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Specific and Nonspecific Antibody Responses in Different Segments of the Respiratory Tract in Rats Infected with *Mycoplasma pulmonis*

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Murine respiratory mycoplasmosis resulting from *Mycoplasma pulmonis* infection in rats provides a useful model for the study of immunological and inflammatory mechanisms operative in the respiratory tract. We have previously shown that LEW rats develop more severe disease than do F344 rats. To further study the production of antibody responses in chronic respiratory disease due to *M. pulmonis* infection, we examined the distribution and development of *M. pulmonis*-specific antibody-forming cells (AFC) in different segments of the respiratory tracts of infected LEW and F344 rats. In these studies, the upper respiratory nodes were the initial site of antibody production after infection and remained the major site for recovery of AFC. Since infected LEW rats had equal or higher numbers of AFC than did infected F344 rats, these results suggest that the level of local antibody production alone is not responsible for the decreased susceptibility of F344 rats to murine respiratory mycoplasmosis. The differences in total antibody responses appear to be due to the greater numbers of cells recovered from the tissues of infected LEW rats compared with those recovered from F344 rats, suggesting that LEW rats may have greater production of chemotactic factors. Also, we demonstrate that nonspecific activation and/or recruitment of B cells occurs in the respiratory tracts of both LEW and F344 rats after infection with *M. pulmonis*.

Murine respiratory mycoplasmosis (MRM) resulting from *Mycoplasma pulmonis* infection in rats provides a useful model for the study of immunological and inflammatory mechanisms operative in the respiratory tract (6). Natural or experimental infection with *M. pulmonis* results in the development of otitis, rhinitis, tracheitis, and bronchopneumonia (16). Like many other infectious respiratory diseases, MRM begins in the nasal passages and progressively spreads downward into the lungs (26). The major feature of the lesions of MRM is the large mononuclear cell accumulation present in all the mucosal regions of the respiratory tract, including hyperplasia of the bronchially associated lymphoid tissue in the lung (8, 16). Eventually, disease of the lung parenchyma can also develop.

We previously demonstrated differences between MRM lesion severity and disease progression in two strains of rats. After infection, LEW rats develop more severe upper and lower respiratory tract lesions than do F344 rats (8). Also, severe lesions in the lung parenchyma often develop in LEW rats, whereas the lung disease in F344 rats is predominately associated with the mucosal surfaces and airways. The lung lesions in F344 rats begin to resolve in 28 days after infection, whereas the lesions in LEW rats continue to worsen (10). In LEW rats, there is an increase in all classes of lymphocytes in the lungs throughout the course of disease. In contrast, the numbers of lung lymphocytes in F344 rats increase only in the first 28 days after infection, and not all lymphocyte classes increase during this time (10).

Specific immune responses also seem crucial to the pathogenesis of MRM. The respiratory lesions of MRM, including lymphocyte infiltration, can be prevented or reduced by intravenous vaccination with killed organisms; however, intranasal immunization with killed organisms reduces the severity of rhinitis but does not affect lesions in any other region of the respiratory tract (7). Thus, this disease provides a unique opportunity to study interactions between the systemic and mucosal immune systems, including interactions between the upper and lower respiratory tracts, in a naturally occurring infection.

In previous studies, we examined the ability of LEW and F344 rats to produce antibody to *M. pulmonis*. First, we evaluated specific immune responses to nonreplicating *M. pulmonis* antigens in both rat strains after parenteral immunization (29). Immunized F344 rats produce higher levels of *M. pulmonis*-specific serum immunoglobulin G (IgG) antibody than do LEW rats, whereas IgM serum antibody responses do not differ. In more recent studies, we examined the temporal sequence of specific serum antibody production in LEW and F344 rats after *M. pulmonis* infection (31). In contrast to the results in the immunization studies, infected LEW rats produce at least as much of each of class of specific antibody to *M. pulmonis* as do infected F344 rats. Although these studies suggest that serum antibody is not responsible for differences in the disease in LEW and F344 rats, antibody production along the respiratory tract may still play a role in disease, because local immunity has not been examined.

