Effect of Different Cell Fractions of *Mycobacterium avium* and Vaccination Regimens on *Mycobacterium avium* Infection

J. O. Falkinham III*, W. B. Gross† & F. W. Pierson†

*Department of Biology; and †College of Veterinary Medicine, Virginia Polytechnic Institute and State University, Blacksburg, Virginia, USA

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Correspondence to: J. O. Falkinham, Department of Biology, Virginia Tech, Blacksburg, VA 24061-0406, USA. E-mail: jofiii@vt.edu

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Abstract

Because of the availability of uniform genetic stocks and the ability to modulate stress levels, chickens were investigated as a host for the development of an antimycobacterial vaccine. The imposition and the timing of stress significantly influenced the outcome of *Mycobacterium avium* infection in chickens. Simple, whole cell or lysate vaccines and combinations of vaccine preparations were identified that led to high levels of protection. In addition, short-term stress at the time of vaccination significantly increased the protective efficacy of *M. avium* vaccine preparations. Post-infection vaccination of *M. avium*-infected chickens was also shown to significantly reduce the number of lesions and colony counts.

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Introduction

*Mycobacterium avium* is an environmental opportunistic pathogen that causes infections in humans, animals (e.g. pigs) and birds (e.g. chickens) [1–3]. For animals of agronomic importance (chickens and pigs), infections result in significant economic losses [1]. *M. avium* infection is also a significant cause of morbidity and mortality in birds in zoos and breeding establishments [1]. Because *M. avium* is present in both natural and drinking water and soils [2], it is difficult, if not impossible, to protect humans, animals and birds from exposure and infection. Furthermore, antimicrobial therapy of animals and birds is not cost-effective. Fortunately, vaccines have been shown to be cost-effective, especially for prevention of mycobacterial infections [4].

The objective of the studies reported herein was to identify inexpensive and simple *M. avium* vaccines and identify conditions leading to protection against *M. avium* infection. After this work was initiated, killed mycobacteria were shown to protect mice from *M. tuberculosis* infection [5]. We have chosen chickens for identifying factors influencing vaccine efficacy for several reasons. First, *M. avium* infects chickens and causes lesions in liver and spleen and eventually death [1, 6, 7]. Second, uniform, inbred lines of chickens are available [8] that reduce the variation in responses to infection and vaccination. Third, stress levels in chickens can be controlled and reproducibly modulated [9]. Stress levels have been shown to influence the course of *M. tuberculosis* infection in mice [10] and *M. avium* infection in chickens [7]. Because stress levels influence vaccine efficacy in humans [11–13] and chickens [14], we also measured the effect of different stress levels on vaccine efficacy. Although the experiments were performed with chickens, the results may have important ramifications in the development of mycobacterial vaccines in humans and may explain, in part, the variable efficacy of bacille Calmette–Guerin (BCG) vaccination [15]. We report a series of robust, yet discrete, observations that offer insight into the determinants of successful mycobacterial vaccination.

Materials and methods

*Mycobacterium avium*. *M. avium* strain A5 [16] was employed for all the experiments. It is an isolate from an acquired immunodeficiency syndrome patient.

*Growth of M. avium*. A single transparent colony of *M. avium* strain A5 on Middlebrook 7H10 agar medium (BBL Microbiology Systems, Cockeysville, MD, USA) containing 0.5% (v/v) glycerol and 10% (v/v) oleic acid–albumin (M7H10) was inoculated into 2 ml of Middlebrook 7H9 broth (BBL Microbiology Systems) containing 0.5% (v/v) glycerol and 10% (v/v) oleic acid–albumin (M7H9). After 7 days of incubation at 37°C, 1 ml was used to inoculate 9 ml of M7H9 broth and that culture was incubated for 7 days at 37°C. Fifty millilitres of M7H9 broth in a 500 ml nephelometer flask was inoculated
with 5 ml from the 10 ml culture. The cultures were incubated at 37°C with aeration (60 r.p.m.) and turbidity (absorbance 580 nm) measured immediately and at twice daily intervals. Log phase cultures were used for vaccine preparation or infection. All cultures were tested for the presence of contaminants and those used here were free from contaminants.

