Preexposure vaccination is the key preventive measure against many infectious diseases. For areas of high endemicity, sporadic outbreaks, emerging infections, and diseases where vaccine efficacy is waning, there is, however, a need for reliable postexposure vaccines (1–3). Postexposure immunoprophylaxis is an accepted approach in a limited number of (mostly viral) infections, exemplified by rabies in humans (4). For bacterial pathogens, there is little experience with postexposure vaccination. Despite increasing occurrence of multidrug-resistant strains, antibiotics are still the mainstay postexposure treatment for bacterial infections in both humans and animals, such as tuberculosis, brucellosis, anthrax, plague, and Q fever.

Transmission of infection through early exposure to pathogens from environmental sources and/or subclinical carriers is an important obstacle for classical preventive vaccination, notably in the case of mycobacterial infections (5–7). This applies to important human pathogens (Mycobacterium tuberculosis, Mycobacterium leprae, and Mycobacterium avium) as well as pathogens of food-producing animals and wildlife (Mycobacterium bovis and Mycobacterium avium subsp. paratuberculosis). Therefore, control of especially this group of pathogens has a particular need for vaccines that can be administered to already-exposed individuals, aiming to reduce clinical disease and population transmission. For bovine paratuberculosis, a bacterin vaccine has been used that slows progression to the clinical stage of the disease, but it has minor effects on infection and shedding of bacteria into the environment (8). In addition, the use of this vaccine interferes with bovine tuberculosis control, and its usefulness is therefore limited (9). The current tuberculosis vaccine BCG (bacillus Calmette-Guérin) has limited efficacy against pulmonary tuberculosis infection (10, 11). Furthermore, there are safety concerns concerning vaccinating immunocompromised individuals with BCG as well as vaccination of exposed individuals with BCG, as it may lead to interleukin 17 (IL-17)-dependent pathology, as was shown in a postexposure murine model (12).

Mycobacterial infections of cattle provide good models for human tuberculosis (13–15). Cattle are within the natural host range of several pathogenic mycobacteria (M. bovis, M. tuberculosis, and M. avium subsp. paratuberculosis). Similarities between bovine and human mycobacterial infections relate to infectious dose, pathology, prolonged latency periods, clinical wasting disease, and immune responses (13–15). Preexposure vaccines have been evaluated in cattle with experimental tuberculosis infection (16–18). But for evaluation of postexposure vaccines, no experimental model is available, and the use of naturally infected cattle is limited because the time of exposure cannot reliably be assessed. Furthermore, they require biosafety level 3 (BSL3) facilities, which limit the number of animals per study. For these reasons, bovine paratuberculosis, the related pathogenic mycobacterial infection of cattle, which causes chronic granulomatous enteritis and is transmitted via shedding of bacteria in feces (19), is a more attractive natural host model for chronic mycobacterial infections. As this disease is endemic and highly prevalent in cattle populations worldwide, naturally infected individuals are readily available for postexposure vaccine trials. Furthermore, animals younger than 6 months are most susceptible to infection; clinical signs do not develop for 4 to 5 years postinfection. As a result, infected individuals have had a much more synchronous exposure than do bovine tuberculosis-exposed individuals.

In this study, postexposure efficacy of an Hsp70 candidate subunit vaccine was studied in cattle with naturally acquired chronic M. avium subsp. paratuberculosis infection to mimic field cond-
tions as closely as possible. Reduction of fecal \textit{M. avium} subsp. \textit{paratuberculosis} shedding and survival were assessed as vaccine efficacy parameters.

**MATERIALS AND METHODS**

**Animals.** Animals originated from Dutch dairy holdings and were selected by Animal Health Services (GD, Deventer, the Netherlands) via their regular paratuberculosis testing program. The inclusion criterion for natural chronic paratuberculosis infection was that animals were shedding the bacterium in their feces. In total, 39 animals were purchased and evaluated for general health. A total of 14 animals were excluded due to pre-existing lameness, udder infection, or poor body condition. The remaining 45 female cattle (average age at time of inclusion, 5.0 years [range, 2.6 to 7.8 years]) were used in this study. After arrival at the experimental farm, cows were vaccinated against bovine herpesvirus type 1 (BHV-1) and ringworm (intranasal Bovilis IBR live marker and Bovilis Ringvac; MSD Animal Health, the Netherlands).