Additional studies on the distribution of antibody-forming cells (AFC) are needed not only to understand the role of antibody in disease progression but to further understand the effect of chronic inflammatory responses on immunity. In a recent study (33), lymphoid tissues of the upper respiratory tract in infected F344 rats were shown to be the major sites of *M. pulmonis*-specific antibody production, whereas the contribution of the lung as a site of antibody production was minimal. In addition to activation of specific immune mechanisms, nonspecific activation of B cells may also occur during disease pathogenesis. We (10, 30, 37) and others (18, 19) have suggested that nonspecific activation of lymphoid

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cells may contribute to the development of disease; this is supported by our studies (9) showing that the LEW lymphocyte response to mitogens in vitro is greater than that of F344 lymphocytes.

To further study the production of antibody responses in chronic respiratory disease due to *M. pulmonis* infection, we examined the distribution and development of *M. pulmonis*-specific AFC in different segments of the respiratory tracts of infected LEW and F344 rats. In these studies, the upper respiratory lymph nodes (URN) were the initial site of antibody production after infection and remained the major site for the recovery of AFC. Also, we demonstrate that nonspecific activation of B cells occurs in the respiratory tracts of both LEW and F344 rats after infection with *M. pulmonis*.

MATERIALS AND METHODS

Animals. Pathogen-free F344 and LEW rats 8 to 12 weeks old were reared and maintained in Trexler-type plastic film isolators (22). The pathogen-free status of animal colonies was monitored by using serological (including an enzyme-linked immunosorbent assay [ELISA] for serum anti-mycoplasmal antibodies) and/or cultural techniques for mycoplasmal, viral (Sendai virus, pneumonia virus, sialodacryoadenitis virus, Kilham rat virus, H-1 virus, reovirus type 3, GD-VII virus, lymphocytic choriomeningitis virus, mouse adenovirus [serological assays by Charles River Biotechnical Services, Wilmington, Mass.]), fungal, and other bacterial pathogens. The rat colony was consistently negative for all pathogens, and the animals were negative for IgG and IgM antimycoplasma antibodies.

Before any experimental manipulation or collection of tissues, rats were anesthetized with a combination of ketamine hydrochloride (Bristol Laboratories, Syracuse, N.Y.) and xylazine (Rhompun, 5 mg/ml; Haver-Lockhart, Shawnee, Kans.). For experimental infection, animals were intranasally inoculated with 50 μl of *M. pulmonis* containing 5×10^6 CFU, unless otherwise indicated. In selected experiments, rats were intraperitoneally immunized with 125 μg of keyhole limpet hemocyanin (KLH).

Mycoplasma and immunizing antigens. *M. pulmonis* UAB 6510 was originally isolated from the lungs of a rat with natural MRM. The isolate was cloned three times with the medium of Hayflick (13), and cultures were identified as pure *M. pulmonis* by using immunofluorescence (11). *M. pulmonis* was grown in modified Hayflick medium (13, 29) and stored in 1-ml aliquots at −70°C. The number of *M. pulmonis* CFU was determined by inoculation of agar plates with serial dilutions of the stock in Hayflick broth medium.

KLH was purchased from Calbiochem-Behring (La Jolla, Calif.). Protein concentration was determined by the micromethod of Bio-Rad Laboratories (Richmond, Calif.) (3). Before immunization, antigens were diluted to 500 μg/ml in phosphate-buffered saline (PBS).

Isolation of lymphocytes. Lung cells were isolated by using Ficoll-Hypaque (LymphPaque; Pharmacia) density gradients after mechanical disaggregation of tissue (9, 10); nearly all of the lung lymphocytes are recovered by this method, since very few cells are retained in the remaining tissue fragments (10). Spleen lymphocytes were isolated as previously described (9). URN and lower respiratory lymph node (LRN) cells were isolated by mechanical disaggregation. URN contained the deep cervical lymph nodes and retropharyngeal lymph nodes, whereas LRN contained the tracheobronchial lymph nodes. Nasal passage (NP) cells were collected after longitudinal section of the head and removal of tissue from the nasal cavity, nasal septa, ethnoid sinuses, and maxillary sinuses by scraping; the tissue was further disaggregated and pushed through 250-μm-pore-size nylon mesh to remove any remaining tissue and bone chips. All cell washes were done with RPMI 1640 (GIBCO Laboratories) supplemented with 10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) and 5% fetal calf serum.

