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Goldfish, *Carassius auratus*, a Novel Animal Model for the Study of *Mycobacterium marinum* Pathogenesis

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We have developed an animal model for studying mycobacterial pathogenesis using *Mycobacterium marinum* and the goldfish, *Carassius auratus*. Goldfish are injected intraperitoneally with doses between 10^2 and 10^9 CFU of *M. marinum* organisms. Depending on the dose of *M. marinum* organisms administered, an acute or chronic disease is produced. The acute disease is characterized by systemic mycobacterial infection, severe peritonitis, tissue necrosis, and a short median survival time. The chronic disease is characterized by granuloma formation in all organs and survival of animals to the end point of the experiment (56 days). Colony counts in organ homogenates showed recovery of mycobacteria from a high percentage of inoculated animals. We believe this well-characterized animal model will be useful for studying mycobacterial pathogenesis.

Although there has been some progress in developing genetic systems to study *Mycobacterium tuberculosis* (2, 4, 12, 21), its slow growth rate (a generation time of more than 20 h) and the necessity of working in a biosafety level-3 facility has led investigators (17, 18) to explore surrogate mycobacterial model systems to study the molecular pathogenesis of this organism.

Mycobacterium marinum, first isolated from saltwater fish in 1926 (1), is an agent of fish tuberculosis. Fish tuberculosis is a disseminated infection reported in more than 150 species of fish (15). The disease is usually accompanied by emaciation and deaths in the infected fish population over a period of months to years. The typical lesion seen with histopathological examination is the granuloma, which may be present in any internal organ (24). The histopathology of the formed granuloma in fish tuberculosis (8, 11, 24) is similar to the histopathology seen in human tuberculosis (10, 13, 14). Unlike *M. tuberculosis*, *M. marinum* can be studied in a biosafety level-2 laboratory with standard bacteriological protocols. The generation time for *M. marinum* is about 4 h in the laboratory (5). Based on an analysis of 16S rRNA sequences of 19 mycobacterial species, *M. marinum* is the mycobacterial species closest to the *M. tuberculosis* complex, with a sequence homology of 99.4% (22).

Two animal models have been described that utilize *M. marinum*. The first is the mouse footpad infection model, which was used to simulate *Mycobacterium leprae* infection (6). Attempts to produce a systemic infection in mice failed even when the inoculum was administered intravenously (5). More recently, a frog (*Rana pipiens*) model for *M. marinum* infection has been proposed (17, 18). With the frog model, granulomas were reported in the livers and spleens of animals sacrificed at 6 weeks postinoculation. No animals died of *M. marinum* infection in the 40-week observation period. Moreover, the *M. marinum* strain used to induce chronic disease in the frog failed to induce overt signs of disease when tested in fish (18).

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In 1963, fish were among 50 species of poikilothermic animals that were found to be susceptible to experimental infection with *M. marinum*. In the model described in this report, the goldfish, *Carassius auratus*, is used to study the pathogenesis of *M. marinum*. Systemic granuloma formation is the characteristic finding in our model of a chronic progressive disease which parallels the pathology seen in human tuberculosis. At higher doses (10^8 to 10^9 CFU), our experimental mycobacterial fish infection becomes an acute model, with systemic dissemination, necrosis, and inflammation, which results in death in 4 to 17 days. The minimum dose to produce systemic granulomas within 8 weeks was 600 CFU per fish. This model should prove useful for studying mycobacterial pathogenesis and for identifying avirulent mutant strains.

MATERIALS AND METHODS

Fish. Goldfish, *C. auratus* (20 to 30 g), were obtained from a local commercial fish farm (Hunting Creek Fisheries, Hunting Creek, Md.). They were acclimated to their new environment (20-gal flowthrough aquaria with a water temperature of 20 ± 2°C and a photoperiod of 16 h light and 8 h dark) in the quarantine area of the fish facility in the Aquatic Pathobiology Center, University of Maryland. After 2 weeks of acclimation, the fish were moved to a negative-air-pressure room, where the experimental infection with mycobacteria was performed. Skin scrapes, gill biopsies, and fecal examinations were performed on a representative sample of fish to determine that they were free of parasitic infestation prior to infection by mycobacteria. The fish were treated with a prophylactic dose, 100 ppm, of formalin to prevent parasitic infestation. The fish were fed pellet trout grower (30% protein; Ziegler Bros., Gardner, Pa.) 3 days a week. Fish inoculated with different doses of mycobacteria and control fish inoculated with phosphate-buffered saline (PBS) were housed in separate aquaria.

