALB/c and C.B-17 mice

To determine the relative contribution of T cells in Cne pulmonary clearance in BALB/c as compared with C.B-17 mice, mice were treated with a combination of anti-CD4 and anti-CD8 mAbs before infection with Cne. T cell depletion was maintained by weekly injections of mAbs and monitored by flow cytometric analysis of splenocytes. Removal of T cells in BALB/c or C.B-17 mice abolished clearance of Cne during the first 21 days of infection (Fig. 1B). The Cne lung burden in T cell-depleted mice of both strains was significantly different from control IgG-treated mice by day 21 and were similar to previously reported levels observed in SCID mice (3, 6). These results indicated that Cne in both BALB/c and C.B-17 mice was T cell mediated.

Cytokine production by LALN cells and lung leukocytes in BALB/c and C.B-17 mice

Although T cells were necessary for clearance, the less efficient Cne clearance in BALB/c mice might reflect a difference in T cell cytokines produced during infection between the two mouse strains. This would be important, because IFN-γ was shown to be required both in the early inductive phase of the immune response (5) and during the effector stage (6). The levels of IFN-γ, IL-4, and IL-5 were determined for both BALB/c and C.B-17 mice by analyzying the cytokines produced by Con A-stimulated LALN cells and lung leukocytes isolated from infected mice both before (7 days) and at the time (14 days) clearance begins. LALNs from uninfected mice were too small to be reliably studied. Con A-stimulated lung leukocytes isolated from uninfected C.B-17 or BALB/c mice secreted low to undetectable cytokine levels, and no differences were detected between strains. Analysis of the cytokines produced by BALB/c cells isolated from infected BALB/c and C.B-17 mice revealed that BALB/c LALN cells secreted significantly lower levels of IFN-γ than by C.B-17 lung leukocytes as compared with BALB/c cells (Fig. 2). LALN cell secretion of IL-4 and IL-5 was not detected in either BALB/c or C.B-17 mice. Cytokines were not detected in cultures of unstimulated LALN cells. Quantitation of cytokines produced by leukocytes isolated from the lung during infection revealed lower levels of IFN-γ produced by BALB/c lung leukocytes at both days 7 and 14 of infection as compared with BALB/c LALN cells (Fig. 3A). Similar levels of IL-4 were produced by lung leukocytes from both mouse strains at day 7, but by day 14, production of IL-4 by BALB/c lung leukocytes was significantly greater than by C.B-17 lung leukocytes (Fig. 3B). Higher levels of IL-5 were also produced by BALB/c lung leukocytes by day 14 as by C.B-17 lung leukocytes (Fig. 3C).

Leukocyte recruitment during a Cne infection in BALB/c and C.B-17 mice

An important role for T cells in the immune response against Cne is to facilitate maximal recruitment of effector cells to the lung (6, 12). To determine whether the difference in cytokine production...
between BALB/c and C.B-17 mice affected cellular recruitment, the numbers and types of leukocytes present in the lung during infection were examined. Quantification of lung cells isolated from BALB/c and C.B-17 mice verified that similar numbers of cells were recruited to the lungs of both mouse strains (Fig. 4A) and that similar numbers of lymphocytes, macrophages, and neutrophils were present at the peak of leukocyte recruitment on day 14 (Fig. 4B). However, significantly greater numbers of eosinophils were present in BALB/c lungs than in C.B-17 lungs at day 14 ($p < .0001$).

**Activation of recruited cells during a Cne infection in BALB/c and C.B-17 mice**

Although there was significantly less IFN-$\gamma$ produced by leukocytes from BALB/c mice, this cytokine was detected in both mouse strains. One important function of IFN-$\gamma$ in protection against Cne is to activate macrophages to express the enzyme, inducible NO synthase, and secrete NO (6). NO production was determined by monitoring urinary nitrate excretion in both Cne-infected BALB/c and C.B-17 mice in parallel. The levels of excreted urinary nitrate observed in BALB/c and C.B-17 mice were similar at all time points (Fig. 5). The levels of inducible NO synthase protein were shown to increase in the lungs of both mouse strains between day 0 and day 14 of infection by Western analysis (data not shown). In addition, isolated lung cells were analyzed by immunohistochemistry for inducible NO synthase, and a similar percentage of positive-staining macrophages were found in both 14-day-infected BALB/c and C.B-17 mice (BALB/c, 36.8 ± 19.4%; C.B-17, 26.6 ± 13.0%; $p > 0.1$, $n = 5$ mice/strain).