The percentages of lymphocytes and other cell types were determined in cell suspensions from infected animals. Cell smears were stained with Diff-Quick (American Scientific Products, McGaw Park, Ill.) and examined under light microscopy. There were no differences in the percentages of lymphocytes in any of the cell suspensions from infected and uninfected rats. The percentages of lymphocytes in infected or uninfected LEW rats were 87, 72, 88, 82, and 86% in URN, NP, spleen, lung, and LRN cells, respectively. In F344 rats, there were 88, 62, 86, 83, and 87% lymphocytes in URN, NP, spleen, lung, and LRN cells, respectively. The remaining cells were mostly macrophages, although 10 to 20% neutrophils were found in NP cell suspensions.

Determination of AFC. The ELISPOT assay for *M. pulmonis*-specific AFC was performed as previously described (33). Note that the presence of neutrophils or passive adsorption of antibody does not affect the results obtained with this assay. Briefly, *M. pulmonis* cell lysate antigen (14) was incubated (5 μg of protein per well) overnight at 4°C in Millititer HH 96-well filtration plates (catalog no. STHA09610; Millipore Corp.). To minimize nonspecific binding, 100 μl of cell culture medium (RPMI 1640, 10 mM HEPES, 1% L-glutamine, 0.2% $NaHCO_3$, 80 μg of gentamicin per ml, and 5% fetal calf serum) was added to each well, and plates were incubated at 37°C for at least 1 h. Serial dilutions of cells were added in triplicate to the plates (100 μl per well) and incubated for 4 h at 37°C in 5% CO_2. The plates were washed with PBS and then PBS–0.5% Tween 20. To remove endogenous peroxidase activity, the plates were incubated for 10 min with 0.5% H_2O_2 in PBS and then washed with PBS–0.5% Tween 20.

Mouse monoclonal anti-rat IgA (MARA-2; BSI/Serotec), anti-rat IgM (MARM-4; BSI/Serotec), horseradish peroxidase (HRP)-labeled rat anti-mouse IgG, $F(ab)_2$ fragments (Jackson Immunoresearch Laboratories, Westgrove, Pa.), and HRP-labeled goat anti-rat IgG antibody (Kirkegard & Perry Laboratories, Inc., Gaithersburg, Md.) were used to reveal the antibody reactions. HRP-conjugated antibody or monoclonal antibodies specific for rat immunoglobulin classes were placed into the appropriate wells at the maximum antibody dilutions previously shown to have minimal background in the ELISPOT assay. After overnight incubation at 4°C, the wells that contained the monoclonal antibodies were reacted with HRP-conjugated rat anti-mouse antibody for 5 h at room temperature. The plates were then washed with PBS, and HRP substrate was added to each well. The HRP substrate was 25 mg of 3-amino-9-ethyl carbazole (Sigma Chemical Co.) in 97 ml of 0.05 M sodium acetate buffer (pH 5.0) plus 0.04% H_2O_2; 3-amino-9-ethyl carbazole was dissolved in 2 ml of *N*,*N*-dimethyl formamide. After the substrate reaction, the plates were thoroughly washed with tapwater.

The spots representing AFC were counted by using a dissecting microscope illuminated indirectly with a high-intensity lamp. The cell concentrations yielding about 20 to 50 spots per well were counted and averaged. The AFC were expressed as the absolute number of AFC in each tissue or