Bacteria. The *M. marinum* strain ATCC 927 (fish isolate) was obtained from the American Type Culture Collection (Rockville, Md.). *M. marinum* M (human isolate) was from Lalita Ramakrishnan, Stanford University (17), while *M. marinum* F-110 was isolated from *Cichlid* sp. fish in the Aquatic Pathobiology Center, University of Maryland (23). All strains were grown with shaking at 30°C as a dispersed culture in 7H9 (Difco, Detroit, Mich.) broth with 10% albumin-dextrose complex enrichment (12). Animal inocula were obtained from mid-exponential-phase cultures (optical density at 600 nm, ~1.0) and adjusted to the appropriate dose. The number of CFU per milliliter was determined by plating on Middlebrook 7H10 agar (Difco). Prior to inoculation in animals, the inocula were disaggregated by sonication for 3 min (power level 3) while cooling, using a cup horn accessory attached to a cell disrupter (model W-220 F; Heat Systems-Ultrasonics, Inc., Farmingdale, N.Y.).

Diagnostic PCR. In brief, the PCR protocol uses genus-specific primers designed from conserved regions of the 16S rRNA sequence of mycobacteria. A 924-bp DNA fragment is amplified from the mycobacterial species known to cause fish mycobacteriosis (*M. marinum*, *Mycobacterium fortuitum*, and *Mycobac-*

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terium chelonae). Following amplification, the DNA product was digested with restriction enzymes, *Ban*I (NEB, Beverly, Mass.) and *Apa*I (GIBCO BRL, Gaithersburg, Md.), to yield unique restriction patterns for each of the mycobacterial species (23).

Animal inoculation. Fish were inoculated intraperitoneally through the lateral abdominal musculature with 0.5 ml of various concentrations of *M. marinum* organisms by using a 25-gauge needle and tuberculin syringe.

Negative-control fish groups were inoculated with sterile PBS coincidentally with the experimentally infected fish to control for environmental conditions (parasitic infestation, changes in water temperature, etc.) in the aquaria.

Fish tissue processing. Fish were sacrificed either in a moribund state or at scheduled 2-week intervals from 2 to 16 weeks postinoculation. At sacrifice, the liver, spleen, and kidneys of each fish were collected and 100-mg portions of these organs were homogenized in PBS with 0.05% Tween 80. Bacterial counts in the organs of the fish were determined by plating serial 10-fold dilutions of organ homogenates on Middlebrook 7H10 agar. The colonies were identified as mycobacterial species by a diagnostic PCR developed in our laboratory (23). A peritoneal wash was performed by intraperitoneal injection of 1 ml of sterile PBS, followed by collection 15 min later. One hundred microliters of the peritoneal wash was plated on 7H10 plates, and the colony count was expressed as CFU per milliliter. Portions of the liver, spleen, and kidneys along with the brain, gills, intestine, gonads, muscle, skin, peritoneum, and heart were fixed in 10% neutral buffered formalin for routine embedding in paraffin (16). Five-micrometer sections of the paraffin-fixed tissue were prepared with a rotary microtome (American Optical, Buffalo, N.Y.). After dewaxing, the sections were stained with hematoxylin and eosin or stained for acid-fast bacilli with modified Ziehl-Neelsen stain (9). To evaluate the extent of goldfish organ involvement after mycobacterial infection, we used an arbitrary scoring scale. The scale was defined as score 0, normal; 1, minimal; 2, mild; 3, moderate; 4, marked; and 5, severe. Lesions were evaluated both in terms of how many were present and how large an area was affected. Lesions which were observed to be few and to have little impact on the organ were classified as minimal. Severe lesions involve an organ to an extent that one can barely recognize the normal tissue. Scores of mild, moderate, and marked are gradations between the two extremes (20). This scale was used to determine the granuloma score (GS). The cumulative GS (CGS) for each fish was the sum of the individual GSs of the peritoneum, liver, heart, spleen, and kidneys.

