Expression of inflammatory cytokine mRNA in lymphoid tissue from swine experimentally infected with *Mycobacterium avium* serovar 2

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Objective—To evaluate in situ expression of inflammatory cytokine mRNA in lymphoid tissue of swine experimentally infected with *Mycobacterium avium* serovar 2.

Animals—7 noninfected pigs and 7 pigs infected with *M avium* serovar 2.

Procedure—Expression of mRNA of inflammatory cytokines such as tumor necrosis factor α (TNFα), interleukin (IL)-1β, IL-6, and IL-8 in formalin-fixed paraffin-embedded blocks of lymphoid tissue (lymph nodes and tonsil) of swine experimentally infected with *M avium* serovar 2 was compared with that of noninfected pigs. Tissues were evaluated by use of morphologic localization of cytokine mRNA, using in situ hybridization at 160 days after inoculation.

Results—A noticeable increase in mRNA expression for TNFα and mild increases in mRNA expression of IL-8 and IL-1β were detected in mandibular lymph nodes from infected swine, compared with noninfected swine. Mild increase in mRNA expression for IL-6 also was observed in tonsils from infected swine. Cytokine mRNA was detected in macrophages and lymphocytes, primarily within cortical follicles and adjacent mantle zones.

Conclusions and Clinical Relevance—Expression of mRNA for inflammatory cytokines was increased in lymphoid tissue of infected swine, possibly resulting from local factors on, or secreted by, *M avium*. These results suggest that alterations in cytokine mRNA expression are important in the pathogenesis and clinical course of mycobacteriosis in swine. Modulation of the immune response by vaccines that selectively target cytokine expression and secretion in response to mycobacterial challenge may be effective in prevention of mycobacteriosis in swine. (*Am J Vet Res* 2000;61:1487–1491)

Mycobacteriosis (tuberculosis) is a common cause of carcass condemnation in swine. Clinical signs usually are not apparent in infected pigs, and the disease rarely is fatal. This lack of clinical signs makes it difficult to make an antemortem diagnosis of the disease. Lesions usually regress over time (months to years); however, the highest incidence of detectable lesions often corresponds with the traditional time of slaughter (ie, when pigs weigh 100 to 120 kg), resulting in carcass condemnation and substantial financial loss to producers. Once infection is established in a swine herd, it is difficult to effectively prevent or eliminate the disease.

Typically, macrophages phagocytize and kill most pathogens; however, mycobacteria and other intracellular pathogens have developed methods to survive within macrophages.[1] Organisms of the ***Mycobacterium avium-intracellulare* complex (MAIC)** commonly infect birds, swine, cattle, and sheep and infrequently infect horses and captive monkeys.[2] Factors that affect pathogenicity and the mechanisms by which mycobacteria survive and reproduce within host macrophages have not been completely described.

Infections in swine attributable to MAIC are of considerable economic importance to many swine producers.[3-7] The USDA regulations require that carcasses with gross granulomatous lesions in > 1 lymphatic region undergo a special cooking process, which substantially decreases the value of those carcasses. When lesions are more extensive, those carcasses must be condemned.[8] In 1 swine herd, 90% of carcasses had granulomatous lesions in mesenteric lymph nodes, and 60% of those carcasses had lesions in additional sites, resulting in them being condemned or passed with the requirement for cooking.[9] A study of 78 lymph nodes from swine originating from 12 abattoirs revealed that 97% of lymph nodes were infected with mycobacteria, and the predominant isolates were *M avium* serovar 1 (22%) and ***M avium* serovar 2 (Mav2**; 30%).[3] In a recent study, it was indicated that the condemnation rate in the United States was < 8.0 carcasses/100,000 swine slaughtered, and the rate of carcasses passed but requiring cooking was 13.6 carcasses/100,000 swine slaughtered, causing an estimated loss of $970,000/y.[10]

Infections in swine attributable to MAIC are not uniformly distributed throughout the world. Regions exist in which the incidence is much higher than national averages. Georgia, Arkansas, Missouri, and Iowa have a higher incidence within the United States. Incidence of the disease in most countries and states is currently unknown and is not reported to state or federal agencies by abattoir meat inspectors. New management practices involving swine raised in facilities previously used for poultry and increased use of wood by-products for bedding have resulted in an increased incidence of MAIC infections in swine. Immuno compromised people, such as patients with acquired immunodeficiency syndrome, transplant recipients, and patients with untreated hairy cell leukemia, often become infected with opportunistic members of the MAIC.[11-14] Improperly handled infected meats may be potential sources for human infection and are becoming a major concern for food safety.