Preparation of M. avium vaccines. The following candidate vaccines were prepared: (i) Autoclaved culture: a 7-day culture of M. avium strain A5 grown in M7H9 broth was autoclaved; (ii) Boiled culture: a 7-day culture of M. avium strain A5 was boiled at 100°C for 10 min; (iii) Cell lysate: cells from a 7-day culture of M. avium strain A5 were collected by centrifugation (5000 g for 20 min) and suspended in 10 ml of water. To 1 ml of cell suspension was added 1 g of 0.1 mm diameter glass beads and the cells broken by bead beating for five periods of 1 min each in a Mini-Bead Beater (BioSpec Products, Bartlesville, OK, USA). Cells were cooled in ice water between each breakage period. The lysate was separated from the glass beads by centrifugation (2000 g for 20 min) and suspended in 10 ml of water. To 1 ml of cell suspension was added 1 g of 0.1 mm diameter glass beads and the cells broken by bead beating for five periods of 1 min each in a Mini-Bead Beater (BioSpec Products, Bartlesville, OK, USA). Cells were cooled in ice water between each breakage period. The lysate was separated from the glass beads by centrifugation (2000 g for 10 min); (iv) Heat-shock culture filtrate: a 7-day culture of M. avium strain A5 was incubated at 50°C for 20 min and then the heat-shocked culture incubated for 2 h at 37°C. The culture was filter sterilized by passage through a 0.22 μm pore size filter. The protein concentration of each of the vaccine preparations was measured [17] and adjusted to 0.5 mg/ml and aliquoted in 1 ml volumes in 2 ml screw-capped tubes and frozen at −70°C.

Chickens. Male and female white leghorn chickens were from stocks selectively bred for a high antibody (HA) response to sheep erythrocytes [8]. Female, HA line chickens were used for the majority of experiments. They were the most susceptible to M. avium infection compared to low antibody line females and high and low antibody line male chickens [7]. These stocks were selected because they were propagated in the Department of Animal and Poultry Science, were free from infectious disease and could be socialized [9].

Heterophil and lymphocyte counts. A haemocytometer was used to count heterophils (H) and lymphocytes (L) [7].

Vaccination. Chickens were vaccinated with 0.1 ml of a vaccine by the intramuscular route. For combinations of vaccines, an equal volume of individual vaccines were mixed and 0.1 ml of the mixture injected to ensure that the amount of injected protein was the same for all vaccinations.

Modulation of stress. Long-term stress in chickens was imposed by incorporation of 30 mg of corticosterone (Sigma Chemical Co., St. Louis, MO, USA) per kilogram feed. Short-term stress was created by injection of 0.5 IU of the synthetic adrenocorticotropic hormone Cortrosyn® (Organon, Inc., Bedford, OH, USA) per kilogram of body weight at a specific time. The duration of the high level of stress resulting from Cortrosyn® at the dose used was 4 h with a peak between 2 and 4 h following injection. Ascorbic acid (100 mg/kg feed) was used to reduce stress levels [18].

Infection. Cells of M. avium strain A5 were grown in M7H9 broth to mid-log phase and collected by centrifugation (5000 x g for 20 min) and washed twice and suspended in phosphate-buffered saline at the same concentration as the culture. Dilutions of the suspension were prepared to examine the response to different infecting doses. When chickens were 4 weeks old, chickens were infected via the intravenous route with 0.1 ml of a single dilution of a particular suspension in triplicate. During the course of the infection, blood was withdrawn for counts of monocytes and other blood cells. At 12 weeks following infection, the chickens were killed, weighed and their livers removed for enumeration of M. avium colony-forming units (CFU) and histopathology. In some experiments (e.g. intramuscular infection), surviving chickens were challenged by the intravenous route to determine whether they were still susceptible to infection. For vaccine comparisons, preparations were injected via the intramuscular route with or without the imposition of stress and 4 weeks later challenged by intravenous injection of M. avium.

Recovery of liver from infected chickens. Livers were aseptically removed from infected chickens, and a portion was removed for histopathology. The portion removed was taken from the same region of the liver in all chickens to avoid bias because granulomas are visible on the surface. The remaining liver was transferred to a sterile plastic bag for recovery and enumeration of M. avium.

Histopathology. A cut section of liver (block face of 1.5 x 1.5 cm) was fixed and stained with haematoxylin and eosin. The number of granulomatous and caseous lesions/cm² tissue section was recorded.