The cows were housed together in a conventional animal house, fed according to requirements, and checked daily for general health. Local side effects after vaccination were recorded. The decision to cull an animal due to end-stage paratuberculosis was blinded and carried out by an independent veterinarian. Clinical features of end-stage paratuberculosis were cachexia, submandibular edema, and chronic diarrhea.

The use of animals in the present study was approved by the experimental animal committee of MSD Animal Health and conducted according to existing regulations.

**Hsp70 antigen.** Recombinant \textit{M. avium} subsp. \textit{paratuberculosis} Hsp70 was produced according to methods described in detail earlier (20). The purity of the recombinant Hsp70 was checked using SDS-PAGE, and the endotoxin concentration was below the detection limit of the assay (\textit{Limulus} amebocyte lysate assay; Pierce).

**Experimental design.** The animals were assigned to one of three experimental groups of 15 animals each. Assignment of animals to groups was stratified based on age and anti-mycobacterial T and B cell responses to purified protein derivative from \textit{M. avium} (PPDA) and purified protein derivative from \textit{M. bovis} (PPDB), using previously described lymphocyte proliferation assays (21) and antibody enzyme-linked immunosorbent assay (ELISA) (20). Control cattle were sham immunized (group 1 [G1]) at day 28. Recombinant \textit{M. avium} subsp. \textit{paratuberculosis} Hsp70 protein vaccine was formulated as previously published (22). Immunization consisted of subcutaneous administration of Hsp70/DDA, 200 \textmu g recombinant \textit{M. avium} subsp. \textit{paratuberculosis} Hsp70 in 1 ml phosphate-buffered saline (PBS) containing 10 mg/ml dimethyl diocadexyl ammonium bromide (DDA) adjuvant (Sigma-Aldrich, United States), in the neck. Group 2 (G2) animals were vaccinated at day 28. Group 3 (G3) animals were vaccinated at day 0, followed by a booster vaccination at day 28. G2 and G3 received an additional booster vaccination with Hsp70/DDA at days 112 and 196.

Heparinized blood was collected aseptically from the tail vein of all animals every 3 weeks throughout the experiment, starting at day 84 prior to vaccination. Serum samples were taken at the same time points.

Fecal samples (approximately 50 g) were collected from the rectums of all animals every 3 weeks, stored in a sterile container, and processed for fecal culture (see below). In the postvaccination observation period, all animals were sampled every 3 weeks. From the animals that were present during the entire postvaccination period (42 weeks), all 15 samples were collected and analyzed. Nine animals that reached predetermined humane endpoints were removed from the study in the postvaccination period; hence, we could not collect the maximum of 15 samples from these animals.

**Diagnosis of paratuberculosis infection.** Diagnosis of paratuberculosis infection was performed by fecal culture in the automated TREQ-DS paraEM ESP egg yolk-based liquid culture system. Samples (2 g feces) were processed according to the manufacturer’s protocol and incubated for 42 days. After the incubation time, all the samples were processed for Ziehl-Neelsen staining of culture broth, and bacterial growth was confirmed to be \textit{M. avium} subsp. \textit{paratuberculosis} based on PCR for the specific IS900 insertion sequence (23).

**Hsp70-specific serology.** Serological responses to recombinant \textit{M. avium} subsp. \textit{paratuberculosis} Hsp70 protein were measured using an ELISA technique as described previously (24).

**IBR gB ELISA.** Glycoprotein B (gB)-specific antibodies to BHV-1 were detected in bovine serum with the HerdCheck IBR gB ELISA kit (Idexx, United States).

**Antigen-specific IFN-\gamma ELISA.** Antigen specific gamma interferon (IFN-\gamma) responses were measured using the whole-blood culture Bogvam assay (Prionics, Switzerland) according to instructions provided by the manufacturer. In brief, 1.5 ml heparinized whole blood was incubated with bovine and avian tuberculin antigens (PPDA and PPDB) in a 24-well tissue culture plate for 24 h in a humidified incubator at 37°C. Nil antigen (PBS) was used to determine spontaneous release of IFN-\gamma in the blood culture. Subsequently, the supernatant plasma was collected and stored at −20°C until analysis. In addition, recombinant \textit{M. avium} subsp. \textit{paratuberculosis} Hsp70 was used as antigen at a final concentration of 20 \mu g/ml. The production of bovine IFN-\gamma was measured using a monoclonal antibody-based sandwich enzyme immunoassay. Results were expressed as S/P ratio, calculated as optical density at 450 nm (OD_{450}) of antigen-stimulated plasma/OD_{450} of positive-control plasma.