Statistical analysis. To investigate the relationship between colony recovery and time postinoculation, three separate linear regression analyses (liver, spleen, and kidney) of log-transformed CFU were performed. Analyses were performed with SAS software; statistical significance was assessed at the 5% level.

RESULTS

MST and LD_{50}. To determine the median survival time (MST) of goldfish after inoculation with *M. marinum* ATCC 927, groups of 20 to 32 fish were inoculated with 10^9, 10^8, or 10^7 CFU. The MST of goldfish inoculated with *M. marinum* was dose dependent, with survival time decreasing with increasing doses of bacteria. The MST of the fish was 4, 10, and >56 days (the end point of the experiment) with inocula of 10^9, 10^8, or 10^7 *M. marinum* organisms, respectively. All fish inoculated with 10^7 CFU or less survived to the end point of the experiment (56 days). The control fish group, inoculated with PBS in five separate experiments, had a total of two premature deaths, one at 8 and one at 19 days postinoculation, from a total of 55 fish. The remainder of the control fish survived to 56 days, the end point of the experiment (Fig. 1). The 50% lethal dose (LD_{50}) at 1 week postinfection with *M. marinum* was 4.5×10^8 and was calculated by the method of Reed and Muench (19).

Mycobacterial recovery from fish organs. To assess the ability of *M. marinum* to persist in goldfish tissue, the liver, spleen, and kidneys from each sacrificed fish were collected for bacteriological examination. *M. marinum* was recovered from all organs of fish in the 10^9- or 10^8-CFU-inoculum groups. In fish inoculated with 10^7 CFU, *M. marinum* was recovered from 96% of the examined organs.

Figure 2 shows the fate over an 8-week period of the *M. marinum* ATCC 927 strain in the livers, spleens, and kidneys of fish inoculated with 10^7 CFU. There was a significant positive linear relationship between time postinoculation and colony recovery in the liver ($P < 0.001$); for the spleen and kidneys, the relationship was positive but did not reach statistical significance ($P = 0.054$ and $P = 0.091$, respectively). Between 8 and 16 weeks postinoculation, *M. marinum* persisted in the tissue with no significant change in the colony counts.

M. marinum was recovered from the peritoneal wash of 40% of the inoculated animals. Colony counts were lower in the peritoneal washes than in the respective liver and spleen in 9 of 10 animals (data not shown). In addition, in the 10^2- to 10^6-CFU-inoculum groups, *M. marinum* was isolated from at least one organ from all infected fish.

No *M. marinum* was isolated from control fish tissue. However, in 2 of 55 control fish, *M. fortuitum*, another agent of fish mycobacteriosis, was isolated. None of the fish from which *M.*

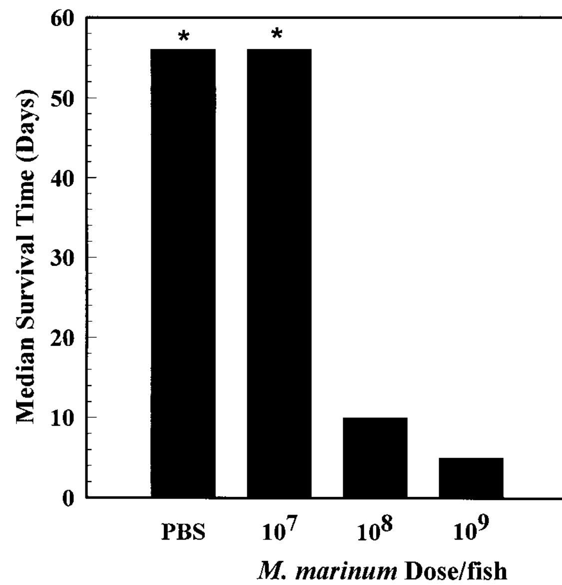

FIG. 1. MST of fish inoculated with *M. marinum* at indicated doses per fish compared to that of control fish inoculated with PBS. *, survival to the end point of the experiment, 56 days.