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To our knowledge, evaluation of in situ cytokine expression for infected lymphoid tissue has not been performed for swine with mycobacteriosis. This information should help elucidate pathogenic mechanisms of mycobacteriosis in pigs and aid in the development of vaccines that modulate cytokine expression, resulting in enhanced mycobacterial clearance. In the study reported here, mRNA expression of inflammatory cytokines such as **tumor necrosis factor α (TNFα)**, **interleukin (IL)**-1β, IL-6, and IL-8 in lymphoid tissue of pigs experimentally infected with Mav2 was evaluated.

Materials and Methods

Animals—Specimens were obtained from 2 of 4 groups of pigs involved in an efficacy trial of a *M avium* vaccine.[15] For that efficacy trial, all pigs shared the same sire, and 8 pigs were obtained from each of 4 litters. The pigs from each litter were randomly distributed to each group in that study, resulting in 2 pigs from each litter in each of 4 groups (n = 8 pigs/group). Group 1 (negative-control pigs) consisted of noninfected pigs that were sham vaccinated. Group 2 consisted of pigs vaccinated with a Mav2 subunit vaccine and challenge-exposed with virulent Mav2. Group 3 consisted of pigs vaccinated with a whole-cell Mav2 vaccine and challenge-exposed with virulent Mav2. Group 4 consisted of pigs sham vaccinated and challenge-exposed with virulent Mav2. Specimens were obtained from pigs in groups 1 and 4 for the study reported here.

During the vaccine efficacy trial, all pigs were housed separately in identical pens and fed the same feed. Pigs were vaccinated with the relevant vaccine when they were 10 days old and again when they were 31 days old (3-week interval). Challenge-exposed pigs were inoculated orally on 2 consecutive days with 75 mg of virulent Mav2 (pelleted wet weight, 2.25×10^9 organisms/dose) 2 weeks after the last vaccination.

Specimens—Serum samples were obtained monthly from all pigs and frozen for subsequent analysis. At 157 days after inoculation, all pigs were given a skin test, using *M avium* **purified protein derivative (PPD)**. All challenge-exposed pigs had positive results for skin tests, whereas all negative-control pigs had negative results. At 160 days after inoculation, blood samples were obtained from all pigs, and pigs then were euthanatized and necropsied. Mycobacterial culture, histologic examination, Ziehl-Neelsen acid-fast staining, and **polymerase chain reaction (PCR)** for IS902, a specific insertion for *M avium*, were performed on all specimens.

One pig from group 1 and 1 pig from group 4 were not available because of disease (joint abscess) during the vaccine efficacy trial[15]; therefore, specimens from 7 pigs/group were evaluated for cytokine expression in the study reported here. Specimens obtained consisted of formalin-fixed (neutral-buffered 10% formalin) mandibular lymph node and tonsil from sham-vaccinated noninfected control pigs and sham-vaccinated Mav2-infected pigs. All specimens were formalin fixed for ≤ 12 hours. Positive-control specimens for cytokine mRNA were used with each batch of specimens and consisted of formalin-fixed paraffin-embedded spleen obtained from a neonatal pig that died because of septicemia attributable to infection with *Escherichia coli*.