**Statistical analysis.** Fecal shedding was analyzed with generalized linear models, available in R statistical software (2.10.1; http://www.R-project.org). The dependent variable was the proportion of negative fecal culture samples relative to the total number of samples per animal in the postvaccination observation period (maximum of 15 time points). Variability in shedding pattern was corrected for by inclusion of a fixed factor that described whether animals had a negative fecal culture in the prevaccination observation period (six or seven time points). The first model evaluated the question of whether the original experimental groups influenced fecal shedding, which included group as a fixed factor. The second model evaluated the question of whether low- or high-Hsp70 antibody responders in the vaccinated animals influenced fecal shedding levels compared to those of controls. Models were selected based on the lowest Akaike information criterion (AIC).

The stratification parameters were compared with analysis of variance (ANOVA). BHV-1 antibody, IFN-\gamma, and lymphoproliferative responses were analyzed with a nonparametric Kruskal-Wallis test.

Survival was analyzed by a Cox proportional-hazard model, available in the survival package of R statistical software (2.10.1). An event was defined as the time an animal reached predetermined humane endpoints, which was observed by an independent, blinded veterinarian. Explanatory factors included the original experimental groups and, in a subsequent model, the Hsp70 antibody responder types. Age, as potential confounding factor, did not significantly impact the outcome. The best model fit was selected based on the lowest AIC.

The level of significance was set at a P value of <0.05.

**RESULTS AND DISCUSSION**

The study design included two vaccination groups that differed in the primary vaccination scheme (G2, day 28; G3, days 0 and 28), which both received additional vaccinations at days 112 and 196, and a control group (G1); each group included 15 animals. The vaccination scheme was based on previous studies (22) which indicated beneficial effects of more prolonged intervals between boost vaccinations (25). In view of antigen-specific immunosuppression known to occur in the course of chronic mycobacterial infections (24, 26), particular care was given to control for the effect of known confounding factors, like age and mycobacterium-specific T and B cell responses, in stratification of animals into homogeneous groups (Table 1). Experimental groups created did not show significant differences in age, \textit{M. avium} subsp. \textit{paratuberculosis} subsp. \textit{M. avium} subsp. \textit{paratuberculosis} Hsp70 protein was used as antigen at a final concentration of 20 \mu g/ml. The production of bovine IFN-\gamma was measured using a monoclonal antibody-based sandwich enzyme immunoassay. Results were expressed as S/P ratio, calculated as optical density at 450 nm (OD_{450}) of antigen-stimulated plasma/OD_{450} of positive-control plasma.

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irrespective of vaccination status (Fig. 2B). There were no differences between this nonresponsiveness to vaccination, immune responses were calculated IFN-γ levels significantly (P = 0.02, logistic regression), Combined, the two vaccination groups had a significantly higher proportion of negative fecal cultures than did the controls (OR, 1.9; 95% CI, 1.1 to 3.6; P = 0.04). This indicates that Hsp70 vaccination likely has direct effects on the chronic intestinal inflammation and subsequent shedding of mycobacteria in feces.

In addition, the contribution of vaccination to survival was assessed. In total, five (33.3%) control animals and two (13.3%) vaccinated animals in each group reached predetermined humane endpoints due to end-stage clinical signs of paratuberculosis. Survival analysis did not indicate these differences to be statistically significant between groups, which can partly be explained by the limited number of animals and events (Fig. 1A).

Hsp70 vaccination in chronically infected cattle induced antibody titers. In contrast to previous experiments with uninfected animals (25), considerable variation in the magnitude of antibody titers was observed (Fig. 2A). Control animals had limited Hsp70-specific antibody titers, which fits previous observations in naturally infected animals (20). Vaccination did not induce or boost Hsp70-specific IFN-γ responses in peripheral blood, although circulating IFN-γ-positive T cells were present in a subset of animals irrespective of vaccination status (Fig. 2B).

Some vaccinated animals were not able to produce high Hsp70-specific antibody titers. To pinpoint the background of this nonresponsiveness to vaccination, immune responses were characterized to find evidence for a potential role for disease-induced immunosuppression. There were no differences between low-antibody responders and high-antibody responders in their ability to produce antibodies against a viral vaccine administered at the start of the experiment (Fig. 2C) (see Materials and Methods), and both responder groups did not significantly differ from the control group and the animals that reached end-stage clinical paratuberculosis in the course of the experiment. Furthermore, there were no differences between antibody responder types in their lymphocyte proliferation responsiveness toward a complex mycobacterial antigen (PPDA) (Fig. 2D), nor did they differ in general health status other than paratuberculosis status. This indicated that low-antibody responders were not likely to have general immunosuppression and also were still capable to mount and maintain specific antimycobacterial responses, but their ability to mount a robust Hsp70-specific antibody response was limited in an antigen-specific manner.