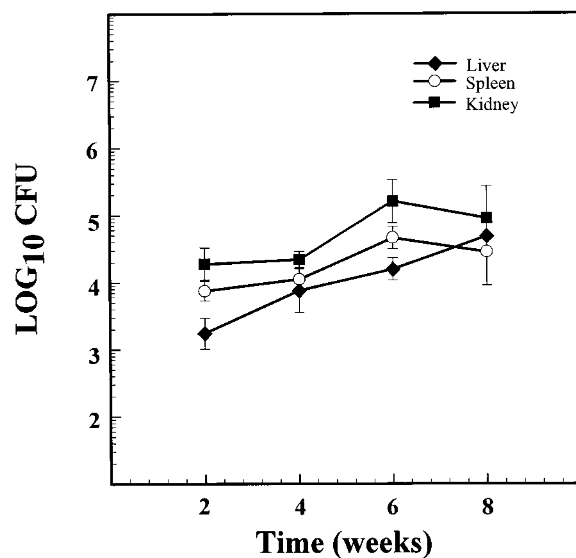

FIG. 2. Comparison of *M. marinum* recovery from liver, spleen, and kidney with an inoculum of 10^7 CFU/fish. The results are given as geometric means and standard errors for eight fish per time point.

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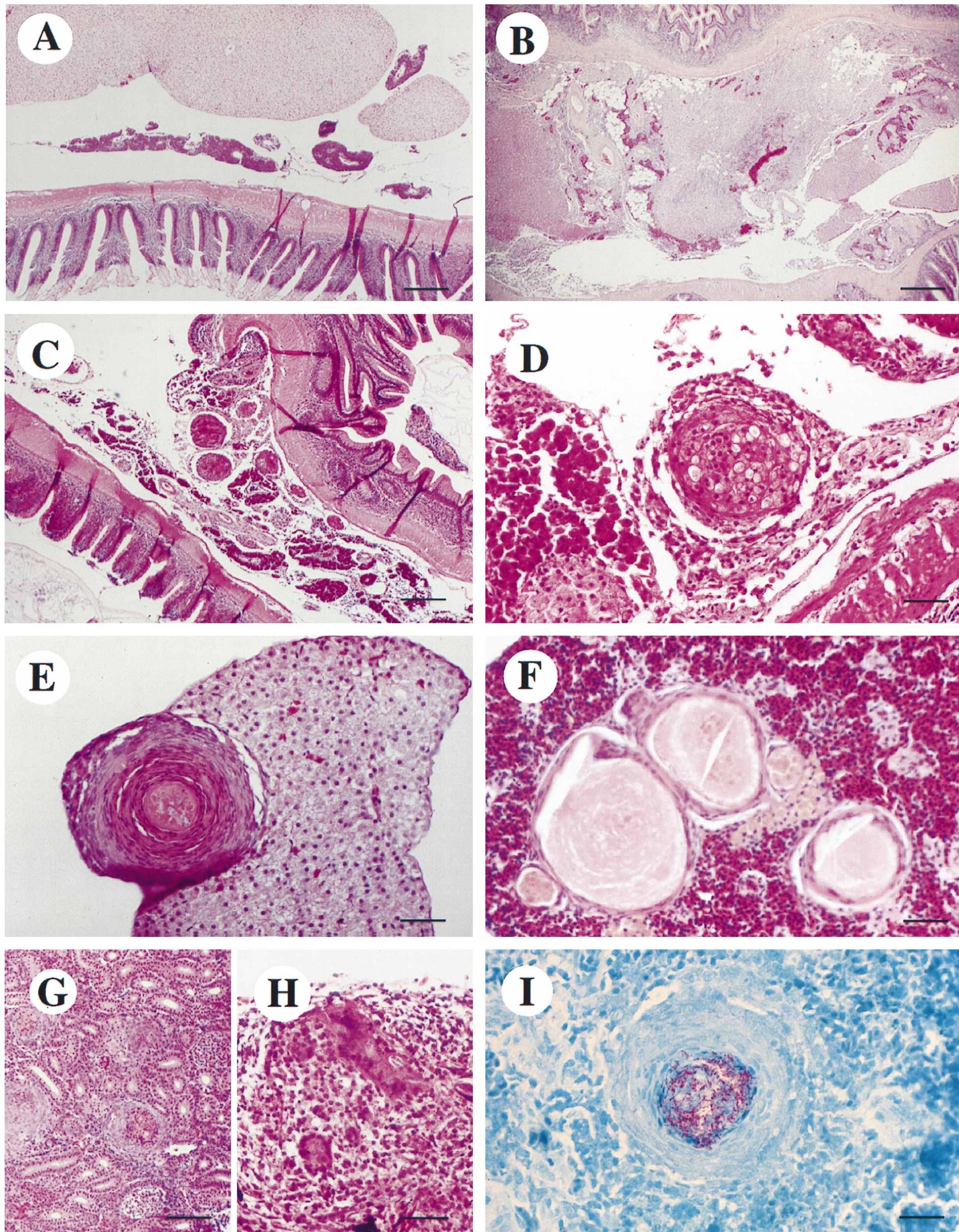