In situ hybridization—Antisense oligonucleotides complementary to bases 573 to 607 of porcine TNF (5'-ATGGTGTGAGTGAGGAAAACGTTGGTGGAAGGGCA-3'), bases 354 to 308 of IL-1β (5'-TGCACGTTTCAAGGATGATGGGCTCTTCTTCAAAG-3'), bases 358 to 392 of IL-6 (5'-GTCTCCTGATTGAACCCAGATTGGAAGCATCCGTC-3'), and bases 201 to 248[a] of IL-8 (5'-AATCACTCTCAGTTCCTTGATAAATTTGGGGTGGAAAGGTGTGGAATG-3'), which can be used to detect these respective cytokines in porcine tissues,[16] were synthesized at a university genetics facility.[b] A negative-control oligoprobe consisting of sense oligonucleotides complimentary to bases 201 to 248 of IL-8 (5'-TTAGTGAGAGTCAAGGAACTATTTAAACCCCACCTTTCCACACCTTAC-3'5') was used to evaluate nonspecific background staining of tissue specimens.[16] Digoxigenin end-labeled oligoprobes were constructed, using a commercial kit[c] in accordance with manufacturer's instructions. Tissues were sectioned at a thickness of 2 µm and placed on slides coated with 3-aminopropyltriethoxysilane. Slides were dried overnight at 37 C, deparaffinized in xylene (2 immersions for 5 min/immersion), and then rehydrated in serial solutions of ethanol (95, 70, and 50%), using 2 minutes for each rehydration. After soaking in buffered saline (0.9% NaCl) solution for 5 minutes, sections were digested in proteinase K (20 µg/ml) and rinsed in PBS solution for 5 minutes before being fixed in 4% paraformaldehyde for 20 minutes. Sections then were rinsed twice in PBS solution (5 minutes/rinse), dipped in 50% ethanol for 30 seconds, incubated for 20 minutes in 0.2*M* HCl acetylated in 0.1*M* triethanolamine (pH 8), and then incubated in 0.5% acetic anhydride for 20 minutes. A final rinse was performed in a solution (0.2*M* tris [pH 7.4] and 0.1*M* glycine) for 10 minutes. Prior to incubation, slides were placed in 2X SSC (0.9% NaCl, 0.45% Na citrate [pH 7.0]) at 70 C for 10 minutes, which was followed by incubation in a humidified chamber with 100 µl of prehybridization buffer (50% formamide, 4X SSC, 1X Denhardt solution, and 10% dextran sulfate) at 37 C for 30 minutes. Slides were overlaid with 2 pmol of labeled oligoprobe in 40 µl of hybridization buffer, a coverslip was placed on each slide, and slides were incubated at 37 C for 14 hours. Washes consisted of 4X SSC, 2X SSC, and 0.2X SSC at 37 C for 5 minutes/wash. Detection was performed with an oligonucleotide detection kit,[c] using a 30-minute incubation in blocking solution, three 10-minute rinses in **tris-buffered saline (TBS;** pH 7.5), incubation with anti-digoxigenin-alkaline phosphatase for 1 hour, 3 additional 10-minute washes in TBS, and staining in detection solution (50 µl of nitroblue tetrazolium chloride, 37.5 µl of 5-bromo-4-chloro-3-indolyl-phosphate, 10 ml of Tris, pH 9.5) for 2 to 4 hours, as determined by microscopic observation of staining. Slides were dipped once briefly (2 seconds) in Gill III hematoxylin as a counterstain and rinsed prior to placement of a coverslip.

Microscopic evaluation—Twenty randomly chosen lymphoid follicles from each specimen of mandibular lymph node (7 infected, 7 noninfected) or tonsil (6 infected, 7 noninfected) were evaluated, and cells with positive staining were quantified independently by 2 microscopists (MEH, KSF) for each set of tissues and each cytokine of interest. Counts of each microscopist were tallied and averaged to reduce the potential for bias.

Statistical analysis—Statistical analysis was performed, using a 2-tailed *t*-test to compare values for noninfected control lymphoid tissues with values for Mav2-infected lymphoid tissues. A computer program[d] was used for all analyses. Correlation between results for the 2 microscopists was evaluated by use of linear regression. Values of $P < 0.05$ were considered to be significant.