**Presence of anti-Cne Ab in BALB/c mice**

The finding that systemic NO levels were similar between BALB/c and C.B-17 mice suggested that the difference in Cne clearance could not be completely explained by IFN-$\gamma$ secretion differences, because our previous studies had shown the dependence of clearance on IFN-$\gamma$-dependent NO production (6). Another possible explanation was that Ab influenced Cne clearance. Sera from uninfected and Cne-infected mice were incubated with Cne strain 52D, and the presence of bound IgM and IgG1, IgG2a, and IgG3 isotypes was detected by indirect immunofluorescence. Surprisingly, sera from all uninfected BALB/c mice possessed IgM, and many also had IgG3 Cne-reactive Ab depending on the age of the mice (Fig. 6, A and B). In contrast, sera from uninfected C.B-17 mice exhibited little Cne-binding activity. During infection, the levels of IgM Abs in BALB/c mice remained stable for the first week of infection and then rose 3- to 4-fold during the second week.
week before declining, but were not detectable in C.B-17 mice (Fig. 6A). Although some increase in IgG anti-Cne Ab levels was observed in C.B-17 serum during infection, BALB/c mice had higher levels of IgG anti-Cne Abs of all three isotypes (Fig. 6, B–D). The predominant IgG isotype in both mouse strains was IgG3.

Discussion

The ability to develop a protective immune response against Cne is influenced by many factors and is likely a complex genetic trait, as indicated by the varying ability of different mouse strains to clear a pulmonary Cne infection. In the current studies, we show that a genetic difference at the telomeric end of chromosome 12 is associated with the efficacy of immune-mediated pulmonary clearance of Cne. Although BALB/c and C.B-17 mice can significantly reduce Cne lung burdens, BALB/c mice cleared the organism less efficiently than C.B-17 mice as demonstrated by a significantly higher lung burden from the second to the seventh week of infection. We have previously shown that clearance of Cne requires the development of a Th1 response with production of IFN-γ and subsequent NO production (4–6). We found in the current study that clearance of Cne in both BALB/c and C.B-17 mice was dependent on the presence of T cells, and both strains developed a Th1-type immune response as shown by production of IFN-γ by LALN cells and lung leukocytes, recruitment of similar numbers of lymphocytes and macrophages into the lung during infection, and activation of recruited macrophages to produce NO. However, BALB/c mice also demonstrated characteristics of an accompanying Th2 response to Cne with increased lung leukocyte secretion of IL-4 and IL-5, a higher number of eosinophils in their lungs, and higher anti-Cne Ab levels (including increased IgG1 anti-Cne Abs). Another difference between the two mouse strains was the presence of Cne-reactive Abs in uninfected BALB/c mice, but not in uninfected C.B-17 mice. The results indicate that a gene(s) on chromosome 12 controls expansion of Cne-reactive B cells and Ab production in uninfected mice and influences humoral and cellular immune responses during a Cne pulmonary infection and fungal lung clearance.

The region of chromosome 12 that differs between BALB/c and C.B-17 mice encompasses many genes including a minor histocompatibility Ag (H40) (13), some alloantigens (TSU, Lm-1) (14, 15), the bacillus Calmette-Guérin-anergy gene (16), the IgH complex (7), and others with unknown functions. Although the bacillus Calmette-Guérin-anergy gene can negatively affect immunity, this gene is unlikely responsible for the reduced ability of BALB/c mice to clear Cne, because anergy occurs in mice with the IgHb haplotype.
haplotype and BALB/c mice have the IgH<sup>+</sup> haplotype (16). Preliminary studies using C.B-reduced congenic strains with smaller chromosome 12 region disparities than exist between C.B-17 and BALB/c mice suggests that the Cne clearance differences between these two strains map to the IgH locus (J.A.L., un unpublished observations). Molecular characterization of several mAbs against cryptococcal polysaccharide (CNPS) has revealed a relatively limited Ab repertoire with restricted Ig variable gene element usage for both H and L chains (17–19). The apparent oligoclonal Ab response to CNPS suggests that very few dominant, immunogenic epitopes of CNPS are recognized. Thus, genetic differences at the IgH locus could affect the potential of mouse strains to generate Ab capable of recognizing Cne and explain the finding of higher anti-CNPS Ab levels in BALB/c mice as compared with C.B-17 mice. We believe that uninfected BALB/c mice exhibit CNPS-reactive IgM and IgG3 Ab, because they are exposed to a foreign, cross-reactive polysaccharide in their environment; and that CNPS, as a T-independent type-2 Ag (20, 21), directly restimulates these cross-reactive B cells upon exposure to the organism during infection. It is also possible that the Ab and reactive B cells are the result of exposure to low levels of the ubiquitous cryptococcal yeast in their bedding, but in any case, both mouse strains are housed in the same animal care facilities, and only the BALB/c mice are able to respond in this fashion.