FIG. 3. Representative histopathology of fish infected with *M. marinum* ATCC 927. All histopathology sections were stained with hematoxylin and eosin stain except that shown in panel I, which was stained with modified Ziehl-Neelsen stain. (A) Light micrograph of normal peritoneum, liver, pancreas, and intestine of control group fish. Magnification, ×64; bar = 156 μm. (B) Peritoneum, pancreas, and intestine of fish infected 3 days earlier with 10^9 CFU of *M. marinum* organisms. There is a region with extensive necrotic tissue, bacterial cells, and exudate. Magnification, ×64; bar = 156 μm. (C to I) Histopathology sections taken from fish infected 8 weeks earlier with 10^7 CFU of *M. marinum* organisms. (C) Dense granuloma formation in the pancreas and peritoneum surrounding the intestine. Magnification, ×64; bar = 156 μm. (D) Granuloma formation with foamy macrophages in the peritoneum. Magnification, ×320; bar = 31 μm. (E) Liver with onion ring granuloma composed of epithelioid macrophages surrounding a necrotic center. Magnification, ×160; bar = 63 μm. (F) Multiple caseous granulomas occupying a large portion of the spleen. Magnification, ×320; bar = 31 μm. (G) Early granuloma formation in the trunk kidney. Magnification, ×160; bar = 63 μm. (H) Both Langhans and foreign-body-type giant cells in the head kidney. Magnification, ×320; bar = 31 μm. (I) Granuloma with a thick wall and acid-fast bacilli in its center in the spleen. Magnification, ×640; bar = 16 μm.

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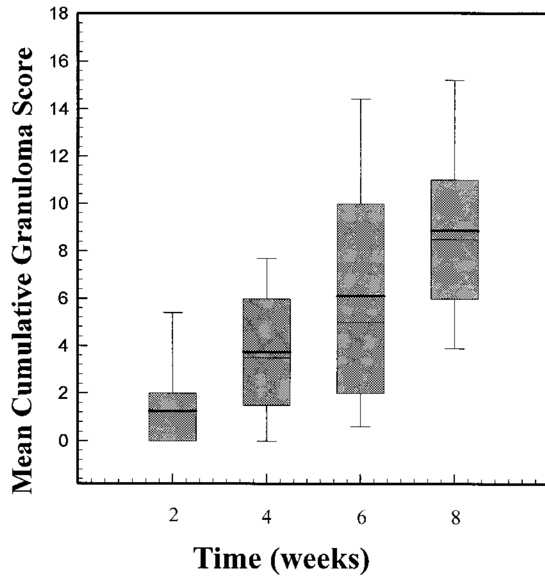