Results

A noticeable increase in expression of TNFα mRNA and mild increases in expressions of IL-8 and IL-1β mRNA expression were detected in mandibular lymph nodes from infected pigs (**Table 1**). A mild increase in expression of IL-6 mRNA was observed in

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Table 1—No. (mean ± SEM) of cells that stained positive for mRNA of various inflammatory cytokines by use of in situ hybridization, using DNA oligoprobes, of lymphoid tissue* obtained from pigs experimentally infected with *Mycobacterium avium* serovar 2 (Mav2)

Tissue	Group	IL-1β	IL-6	IL-8	TNFα
Mandibular lymph node	Control	223 ± 17	42 ± 4	84 ± 9	189 ± 17
	Infected	298 ± 29[a]	54 ± 9	126 ± 12[b]	348 ± 30[c]
Tonsil	Control	243 ± 17	46 ± 8	100 ± 11	72 ± 11
	Infected	292 ± 37	181 ± 42[d]	151 ± 25	98 ± 24

*Values represent data for 20 randomly chosen lymphoid follicles per tissue (7 experimentally infected pigs, 7 control pigs). [a]Significantly ($P = 0.034$) different from the value for the same tissue obtained from control pigs. [b]Significantly ($P = 0.009$) different from the value for the same tissue obtained from control pigs. [c]Significantly ($P < 0.001$) different from the value for the same tissue obtained from control pigs. [d]Significantly ($P = 0.02$) different from the value for the same tissue obtained from control pigs.
IL = Interleukin. TNF = Tumor necrosis factor.

tonsils from infected swine. Expression of TNFα mRNA was compared with score of histologic lesions, number of Mav2 cultured, detection of acid-fast bacilli, and results of PCR for IS902 in mandibular lymph nodes (**Table 2**). All noninfected control pigs had negative results for mycobacterial cultures, detection of acid-fast bacilli, and PCR, and all had lesion scores of 0. Increased numbers of positively stained cells were detected in most specimens, with higher histologic lesion scores, higher numbers of Mav2 in cultures, and positive results for PCR. The magnitude of expression for all cytokines evaluated was greatest for TNFα mRNA, and TNFα has been associated with mycobacterial clearance in other species.[5,17-19]

Specimens from noninfected control pigs did not have gross or microscopic lesions consistent with mycobacteriosis (mean ± SD lesion score, 0.0 ± 0.0). Mycobacteria were not obtained on culture, acid-fast stains had negative results, and PCR evaluation for IS902 was negative on all control specimens. Results of *M avium* PPD skin tests were negative for all 7 pigs prior to necropsy.[15] Specimens from the Mav2-infected pigs had small pinpoint granulomas in mandibular lymph nodes and microscopic lesions consistent with mycobacteriosis (mean lesion score, 4.93 ± 2.21; maximum score possible was 9.5). Results of skin tests with *M avium* PPD were positive on all 7 challenge-exposed pigs. *Mycobacterium avium* was cultured from mandibular lymph nodes in 6 of 7 infected pigs and correlated to a mean of 100 ± 64.5 colony-forming units (CFU)/g of tissue. Acid-fast stains yielded positive results for 3 mandibular lymph node specimens, whereas 5 specimens were positive on PCR, and 4 had microscopic lesions consistent with mycobacteriosis. All tonsil specimens (n = 6) for experimentally infected pigs had negative results for microbial culture and PCR, but 4 had positive results for acid-fast bacilli, and 4 had microscopic lesions consistent with mycobacteriosis.

Background staining from the negative-control oligoprobe (IL-8 sense) was lacking in all specimens (data not shown). Tissue sections with antisense oligoprobes for IL-1β, TNFα, IL-8, and IL-6 had minimal background staining, and the intense staining of positive cells was easy to delineate in all sections evaluated. Approximately 50% of positively staining cells had morphologic features compatible with macrophages or monocytic lineage. The remaining positively stained cells appeared to be lymphocytes. Staining was evident within the nucleus and cytoplasm of positively stained cells, but staining was most intense in the nuclei. The number of positively stained cells in lymph nodes was much greater within germinal centers and the adjacent

Table 2—Comparison of the No. of cells that stained positive for TNFα mRNA determined by in situ hybridization that used DNA oligoprobes with histologic lesion score, results of mycobacterial culture, detection of acid-fast bacilli (AFB), and results of polymerase chain reaction (PCR) for IS902, a specific insertion for *M avium*, in mandibular lymph nodes obtained from specimens collected from each of 7 pigs experimentally infected with Mav2*

Pig	Count	Lesion score	CFU	AFB	PCR
A	537.5	5.0	100	+	B
B	509.0	5.0	100	+	+
C	365.0	4.0	150	B	B
D	274.0	5.0	200	B	+
E	354.0	3.0	0	B	+
F	357.0	3.0	50	B	+
G	396.5	9.5	100	+	+

*Noninfected control pigs had negative results for mycobacterial culture, detection of acid-fast bacilli, and PCR and had lesion scores of 0. Mean ± SEM No. of positively stained cells in mandibular lymph node of noninfected control pigs was 189 ± 17.
MLN = Mandibular lymph node. Count = Mean count of cells expressing cytokine mRNA. CFU = Mean No. of colony-forming units of Mav2/g of specimen. B = Negative result. + = Positive result.