Recovery and enumeration of M. avium from chicken liver samples. A portion of liver tissue was cut from the sample, weighed under aseptic conditions (0.1–0.2 g) and transferred to a sterile tissue homogenizer. Ten millilitres of buffered saline gelatin (BSG; 0.1 g gelatin, 8.5 g NaCl, 0.3 g KH₂PO₄, 0.6 g Na₂HPO₄ per litre) was added and the tissue homogenized to produce a uniform suspension. The suspension was diluted using BSG and 0.1 ml samples of the undiluted and diluted suspensions spread in triplicate on M7H10 agar medium. The agar medium was incubated for 14 days at 37°C and colonies counted. The data are reported as CFU/g of liver tissue.

Statistical analysis. Statistical analysis was performed using INSTAT VERSION 3.0 (GraphPad Software, Inc., San Diego, CA, USA).

Results

Effect of sex on M. avium infection in chickens
HA line male and female chickens were infected intravenously at 4 weeks of age and outcome of the infection
measured at 10 weeks. Female chickens had significantly ($P<0.009$) more caseous lesions/cm$^2$ of liver tissue (24) than did males (10). For the remaining experiments, only females were used.

Dose–response of *M. avium* infection in chickens

There was a highly significant linear relationship between the number of CFU of infecting *M. avium* strain A5 cells via the intravenous route and the number of lesions in the female HA line chicken livers. The correlation coefficient ($r$) was 0.999. Over a range of infecting dose of $10^3$–$10^6$ CFU per chicken, the number of lesions in the liver samples was 3.5–1200. Based on that data, an infecting dose of $10^4$ CFU per chicken was chosen for the remaining experiments. For a 10-fold increase in infecting dose of *M. avium* strain A5 cells, there was a corresponding 6.4-fold increase in the number of lesions. The lesions fell into one of two types: small granulomas (average diameter about 2.2 $\mu$m) and large caseous granulomas (average diameter about 6.5 $\mu$m). Acid-fast cells could be observed in the centre of the large caseous granulomas. There was also a highly significant correlation ($r=0.999$) between the number of lesions in the liver samples and the number of circulating monocytes. This correlation meant that it was possible to monitor the course of *M. avium* infection in chickens without killing the birds by measuring blood monocyte counts.

Course of *M. avium* infection in chickens

The weight of chickens and their liver, the numbers of *M. avium* and lesions in the liver and the number of blood monocytes following infection in chickens are summarized in Table 1. There was no effect of infection on the weight of the chickens and their livers for up to 13 weeks following infection (Table 1). It was only after 20 weeks that the chickens lose weight and liver weight increased disproportionately (data not shown). After the initial rapid increase in *M. avium* CFU/g liver in the first 6 weeks, the rate of increase slowed. There was a steady increase in lesions in the liver and blood monocyte counts (Table 1).

Effect of route of infection on *M. avium* infection in chickens

The route of *M. avium* infection influenced the number of lesions and *M. avium* colony counts in the liver (Table 2). Chickens were infected at 4 weeks and killed 8 weeks later. The number of lesions and the CFU/g in liver tissue samples were significantly higher in chickens infected via the intravenous route compared to the intramuscular route ($t$-test with Welch correction, $P<0.005$). For all ensuing experiments, female HA line chickens were infected via the intravenous route. The number of blood monocytes/mm$^3$ in the chickens infected intramuscularly (Table 2) was close to the number in normal, uninfected birds (i.e. 1500 monocytes/mm$^3$) [9].

Intramuscular infection of chickens with *M. avium* results in protection against subsequent challenge

Because chickens that had been infected via the intramuscular route had low numbers of both lesions and *M. avium* cells in liver samples (Table 2), we sought to determine whether they were protected against subsequent infection. Chickens were infected with *M. avium* strain A5 cells intramuscularly at 4 weeks of age, and 8 weeks later they were infected intravenously with *M. avium* strain A5 and killed 8 weeks later (20 weeks old). The results demonstrated that the chickens infected via the intramuscular route were protected against subsequent *M. avium* infection (Table 3). For the comparisons between different vaccine preparations, all vaccines were injected intramuscularly in female HA chickens and subsequently challenged by intravenous infection.