Given the observation that some animals were less able to mount Hsp70-specific antibodies, the major immune parameter associated with protection in our previous study, one could argue that in these low responders decreased vaccine efficacy may be expected, because antibodies might be correlated with or instrumental in the protective response. To test this hypothesis, the vaccinated animals were divided into low- and high-antibody responders based on the median value of the Hsp70-specific antibody level (Fig. 2A). Subsequently, the effect of responder types on vaccine efficacy parameters was compared with the control animals, under the assumption that there is no strong correlation between the inability to produce Hsp70-specific antibodies and disease-induced immunosuppression with subsequent disease progression. There is no direct way of assessing this correlation, because nonvaccinated paratuberculosis-infected animals have limited Hsp70-specific antibodies (20). We did not detect measurable differences in immune responses between high- and low-antibody responders for complex mycobacterial and viral antigens at the start of the experiment, 3 months before the time of defining antibody responder types. High Hsp70-specific antibody levels significantly (P = 0.01, logistic regression) correlated with reduced fecal shedding compared to that of controls. In contrast, fecal shedding in low-antibody responders did not differ from that of controls. Furthermore, high-antibody responders had a significantly (P = 0.047, likelihood ratio test) higher survival rate (Cox regression) than did controls (Fig. 1B). Since the magnitude of the antibody response generated by Hsp70 vaccination was significantly associated with reduced fecal shedding and higher survival rates, this could indicate that the generation of Hsp70-specific antibodies after vaccination is needed for vaccine efficacy.

The composition of the Hsp70 vaccine and its present route of application did not lead to induction of a Th1-type response. Repeated vaccination with Hsp70 did not induce IFN-γ-positive

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### TABLE 1 Stratification parameters

<table>
<thead>
<tr>
<th>Stratification *</th>
<th>Mean value for group (SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>G1</td>
</tr>
<tr>
<td>Age (mo)</td>
<td>62.60 (15.16)</td>
</tr>
<tr>
<td>Pourquier ELISA (S/P)</td>
<td>239.00 (32.92)</td>
</tr>
<tr>
<td>LST-PPDA (SI)</td>
<td>8.67 (7.49)</td>
</tr>
<tr>
<td>LST-PPDB (SI)</td>
<td>4.53 (4.49)</td>
</tr>
</tbody>
</table>

* Stratification parameters. Animals were assigned to three experimental groups (n = 15). Pourquier ELISA is an M. avium subsp. paratuberculosis-specific antibody ELISA. No significant differences between the groups were found as analyzed by ANOVA for all parameters. S/P, sample-to-positive ratio; SI, stimulation index; LST-PPDA, lymphoproliferative response to tuberculin from M. avium subsp. avium; LST-PPDB, lymphoproliferative response to tuberculin from M. bovis.

### TABLE 2 Fecal culture of Mycobacterium avium subsp. paratuberculosis

<table>
<thead>
<tr>
<th>Group</th>
<th>Before vaccination</th>
<th>After vaccination</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>FCₙ₀/n %</td>
<td></td>
</tr>
<tr>
<td>G1</td>
<td>3/100</td>
<td>3.00</td>
</tr>
<tr>
<td>G2</td>
<td>4/101</td>
<td>3.96</td>
</tr>
<tr>
<td>G3</td>
<td>4/88</td>
<td>4.55</td>
</tr>
</tbody>
</table>

* The number of negative fecal cultures (FCₙ₀) for M. avium subsp. paratuberculosis is shown in the periods before and after the start of vaccination for the control group and two vaccination groups. Animals were vaccinated with recombinant M. avium subsp. paratuberculosis Hsp70 in DDA adjuvant.

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Some vaccinated animals were not able to produce high Hsp70-specific antibody titers. To pinpoint the background of this nonresponsiveness to vaccination, immune responses were characterized to find evidence for a potential role for disease-induced immunosuppression. There were no differences between low-antibody responders and high-antibody responders in their ability to produce antibodies against a viral vaccine administered at the start of the experiment (Fig. 2C) (see Materials and Methods), and both responder groups did not significantly differ from the control group and the animals that reached end-stage clinical paratuberculosis in the course of the experiment. Furthermore, there were no differences between antibody responder types in their lymphocyte proliferation responsiveness toward a complex mycobacterial antigen (PPDA) (Fig. 2D), nor did they differ in general health status other than paratuberculosis status. This indicated that low-antibody responders were not likely to have general immunosuppression and also were still capable to mount and maintain specific antimycobacterial responses, but their ability to mount a robust Hsp70-specific antibody response was limited in an antigen-specific manner.