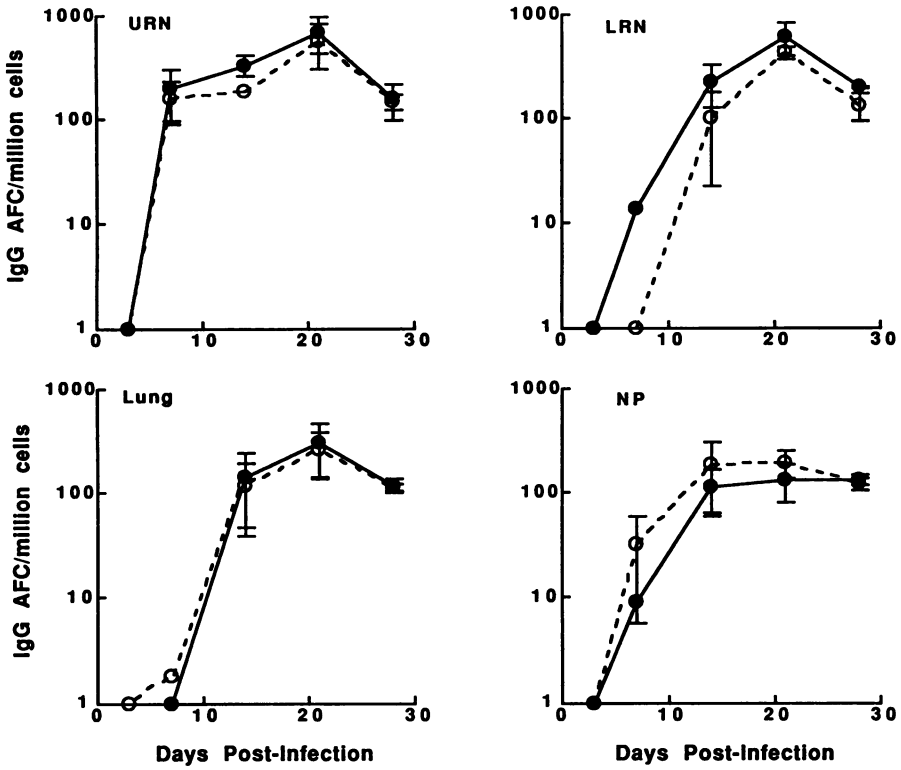

FIG. 1. Development of anti-*M. pulmonis* antibody responses in LEW and F344 rats. IgG AFC responses in LEW (●) and F344 (○) rats are shown. At the 3-day time point, no or very few AFC were detected in any tissue. Similar results were obtained for the other antibody subclasses. The data are expressed as the means ± standard deviations of IgG-producing AFC/10^6 cells within the indicated tissues.

AFC/10^6 cells; the numbers of AFC were corrected for the percentage of lymphocytes in the initial cell population.

Statistics. All experiments were repeated at least twice. Statistical analysis was performed by using the SYSTAT version 5.0 program (Systat, Inc., Evanston, Ill.) on a Macintosh SE computer. The data were analyzed by analysis of variance and Pearson correlation after logarithmic transformation of the data (1), followed by post hoc tests for multigroup comparisons when appropriate. A probability (P) of less than 0.05 was accepted as significant.

RESULTS

Antibody responses in LEW and F344 rats. In previous studies, LEW rats developed higher-than-expected antibody responses, relative to those of F344 rats, to *M. pulmonis* after infection (31). Subsequently, the URN were described as a major site of antibody production in infected F344 rats (33). To further examine the development of antibody responses in rats with MRM, the numbers of AFC in lymphoid tissues were determined in LEW and F344 rats at 3, 7, 14, 21, and 28 days after intranasal infection with *M. pulmonis*. The experiment was done at least twice until data from a total of six animals per rat strain were obtained for each time point, except for four animals at 21 days postinfection. Consistent with the results from previous studies (8, 10), gross lung lesions were observed in about half of the LEW rats at 21 and 28 days after infection, whereas no gross lung lesions were found in F344 rats. At least one uninfected animal per rat strain was used as a control at each time point; no anti-*M. pulmonis* responses were seen in these animals.

In both LEW and F344 animals, the URN appeared to be the initial site of antibody production after infection (Fig. 1). Few or no AFC were found in any tissue 3 days after infection with *M. pulmonis*. At 7 days postinfection, there was a large increase in the number of AFC/10^6 cells in URN, whereas the other tissues had only minor levels ($P < 0.05$). There was no significant difference due to antibody isotype in any of the tissues at 7 days postinfection. The level of AFC activation continued to increase in URN but not as dramatically as in the first 7 days. In all other tissues, there were large increases in AFC activation by 14 days after infection, with less rapid increases in AFC activation at later times.

As shown in Fig. 2, the upper respiratory nodes were a major site of total antibody production in both LEW and F344 rats ($P < 0.05$). There was no statistical difference in the total amount of any antibody isotype produced in the URN of LEW and F344 rats. However, LEW rats had dramatically higher numbers (greater than 6 times) of total AFC in the LRN than did F344 rats ($P < 0.05$). This was true for all classes of antibody. There was also a higher number of total AFC in the URN of LEW rats ($P < 0.05$), but it was less than double that found in F344 rats. There was also a slightly higher number of total IgG-, IgA-, and IgE-producing AFC in LEW lungs than in F344 lungs ($P < 0.05$), and the total number of IgM-producing AFC in lungs was close to being significantly different ($P = 0.052$). Thus, the relative levels of the antibody responses in the LRN were the major difference in the distribution of antibody responses in infected LEW and F344 rats. However, other tissues, especially the URN, of LEW rats also had higher numbers of total AFC.