Effect of stress on vaccine efficacy

To determine whether stress influenced vaccine efficacy in chickens, chickens were injected with Cortrosyn$^R$. Stress was measured by increases in H/L ratio [9]. Cortrosyn$^R$ injection resulted in an increase in the H/L ratio (Table 4). The peak stress response, measured by H/L ratio, occurred between 2 and 4 h following injection (Table 4). Four-week-old chickens were vaccinated via the intramuscular route using the cell lysate vaccine immediately before or 2 h after Cortrosyn$^R$ injection. Four weeks later (8 weeks

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Table 1 Time course of *Mycobacterium avium* infection in chickens

<table>
<thead>
<tr>
<th>Time after infection ($n$)</th>
<th>Weight</th>
<th>Lesions/cm$^2$</th>
<th>CFU/g of liver</th>
<th>Blood monocytes/mm$^3$</th>
</tr>
</thead>
<tbody>
<tr>
<td>3 weeks (4)</td>
<td>Chicken (g)</td>
<td>619 ± 24</td>
<td>0</td>
<td>$1.9 \times 10^7 \pm 0.9 \times 10^4$</td>
</tr>
<tr>
<td></td>
<td>Liver (g)</td>
<td>15 ± 1.5</td>
<td>5 ± 2 (2)</td>
<td>$2.4 \times 10^5 \pm 0.9 \times 10^4$</td>
</tr>
<tr>
<td>6 weeks (4)</td>
<td>Chicken (g)</td>
<td>835 ± 67</td>
<td>19 ± 1.5</td>
<td>$3.2 \times 10^5 \pm 2.2 \times 10^4$</td>
</tr>
<tr>
<td></td>
<td>Liver (g)</td>
<td>19 ± 1.5</td>
<td>13 ± 7 (1)</td>
<td>$3.7 \times 10^5 \pm 2.9 \times 10^4$</td>
</tr>
<tr>
<td>8 weeks (4)</td>
<td>Chicken (g)</td>
<td>965 ± 52</td>
<td>27 ± 2.5</td>
<td>$4.0 \times 10^5 \pm 2.4 \times 10^4$</td>
</tr>
<tr>
<td></td>
<td>Liver (g)</td>
<td>27 ± 2.5</td>
<td>32 ± 8 (6)</td>
<td>$4.0 \times 10^5 \pm 2.4 \times 10^4$</td>
</tr>
<tr>
<td>13 weeks (4)</td>
<td>Chicken (g)</td>
<td>1220 ± 61</td>
<td>31 ± 1.7</td>
<td>$4.0 \times 10^5 \pm 2.4 \times 10^4$</td>
</tr>
<tr>
<td></td>
<td>Liver (g)</td>
<td>31 ± 1.7</td>
<td>32 ± 8 (6)</td>
<td>$4.0 \times 10^5 \pm 2.4 \times 10^4$</td>
</tr>
</tbody>
</table>

All values are expressed as average ± standard deviation. Uninfected chickens have 1500 ± 500 monocytes/mm$^3$ (3–13 weeks) and weigh approximately 600 g (3 weeks), 850 g (6 weeks), 1000 g (8 weeks) and 1400 g (13 weeks). $n$, number of chickens. CFU, colony-forming units.
In a preliminary trial, chickens were vaccinated via the intramuscular route during the induction of stress with corticosterone in feed. Chickens were provided with their normal feed containing corticosterone in the afternoon of the day before vaccination. At a concentration of 30 mg of corticosterone/kg feed, the H/L ratio was 1.25 in birds the following morning which is close to the value attained with injection of Cortrosyn® (Table 4). As with Cortrosyn®, the effect of corticosterone in feed on H/L ratio is transient. H/L ratios returned to normal within 1 day after substitution of normal feed for feed containing 30 mg corticosterone/kg feed. The effect of the four different vaccine preparations alone or in combination on the number of both lesions and \textit{M. avium} colony counts in liver was measured. The histopathology of lesions was also recorded. The results demonstrated that all vaccine preparations significantly reduced \((t\text{-test,} \ P < 0.05)\) the number of \textit{M. avium} CFU/g liver tissue (Table 6). Greatest protection was provided by the autoclaved culture and heat-shock culture filtrate vaccines alone (Table 6). Substantial protection (i.e. >99% protection) was provided by vaccination of the combination of the boiled culture with either the heat-shock culture filtrate or the cell lysate or the autoclaved culture with either the boiled culture or the cell lysate (Table 6). There was a strong correlation between the number of lesions/cm² and \textit{M. avium} CFU/g liver \((r = 0.82)\). The lesions in the vaccinated birds were small, well delineated and were surrounded by large numbers of lymphocytes.