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The composition of the Hsp70 vaccine and its present route of application did not lead to induction of a Th1-type response. Repeated vaccination with Hsp70 did not induce IFN-γ-positive
cells in peripheral blood of our *M. avium* subsp. *paratuberculosis*infected animals (Fig. 3A). This is similar to what was previously found in uninfected animals (24). The immune responses to the bacterium, as indicated by PPDA-specific T cell responses, during the course of infection in this experiment were variable between individual animals but not different between the groups of low- or high-Hsp70 antibody responders. Therefore, it was expected that these groups were comparable with respect to immunocompetence and progression of the disease (Fig. 3B).

The demonstration of an association between antibodies and vaccine efficacy warrants further study into the exact mechanism of the protective immune response. From our study, vaccination-induced antibodies appear to confer the therapeutic modality of the vaccine. This is in contrast to the current dogma that cell-mediated immunity is the most important defense against intracellular infections. We have previously shown that Hsp70 is present in the cell wall of *M. avium* subsp. *paratuberculosis* and accessible for Hsp70-specific antibodies, induced by vaccination (27). *M. avium* subsp. *paratuberculosis* infects macrophages and is able to arrest phagosomal maturation to enable intracellular survival, similar to *M. tuberculosis*. However, following a stage of replication, bacteria need to escape from their infected host cell to infect new cells. In this process, bacteria are present in the extracellular environment and become a target for humoral responses. One defense mechanism could be that Fc receptor engagement of antibodies can protect by altering intracellular trafficking of mycobacteria into lysosomal compartments (28). Moreover, the repeated postexposure vaccination strategy with Hsp70 led to antigen-specific skewing away from IFN-γ-driven Th1-type response (Fig. 3A). These observations are further supported by the fact that Hsp70 vaccination induces production of IgG1 isotype antibodies, a marker for a Th2-type response (29).

In light of the growing incidence of multidrug-resistant mycobacteria, it is anticipated that new postexposure vaccines can be beneficial in a targeted approach complementary to antimycobacterial drugs (30). Apart from the beneficial effects for the vaccinated individual, population effects of vaccination need to be considered, most importantly, reduction of transmission. We show that vaccination reduced fecal shedding, the natural way of transmission, of mycobacteria by individuals with naturally acquired chronic infection, and it improved survival in a subset of vaccinated animals. For bovine and human tuberculosis, transmission of infection is related to the presence of individuals with active pulmonary disease. Postexposure vaccination should ideally limit transmission from active tuberculosis-diseased individuals. It is important that the effect on population transmission of postexposure vaccines, but in fact also of prophylactic vaccines, is evaluated in transmission studies.

Overall, our findings suggest that vaccination of individuals chronically infected by *M. avium* subsp. *paratuberculosis* has a therapeutic effect, improving longevity of infected individuals. We did not observe adverse effects associated with repeated postexposure vaccination at the site of application or systemically in this or previous experiments (24, 25, 27). Recently, Aagaard et al. have shown postexposure efficacy of a subunit vaccine including a latency antigen in an experimental challenge model of tuberculosis in mice, without adverse effects (31). The high conservation of Hsp70 between mycobacterial species (94% amino acid sequence identity between *M. avium* subsp. *paratuberculosis* and *M. bovis* and *M. tuberculosis*), and similarity in pathogenesis, entails the potential that comparable postexposure vaccine-induced protection can be translated to other mycobacterial infections. Bovine tuberculosis is a statutory disease in most developed countries, and test-positive animals need to be destroyed. Thus, the application of a therapeutic vaccine in such countries would currently not be practical or legally acceptable. However, in countries without...
“test and cull” policies, such a vaccine would have major impacts on disease control, food safety, and human and animal health. If the protective effects as observed in this study can be repeated in a field situation, they may not be sufficient for controlling *M. avium* subsp. *paratuberculosis* transmission in the field; therefore, combining Hsp70 with other candidate vaccine antigens into a multistage vaccine would be an attractive pathway to explore in future studies, preferably using a natural host model.
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