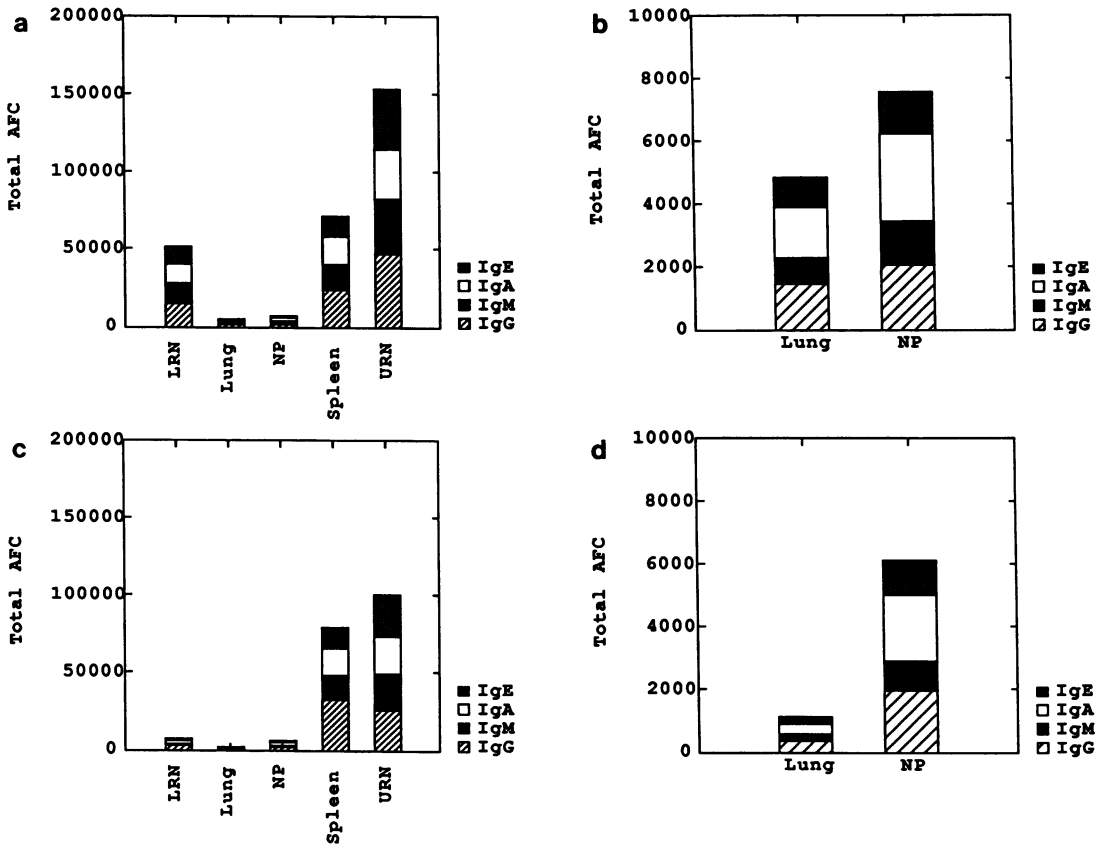

FIG. 2. Contribution of different tissues to total anti-*M. pulmonis* antibody response. The total number of AFC in tissues from LEW (a, b) and F344 (c, d) rats at 14 days postinfection. The distribution of antibody responses was similar at later time points. The stacked bar charts indicate the total number of AFC in each tissue, including the breakdown of the isotypes. Note the different scales used when showing all tissues (a, c) and only lungs and nasal passages (b, d).

Nonspecific activation of B cells in infected rats. Previous studies (10, 18, 19, 37) suggested that nonspecific activation and/or recruitment of B cells may occur in rats infected with *M. pulmonis*. To examine this possibility, LEW and F344 rats were immunized intraperitoneally with 125 μg of KLH and rested for 2 to 3 weeks, allowing the antibody response to KLH to subside (data not shown). Half of these rats then were intranasally infected with *M. pulmonis*, and the remaining control rats remained uninfected to measure the residual anti-KLH response. Two weeks after infection, the numbers of IgG-producing cells specific for KLH or *M. pulmonis* were determined in all lymphoid tissues.