### Table 2: Effect of route of infection on \textit{Mycobacterium avium} infection in chickens

<table>
<thead>
<tr>
<th>Route of infection</th>
<th>Number</th>
<th>Lesions</th>
<th>Monocytes/mm³</th>
<th>CFU/g of liver</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intravenous</td>
<td>4</td>
<td>34 ± 4.0</td>
<td>13,900 ± 2700</td>
<td>5.7 × 10³ ± 2.7 × 10³</td>
</tr>
<tr>
<td>Intramuscular</td>
<td>4</td>
<td>3 ± 1.9</td>
<td>1800 ± 1000</td>
<td>1.7 × 10³ ± 1.3 × 10³</td>
</tr>
</tbody>
</table>

All values are expressed as average ± standard deviation. Four-week-old female high antibody line chickens were infected with \(10^5 \textit{M. avium}\) by either the intravenous or the intramuscular route, and at 10 weeks of age, after removal of blood for heterophil, lymphocyte and monocyte counts, chickens were killed and livers removed for \textit{M. avium} enumeration and histopathology. CFU, colony-forming units.

### Table 3: Evidence that intramuscular infection of chickens results in protection against \textit{Mycobacterium avium}

<table>
<thead>
<tr>
<th>Prior exposure</th>
<th>Number</th>
<th>Lesions*</th>
<th>Monocytes/mm³</th>
<th>CFU/g of liver</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>4</td>
<td>42 ± 7 (5)</td>
<td>9900 ± 2700</td>
<td>1.5 × 10³ ± 0.3 × 10³</td>
</tr>
<tr>
<td>Intramuscular</td>
<td>4</td>
<td>0</td>
<td>2200 ± 800</td>
<td>1.2 × 10³ ± 0.1 × 10³</td>
</tr>
</tbody>
</table>

CFU, colony-forming units.

*Average lesions/cm² ± standard deviation.
†Chickens were infected via the intramuscular route at 4 weeks and after 8 weeks were infected intravenously with \textit{M. avium} and killed 8 weeks following infection.

### Table 4: Time course of change of heterophil/lymphocyte (H/L) ratio in chickens following Cortrosyn® injection

<table>
<thead>
<tr>
<th>Time after injection (h)</th>
<th>Heterophil/lymphocyte ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.50</td>
</tr>
<tr>
<td>2</td>
<td>1.15</td>
</tr>
<tr>
<td>4</td>
<td>1.20</td>
</tr>
<tr>
<td>6</td>
<td>0.60</td>
</tr>
<tr>
<td>8</td>
<td>0.54</td>
</tr>
<tr>
<td>10</td>
<td>0.50</td>
</tr>
</tbody>
</table>

Four female high antibody chickens were injected at 4 weeks age and injected with Cortrosyn® and blood H/L ratios were measured by haemocytometer as described [7].

### Table 5: Comparison of different vaccines for protection against \textit{M. avium} infection

<table>
<thead>
<tr>
<th>Comparison</th>
<th>Number</th>
<th>Lesions</th>
<th>Monocytes/mm³</th>
<th>CFU/g of liver</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>4</td>
<td>40 ± 7</td>
<td>9900 ± 2700</td>
<td>1.5 × 10³ ± 0.3 × 10³</td>
</tr>
<tr>
<td>Intramuscular infection</td>
<td>4</td>
<td>0</td>
<td>2200 ± 800</td>
<td>1.2 × 10³ ± 0.1 × 10³</td>
</tr>
</tbody>
</table>

In a preliminary trial, chickens were vaccinated via the intramuscular route (without stress) with the heat-shock culture filtrate at 4 weeks of age, infected intravenously with \textit{M. avium} at 8 weeks of age and the number of liver lesions counted at 8 weeks later. Vaccinated birds had 19 ± 2.9 and unvaccinated birds had 33.5 ± 14 lesions/cm² of liver tissue \((t\text{-test with Welch correction,} \ P < 0.001)\) the number of lesions in the liver of infected chickens (Table 5).