FIG. 4. Comparison of MCGSs over time of fish infected with 10^7 CFU of *M. marinum* organisms. The results are given as a vertical box plot, with the bottommost and topmost horizontal lines marking the 10th and 90th percentile points, respectively, of GSs for eight animals at each time point. The box encompasses the 25th through the 75th percentiles. Within the box, the light horizontal line represents the median (50th percentile) and the thick line represents the mean for each group. At 2 weeks, the median 50th percentile and mean values are the same.

fortuitum was isolated appeared ill at the time of sacrifice. This isolation of *M. fortuitum* probably represents the indigenous disease present in the fish population.

Acute and chronic forms of mycobacterial infection. The pathology of infected fish was dependent on the inoculum dose and the time postinfection of animal sacrifice. Fish infected with either 10^9 or 10^8 CFU of *M. marinum* organisms suffered from anorexia, sluggish movement, and loss of equilibrium. Fish that survived more than 7 days in the 10^8-CFU-inoculum group suffered from moderate-to-heavy infestation of the ectoparasite *Ichthyophthirius multifiliis*. This was despite two prophylactic formalin treatments and a negative screen for parasites when the fish were introduced into the experimental tanks. Fish infected with 10^7 or fewer CFU displayed normal behavior.

The histopathology of fish infected with 10^9 and 10^8 CFU was characterized by severe peritonitis and necrosis (Fig. 3B) compared to control fish (Fig. 3A). As seen in Fig. 3B, the peritoneum was filled with inflammatory cells consisting of lymphocytes, macrophages, and fibrous connective cells as well as with degenerating cells and bacteria. The mean CGSs (MCGSs) for these two groups were similar (0.2 for the 10^9-CFU group and 0.9 for the 10^8-CFU group). In the 10^8-CFU-inoculum group, granuloma formation was more likely to be found in animals which survived more than 2 weeks postinoculation.

When examined at 2 weeks, six of eight fish in the 10^7-CFU group had moderate-to-severe peritonitis. Unlike the 10^8- and 10^9-CFU-inoculum groups, which succumbed to infection, the 10^7-CFU-inoculum group survived the infection, and by 4 to 6 weeks postinoculation, the acute peritoneal inflammation was replaced by a chronic inflammatory state. Fish inoculated with 10^7 CFU demonstrated granuloma formation in all organs evaluated (MCGS, 5.0), including the peritoneum and pancreas (Fig. 3C and D), liver (Fig. 3E), spleen (Fig. 3F), trunk kidney (Fig. 3G), head kidney (Fig. 3H), heart, and intestine. Pleomorphic granulomas (necrotizing, nonnecrotizing, and caseous) were seen. The necrotizing granulomas were characterized by a central area of necrosis surrounded by macrophages, epithelioid cells, and thin fibrous connective tissue (Fig. 3E). Frequently, caseous necrosis was present in the central area of the granuloma (Fig. 3F). Granulomas containing foamy macrophages were also seen (Fig. 3D). Occasionally, Langhans and foreign-body-type giant cells (Fig. 3H) were observed. In addition, acid-fast bacilli could be demonstrated with the modified Ziehl-Neelsen stain (Fig. 3I). Melanomacrophage centers were seen in a few cases.

The chronic inflammatory response of fish towards *M. marinum* was time dependent, as shown by the increase in MCGSs with time in animals inoculated with 10^7 CFU (Fig. 4) up to 8 weeks. From 8 to 16 weeks postinoculation, there was no significant change in the MCGSs (5.0 and 5.7, respectively).

MID. To estimate the lowest possible dose of *M. marinum* able to establish infection in goldfish, groups of four fish were inoculated with *M. marinum* ATCC 927 at doses of 10^6, 10^5, 10^4, and 10^2 CFU. Granuloma formation was seen in 25% of the goldfish by 4 weeks and in 88% by 8 weeks postinfection with a dose of 6.3×10^2 CFU or higher (Table 1). The minimum number of organisms required to establish infection (MID) in goldfish appears to be approximately 600 CFU.