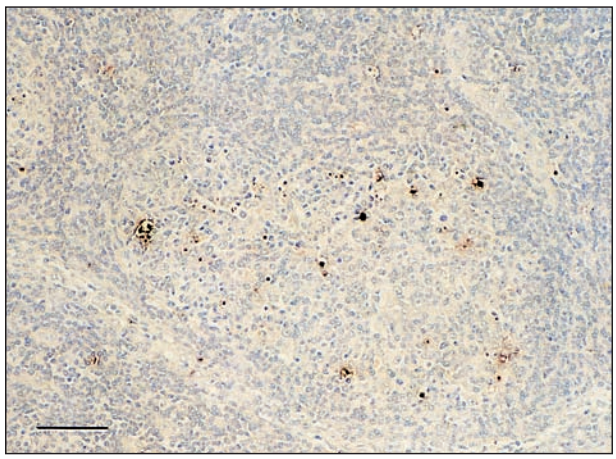

Figure 1—Representative results of in situ hybridization for tumor necrosis factorα mRNA in a section of mandibular lymph node obtained from a pig experimentally infected with *Mycobacterium avium* serovar 2. Notice the lack of background staining, location of positively stained cells, and the generally intense staining of positively stained cells. Gill-III hematoxylin stai; Bar = 100 μm.

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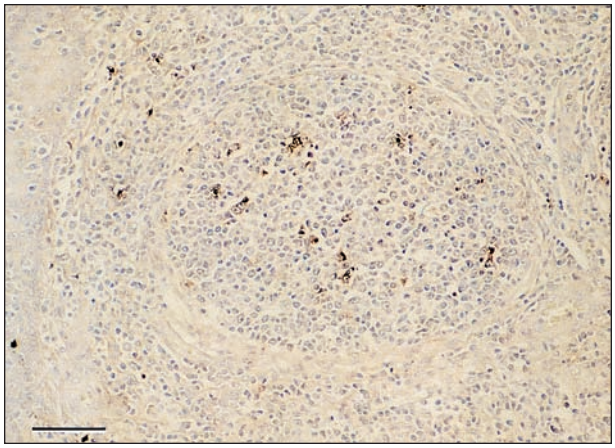

Figure 2—Representative results for in situ hybridization for interleukin-6 mRNA of a section of tonsil obtained from the pig in Figure 1. Notice the location of positively stained cells within lymphoid follicles and lack of background staining. Gill-III hematoxylin stain. Bar = 100 μm.

mantle zones of cortical lymphoid follicles. The medulla of lymph nodes had infrequently scattered positively stained cells. Positively stained cells within tonsils were generally limited to germinal centers and associated mantle zones in cells of lymphoid and monocytic lineage. Cells with increased expression of TNFα mRNA were especially prominent in mandibular lymph nodes (**Fig 1**). Expression of IL-6 mRNA was most prominent in tonsils of experimentally infected pigs (**Fig 2**).

Results for the 2 microscopists were compared, using linear regression and correlation. Although some variation was evident in the counts obtained by each microscopist, patterns were similar. Overall correlation of results (number of positively stained cells) between microscopists for the study was good (R = 0.803; R = 0.710 for mandibular lymph nodes and R = 0.897 for tonsils).

Discussion

Specimens used in the study reported here were obtained from sham-vaccinated noninfected control pigs and sham-vaccinated Mav2-infected pigs (group 1 and 4, respectively) of our previous vaccine efficacy trial.[15] In that study, these specimens were evaluated for gross and microscopic lesions, stained for acid-fast bacilli, cultured for mycobacteria, and analyzed by use of PCR performed for IS902. Lesions were rarely detected in organs other than tonsil and mandibular lymph nodes; therefore, only these tissues were selected for expression of cytokine mRNA in this study. The number of mycobacteria cultured from these tissues was low, making it difficult to establish whether a significant association exists between expression of cytokine mRNA and number of organisms isolated. It is known that it is notoriously difficult to isolate MAIC from infected porcine tissues.