Significantly higher KLH responses were found in both infected LEW and F344 rats as compared with those in the control animals (Fig. 3). This was true whether the data were expressed as total AFC (as shown) or AFC/10^6 cells. The only KLH-specific AFC in the uninfected (control) animals were found in the LRN. No significant difference was found between the responses in LEW and F344 rat strains due to the extremely large variation in these experiments. There was no significant difference in the numbers of KLH-specific AFC in the URN, LRN, and NP in infected animals, whereas the lung contained smaller numbers of AFC. Spleens had lower, but not statistically significant (P = 0.053), numbers of AFC. The distribution of *M. pulmonis*-specific AFC was similar to those described above. However, the distribution of AFC specific for *M. pulmonis* did not correlate well with the tissue distribution of KLH-specific AFC. Thus, nonspecific activation and/or recruitment of B cells occurs in both LEW and F344 rats after infection with *M. pulmonis*.

DISCUSSION

Most studies on respiratory immunity and disease have examined only the presence of antibody in nasal or tracheobronchial secretions or the distribution of AFC within the lower respiratory tract (5, 12, 15, 21, 23, 28, 35, 36); few studies have considered antibody responses in both the upper and lower respiratory tracts (4, 27). However, the elucidation of the immune mechanisms present in all tissues of the respiratory tract is of great importance for the development of optimal vaccination regimens as well as for understanding the mechanisms of pathogenesis of various infectious and allergic diseases.

The results from our laboratory suggest that the upper respiratory tract plays a major role in the development of antibody responses in some respiratory infections. In an earlier study (33), we demonstrated that the upper respiratory tract is the major site of antibody production in F344 rats 28 days after infection with *M. pulmonis*. In the present study, we examined the production of antibody responses in LEW and F344 rats after intranasal infection with *M. pulmonis*. In both LEW and F344 rats, the URN were the initial

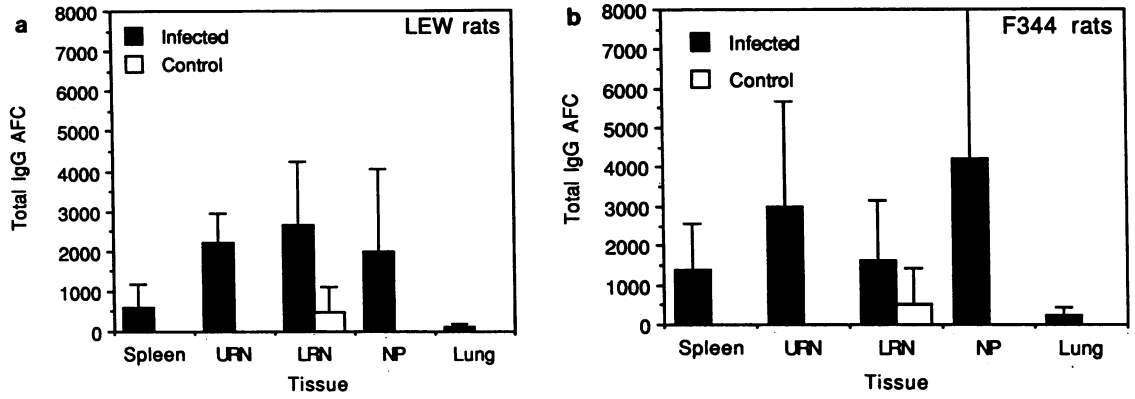

FIG. 3. Nonspecific activation of B cells in *M. pulmonis*-infected animals. LEW and F344 rats were primed with KLH and rested before infection with *M. pulmonis*. There was a significantly higher number of KLH-specific IgG AFC in LEW (a) and F344 (b) rats infected with the organism as compared with those in control animals, which were not infected. The data are expressed as the means ± standard deviations.

site of antibody production after infection. By 14 days after infection, AFC were found in other tissues. As in our previous study (33), the URN remained a major site of AFC through the 28-day period in both LEW and F344 rats. These results suggest that URN may be a significant source of primed or activated B cells, which migrate to other stimulated (infected) tissues.