Mycobacterial virulence assay. To determine the utility of this animal model in evaluating the virulence of different strains of *M. marinum*, we assessed the relative virulence of different mycobacterial strains of both human and animal origin. Three mycobacterial strains, *M. marinum* ATCC 927, M, and F-110, were inoculated into goldfish at 10^8 CFU. The MSTs of *M. marinum* M, ATCC 927, and F-110 were similar, ranging from 4 to 10 days.

DISCUSSION

We have developed a novel animal model for the study of mycobacterial pathogenesis using the goldfish, *C. auratus*, and *M. marinum*. An interesting feature of our model is that, depending on the dose of *M. marinum* inoculated, we can elicit acute or chronic disease. The acute disease is induced by the injection of 10^8 to 10^9 CFU per fish, while the chronic disease is induced by the injection of 10^2 to 10^7 CFU per fish. The heavy infestation by *I. multifiliis*, which occurred during two separate experiments in the 10^8-CFU-inoculum group, may be secondary to immune suppression precipitated by infection with *M. marinum*. Fish had been randomized to inoculum groups from the same population, yet neither the control fish nor the 10^7- or 10^9-CFU group developed an infestation of the parasite. The 10^9-CFU-inoculum group all died within 6 days of infection; if they had lived longer, parasitic infestation most likely would have occurred. We would hypothesize that im-

TABLE 1. MID of *M. marinum* ATCC 927

Inoculum (CFU/fish)	No. positive[a]		MCGS
	4 Wk	8 Wk	
1.2×10^6	1/2	1/2	5.0
3.0×10^5	0/2	2/2	5.5
2.4×10^4	1/2	2/2	1.5
6.3×10^2	0/2	2/2	4.5

[a] Number of granuloma-positive animals per total number of animals at 4 and 8 weeks postinoculation.

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mune suppression occurs about 7 days after *M. marinum* infection in the high-inoculum groups (10^8 or 10^9 CFU).

In contrast, the chronic disease (induced by 10^2 to 10^7 CFU) is characterized by progressive, systemic granuloma formation. Granulomas with different histopathological features (necrotizing, nonnecrotizing, and caseous) were seen in the experimentally infected goldfish, which is consistent with the granuloma types seen in naturally infected animals (3, 11). The fish demonstrating chronic disease appeared healthy until sacrifice (up to 16 weeks postinoculation). The histopathology of caseous granulomas observed in the infected goldfish is similar to the pathology reported in immunocompetent human beings infected with *M. tuberculosis* (13, 14). This contrasts with the mouse model of *M. tuberculosis* (7), where little caseation is observed in granulomas.

Isolation of *M. marinum* from fish tissue was possible throughout the course of the experiment (up to 16 weeks). This persistence in tissue is a feature which parallels human tuberculosis, where organisms may remain dormant in organs for many years. The systemic nature of the disease in the experimentally infected animals was supported by induction of granulomas and isolation of mycobacteria from retroperitoneal organs, such as the kidney and heart. Further experiments extending the length of infection are needed to show whether the fish can eventually eradicate the infection.

With the goldfish model, the MID of *M. marinum* was estimated to be approximately 600 CFU, compared to 10^4 CFU with the frog model (18). We used our animal model to assess the virulence of *M. marinum* strains of either human or fish origin. We found that the goldfish response to *M. marinum* is similar regardless of the origin of the strains tested.

We have calculated the LD_{50} at 1 week for *M. marinum* ATCC 927 as 4.5×10^8 CFU per fish. To our knowledge this is the first determination of the LD_{50} for *M. marinum* in any animal model.

The fish model for mycobacteriosis described in this report is a convenient, easily reproducible model which obviates the need for a biosafety level-3 facility to study mycobacteria and utilizes *M. marinum*, a relatively rapid grower. We plan to use this model to screen for potential virulence mutants of *M. marinum*. We believe that it represents an excellent animal model for studying the genetic basis of mycobacterial pathogenesis.

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