### Table 6: Effect of post-infection vaccination on \textit{M. avium} infection

Recently, it was shown that inoculation of mice with the 30 kDa mycolyl transferase A protein increased the levels of protection of BCG-vaccinated mice to a later challenge with \textit{M. tuberculosis} [19]. This observation suggested that
post-infection vaccination of *M. avium*-infected chickens might reduce the extent of infection by stimulating the immune response. Accordingly, 6-week-old chickens were infected with *M. avium* strain A5 and vaccinated intramuscularly 12 weeks later with the autoclaved culture + cell lysate combination vaccine. To increase the efficacy of the post-infection vaccination, chickens were injected with Cortrosyn® and vaccinated 2 h later. Blood samples were withdrawn for monocyte counts, and chickens were killed at 36 weeks of age and livers removed. The number of lesions and mycobacteria was significantly lower in liver samples (*t*-test, *P* < 0.001), and monocyte counts were also reduced to near normal levels (Table 7). The number of lesions in the control, non-vaccinated chickens was higher than the number in 13-week-old birds (Table 1), demonstrating that the number of lesions increased as the length of time following infection increased. As noted above, the lesions in the post-infection vaccinated chickens were small and surrounded by large numbers of lymphocytes. Based on autopsy of two chickens, maintenance of post-infection vaccinated chickens beyond 36 weeks reduced the number of lesions to near zero.

**Discussion**

**Characteristics of *M. avium* infection in chickens**

In confirmation of earlier work [6, 7], *M. avium* infection in chickens is influenced by infecting dose and results in granulomatous lesions and proliferation of *M. avium* in the liver. The course of the infection, reflected by the CFU/g liver, reported here (Table 1), is similar to that reported previously [6]. The time course of infection and the absence of mortality are similar to that observed in BALB/c mice [20]. Lesions were also observed in the spleen, but not lungs of the infected chickens. Counts of lesions and mycobacteria were measured in liver samples because of the ease of obtaining the whole liver without any other tissue. Females were significantly more susceptible to infection than were males. The reported difficulty in assessing virulence of *M. avium* strains in chickens [20] may have been due to the fact that their chickens were not socialized and may not have come from a genetically homogeneous line as has been employed here [9]. The course of the infection can be monitored by monocyte counts and lesions and colony counts in the liver because they all correlated with one another [7].

**Influence of the route of infection on infection and vaccination**

The route of *M. avium* infection influenced the number of lesions and *M. avium* colony counts in the liver (Table 2). The number of lesions and the CFU/g in liver tissue samples were significantly higher in chickens infected via the intravenous route compared to the intramuscular route. Guinea pigs infected with *M. avium* strains via the

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**Table 5** Effect of timing of stress induction on vaccine efficacy of *Mycobacterium avium* infection in chickens

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Number</th>
<th>Lesions/cm²</th>
<th>CFU/g of liver</th>
<th>Protection (%)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control, none</td>
<td>5</td>
<td>101±8</td>
<td>2.0×10⁶±1.2×10⁶</td>
<td>0</td>
</tr>
<tr>
<td>Intramuscular vaccine 2 h after Cortrosyn®</td>
<td>5</td>
<td>3±1</td>
<td>1.7×10³±0.3×10³</td>
<td>99.0</td>
</tr>
<tr>
<td>Intramuscular vaccine at the time of Cortrosyn®</td>
<td>5</td>
<td>82±11</td>
<td>1.5×10⁵±0.9×10⁶</td>
<td>99.1</td>
</tr>
</tbody>
</table>

Female high antibody chickens were injected with cell lysate vaccine either at the time of Cortrosyn®-induced stress or 2 h later and were infected intravenously 4 weeks later with *M. avium* and killed 12 weeks later. All values are expressed as average ± standard deviation. CFU, colony-forming units.

*Vaccine was cell lysate.

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**Table 6** Efficacy of different vaccine preparations on *Mycobacterium avium* infection in chickens