Data obtained in this study reflected the in situ expression of cytokine mRNA in *M avium*-infected swine lymphoid tissue at 160 days after inoculation, which corresponds to the traditional age of slaughter and associated carcass condemnations. This reflects a stage at which a cell-mediated immune response is active and suggests that TNFα is important in disease resolution.

Tonsils are generally regarded to be the most important portal of entry for *M avium* in swine, whereas gross and microscopic lesions (granulomas, microgranulomas, and lymphoid hyperplasia) usually are more evident in mandibular lymph nodes. In several in vitro studies, increased concentrations of TNFα have enhanced clearance of *M tuberculosis*.[5,17-19] In the study reported here, a noticeable increase in expression of TNFα mRNA was found in mandibular lymph nodes infected with *M avium* but not in infected tonsils. On the basis of studies in mice, it appears that production of TNFα is increased later in the course of disease and eventually results in clearance of the organisms and resolution of disease.

In other in vitro studies, investigators have documented that increased amounts of IL-6 enhance growth of *M avium* within macrophages, acting similar to a growth factor.[17,20,21] In the study reported here, an increase in expression of IL-6 mRNA was found in tonsils but not in infected mandibular lymph nodes. This suggests that tonsillar tissue (via increased expression of IL-6 mRNA) may provide a favorable environment for survival of *M avium* and for subsequent dissemination to regional lymph nodes. Interleukin 6 was the only cytokine altered in infected tonsils in this study. Tonsils continuously are being exposed to infectious microorganisms in the environment. Although a low number of *M avium* are excreted in feces, coprophagia (common in pigs) may provide a route of continuous reinfection.

A mild increase in expression of IL-8 mRNA was detected in mandibular lymph nodes, but not in tonsils, from experimentally infected pigs. Interleukin 8 is secreted by macrophages and activated T cells, and in addition to its role as a primary chemotactic chemokine for recruitment of neutrophils, it stimulates monocyte adhesion at inflammatory sites, thus enhancing host defenses against pathogens.[22] A mild increase in expression of IL-1β mRNA was found in mandibular lymph nodes, but not in tonsils, from experimentally infected pigs. Interleukin 1β evokes a number of proinflammatory biological effects, including upregulation of TNFα secretion, enhancement of specific activity of T-helper cells, and induction of a cell-mediated response.[22] Although we detected a significant increase in expression of IL-1β mRNA, the magnitude was mild and may not be biologically important.

Only a small number of inflammatory cytokines was evaluated in this study, because only a few genetic sequences for porcine cytokines have been published. Genetic sequences for murine cytokines are more readily available but have not been proven to be effective in porcine tissues. In studies involving mice, investigators have evaluated expression of cytokine mRNA and provided valuable insight into the pathogenesis of mycobacteriosis in mice[19,20]; however, the course and resolution of infection in mice differs substantially from that of swine, and it is unclear whether the mechanisms suspected in the murine model are valid for

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swine. As more probes become better defined, other cytokine alterations also may be detected in *M avium*-infected swine. Analysis of other studies of murine cytokines suggests that increased amounts of TNFα and IL-8 are involved in clearance of organisms, whereas increased tonsilar IL-6 secretion likely enhances susceptibility to infection with *M avium*.[5,17-21] Analysis of our results also documented alterations in mRNA expression of these cytokines. Modulation of the immune response by vaccines that selectively increase TNFα secretion and suppress IL-6 secretion in response to mycobacterial challenge may be effective in prevention of mycobacteriosis in swine.

[a]Oligoprobe tailing kit, Boehringer Mannheim, Indianapolis, Ind.
[b]Molecular Genetics Facility, University of Georgia, Athens, Ga.
[c]Oligo detection kit, Boehringer Mannheim, Indianapolis, Ind.
[d]Graphpad Prism, version 2.01, GraphPad Software, San Diego, Calif.

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