Our earlier studies suggested that the development of a Th1 response against Cne was not linked to the IgH-linked region of chromosome 12, because C57BL/6 mice, which are incapable of either clearing Cne or developing a Th1 immune response to pulmonary Cne (4), are identical with C.B-17 mice at this region (16). The data in the current paper, coupled with our previous studies, indicate that genes outside of the chromosome 12 region are the most important in defining whether cryptococci will be cleared from the lung or not. If the resistance phenotype is present as in C.B-17 and BALB/c mice, then the presence of IgH-linked genes from either the BALB/c or the C57BL/6 mouse should define the rate of pulmonary clearance. Therefore, the C.B-17 mouse represents a strain that has the full complement of resistance genes of the BALB/c mouse with the replacement of its IgH-linked genes by C57BL/6 chromosome 12, and a more favorable clearance pattern occurs than in the wild-type BALB/c.

The ability of BALB/c mice to make Cne-cross-reactive B cells and Ab before and early in infection in BALB/c mice may explain the difference in T cell subset development between BALB/c and C.B-17 mice. The differentiation of naive, Ag-specific CD4<sup>+</sup> Th cells into mature Th1- or Th2-type cells depends on several factors including the type of APC, the cytokines present in the milieu at the time of Ag presentation, and the Ag dose. Although dendritic cells are the major APCs involved in initiating a primary immune response (22), primed Ag-specific B cells can also function as APC for naive T cells (23–25). Dendritic cells secrete IL-12 and tend to induce development of Th1 cells (26), while Ag presentation by B cells favors development of Th2 cells (24). Although dendritic cells likely constitute the majority of cells presenting Cne Ag to naive T cells in LALNs and induce the development of Th1 cells, we cautiously propose that Ag presentation by an expanded population of Cne-reactive B cells in BALB/c mice could alter the ratio of Cne-specific Th1:Th2 cells and explain the higher levels of Th2 cytokines produced by LALN and lung leukocytes from BALB/c mice. A second possibility to explain how the presence of Ig could affect T cell development is that cross-reacting Ab results in sequestration of cryptococcal Ag, thereby reducing the concentration of Ag in lymph nodes during the developing response in BALB/c mice as compared with C.B-17 mice and favoring Th2 subset development (27).

We have offered postulates for how the chromosome 12 locus could influence Ig levels and T cell cytokine profiles, but the mechanism responsible for the less efficient clearance of Cne in BALB/c mice remains uncertain. However, at least three possibilities exist. First, although NO production is necessary, it may not be sufficient for Cne clearance, and the lower levels of IFN-γ produced by BALB/c leukocytes may reflect a decrease in additional IFN-γ-dependent mechanisms that synergize with NO for efficient reduction of Cne. Second, the generation of Th2 cytokines may adversely affect the ability of BALB/c mice to clear Cne by suppressing the aforementioned IFN-γ-dependent clearance mechanisms or activating effector cells, such as eosinophils, to inhibit clearance. A third possibility is that the anti-Cne Abs generated in BALB/c mice have a direct inhibitory effect. Studies by others examining individual anti-CNPS mAbs have shown that certain mAb prolonged survival and others decreased survival when passively administered before Cne inoculation in lethal i.v., i.p., or pulmonary Cne infection models (19, 28–31). In these studies, the capacity of a particular mAb to be protective depended on both epitope specificity and isotype (28–30, 32). Interestingly, particular IgG3 anti-CNPS mAbs were not protective and could even interfere with the efficacy of a protective Ab (33); perhaps by sequestering Cne in unactivated macrophages via Fc receptor-dependent phagocytosis (32, 34). Further studies are underway to analyze the immune response to Cne in C.B-reduced congenic strains to identify the specific gene(s) that determine the difference between the two strains. In addition, we will examine IgH knockout mice on the BALB/c background to determine whether these mice demonstrate cryptococcal lung clearance curves identical with C.B-17 mice.

Overall, these studies reveal that an IgH-linked gene(s) on chromosome 12 can influence humoral and cellular immune responses to Cne and subsequently affect the ability of fully immunocompetent mice to respond to this opportunistic organism. By extension, the increased susceptibility to Cne infections of some apparently immunocompetent humans may reflect subtle, genetically determined differences in their immune response to this pathogenic yeast.

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References