Previous studies from our laboratory have compared serum antibody responses in LEW and F344 rats after immunization with *M. pulmonis* antigen (29) and intranasal infection with the organism (31). After immunization with nonreplicating organisms, F344 rats produce higher levels of serum IgG, particularly the IgG2b subclass, than do LEW rats (29). In contrast, infected LEW rats develop serum antibody responses, including IgG2b responses, equal to or greater than those of F344 rats (31). However, the subclass distribution of the antibody response is consistent with the immunization studies. IgG2b contributed the most to the total IgG in infected F344 rats, whereas IgG1 and IgG2a were the major components of the LEW IgG response. From these results, we suggested that the total IgG response to *M. pulmonis* in infected LEW rats is much higher than expected because of infection without affecting the IgG subclass distribution.

In the present study, there was a major difference between LEW and F344 rats in the number of *M. pulmonis*-specific B cells in the draining lymph nodes of the lungs (LRN). LRN from infected LEW rats contained a larger proportion of *M. pulmonis*-specific B cells (AFC/10^6 cells) than did LRN from F344 rats. This difference was amplified by a difference in the total number of cells recovered from LRN after infection, resulting in a much higher number of AFC in LEW rats compared with that in F344 rats. There was also a larger antibody response in the lungs and URN of LEW rats. The antibody response within the lungs was minor compared with that of the LRN. Thus, the results from the present study are consistent with the higher-than-expected serum antibody responses in infected LEW described in the previous studies (31) and suggest that the antibody production in the LRN and URN contributes significantly to the differences between LEW and F344 responses.

Although earlier in vivo and in vitro studies suggest that polyclonal activation of B cells can occur as a result of *M. pulmonis* infection (18, 19, 37), the present study is the first to demonstrate that polyclonal activation of B cells does actually occur in rats with MRM. More KLH-specific IgG AFC were found in animals immunized with KLH and infected 2 weeks later with *M. pulmonis* than were found in similarly immunized, uninfected animals. This was true for both LEW and F344 rats. Unfortunately, we cannot directly compare the levels of polyclonal activation of B cells in these two strains because the numbers of primed B cells probably differ. F344 rats produce higher levels of serum IgG to KLH than do LEW rats after immunization (29), supporting the idea that there was probably a difference in the number of KLH-primed B cells before infection with *M. pulmonis*.

Nonspecific recruitment of AFC to lymphoid tissues during MRM may also occur. Bice et al. (2) demonstrated that B cells are recruited to the lung without regard to antigen specificity when an inflammatory stimulus is present. In our study, a significant number of KLH-specific AFC were found in the URN after infection. Antibody responses to KLH were never found during this study in URN after primary intraperitoneal immunization (data not shown). In addition, we found that primed B cells develop in the URN only after intranasal immunization with an antigen (32). Overall, these data suggest that nonspecific recruitment of B cells to URN, and probably other tissues, occurs during the pathogenesis of MRM.

The mechanisms of nonspecific activation or recruitment of B cells during MRM are presently unknown. One likely possibility is that cytokines associated with specific immune and/or inflammatory responses play a role. B-cell chemotactic factors are present in delayed-type hypersensitivity lesions (17) and may be present in the lesions associated with MRM. In addition, polyclonal activation of B cells can occur in vivo after passive transfer of activated Th2 helper T lymphocytes, which produce interleukin-4 (34). Most likely, other cytokines contribute to the polyclonal activation and nonspecific recruitment of B cells during disease. In addition to cytokines, the direct interaction of *M. pulmonis* and B cells may contribute to the effects seen during MRM. *M. pulmonis* membranes are mitogenic for B cells in vitro (20, 24). We have also found that a major cell surface antigen of *M. pulmonis* is chemotactic for naive B cells (25). Thus, cytokine and/or *M. pulmonis* interactions with B cells most likely result in the activation and recruitment of these cells.

In summary, antibody responses first develop in the URN after infection of rats with *M. pulmonis*. The URN remains a major site of antibody production throughout the course of

disease. Since infected LEW rats had equal or higher numbers of AFC compared with those in infected F344 rats, these results suggest that the level of local antibody production alone, like that of serum antibody (31), is not responsible for the decreased susceptibility of F344 rats to MRM. The differences in total antibody responses appear to be due to the greater numbers of cells recovered from the tissues of infected LEW rats compared with those in F344 rats, suggesting that LEW rats may have greater production of chemotactic factors. Our data also demonstrate that nonspecific activation and recruitment of B cells occur during the development of MRM. Although further studies are needed, the mechanisms involved in the nonspecific effects on B cells probably influence the distribution and levels of specific antibody responses and thus may actually contribute to the development of disease.

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