<table>
<thead>
<tr>
<th>Vaccine</th>
<th>Birds</th>
<th>Lesions/cm²</th>
<th>CFU/g</th>
<th>Protection (%)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control, no vaccine</td>
<td>4</td>
<td>16.3±2.5</td>
<td>2.5×10⁶±1.3×10⁶</td>
<td>0</td>
</tr>
<tr>
<td>Autoclaved culture</td>
<td>4</td>
<td>3.5±1.3</td>
<td>2.5×10³±2.2×10³</td>
<td>99.0</td>
</tr>
<tr>
<td>Boiled culture</td>
<td>4</td>
<td>6.0±3.8</td>
<td>1.7×10³±2.5×10³</td>
<td>93.2</td>
</tr>
<tr>
<td>Cell lysate</td>
<td>4</td>
<td>3.3±1.9</td>
<td>7.0×10¹±5.7×10²</td>
<td>97.2</td>
</tr>
<tr>
<td>Heat-shock culture filtrate</td>
<td>4</td>
<td>7.8±3.0</td>
<td>2.3×10⁴±0.9×10⁴</td>
<td>99.1</td>
</tr>
<tr>
<td>Autoclaved + boiled culture</td>
<td>3</td>
<td>1.0±0</td>
<td>1.5×10⁴±2.4×10⁴</td>
<td>99.4</td>
</tr>
<tr>
<td>Autoclaved + cell lysate</td>
<td>3</td>
<td>0.3±0.6</td>
<td>2.0×10³±1.6×10⁴</td>
<td>99.2</td>
</tr>
<tr>
<td>Autoclaved + heat-shock filtrate</td>
<td>4</td>
<td>8.8±2.6</td>
<td>1.5×10⁴±1.5×10⁴</td>
<td>94.0</td>
</tr>
<tr>
<td>Boiled + heat-shock filtrate</td>
<td>4</td>
<td>5.8±2.5</td>
<td>7.3×10³±0.7×10⁴</td>
<td>99.7</td>
</tr>
<tr>
<td>Cell lysate + heat shock filtrate</td>
<td>4</td>
<td>6.3±1.9</td>
<td>4.1×10⁴±2.4×10⁴</td>
<td>98.4</td>
</tr>
<tr>
<td>Boiled + cell lysate</td>
<td>2</td>
<td>5.0±1.4</td>
<td>1.9×10⁴±2.4×10⁴</td>
<td>99.2</td>
</tr>
</tbody>
</table>

Female, 4-week-old female chickens were vaccinated intramuscularly without stress, infected intravenously with *M. avium* at 8 weeks of age and killed at 16 weeks of age. All values are expressed as average ± standard deviation. CFU, colony-forming units.

*Protection is per cent decrease in CFU/g liver compared to control.
intramuscular route (1–2.5 × 10^7 injected) all became sensitized to avian tuberculin, and no lesions in the liver were found when the animals were killed 12 weeks after infection [20]. Similarly, we demonstrate that intramuscular infection with M. avium results in few lesions in the liver (Table 2) and protection against subsequent infection with M. avium (Table 3). Thus, it would appear that the course of infection in chickens offers a model for M. avium infection that is no different than the course in other laboratory animals. Furthermore, chickens may serve as a superior model for infection because of the genetic uniformity of the lines used here and the ability to modulate stress levels easily.

Efficacy of different vaccines for protection against M. avium infection

As has been shown by others [5], a variety of different vaccine preparations significantly protect against M. avium infection. Heat-shock proteins, including those of mycobacteria, have been shown to stimulate immune responses to other proteins [21]. Highest level of protection resulted from vaccination with combinations of preparations, including the combinations of the boiled culture with either the autoclaved culture or the heat-shock filtrate (Table 5). The pattern of small, well-delineated lesions surrounded by large numbers of lymphocytes in vaccinated chickens is the same as that seen in BCG-vaccinated guinea pigs [22]. The vaccines described here offer substantial levels of protection (>99% reduction in liver colony counts) and can be prepared cheaply.

The increased level of protection provided by the combinations reached values of greater than 2 logs. These data suggest that candidate mycobacterial vaccines consisting of components recovered from different cell fractions (cells and extracellular components) isolated under different conditions (with and without heat-shock) would have high efficacy.

Post-infection vaccination reduces the extent of M. avium infection in chickens

Post-infection vaccination of M. avium-infected chickens significantly reduced the number of lesions and mycobacteria in liver samples (Table 6). It may be important that post-infection vaccination abolished the presence of large caseous granulomas in the livers, because they may serve as foci for the spread of the mycobacteria beyond the liver. Inoculation of mice with the 30 kDa mycolyl transferase A protein increased the levels of protection of BCG-vaccinated mice to challenge with M. tuberculosis [19]. It is possible that post-infection vaccination of other animals and humans may also reduce the extent of M. avium infection, consequently offering an alternative to multiple antimycobacterial drug therapy for treatment of infection. Certainly, the vaccines described here are less expensive than antibiotic therapy.

Effect of stress on vaccine efficacy

Earlier, we showed that increased levels of stress for long periods of time (chronic stress) increased the severity of disease and led to higher numbers of M. avium in infected chickens [7]. Similar effects of stress, brought about by crowding, were reported for M. tuberculosis infection in mice [10]. Herein, we report that induction of stress at the time of vaccination significantly increased the efficacy of M. avium vaccines (Table 4). Stress must be limited to a short period of time because long-term imposition of stress reduces vaccine efficacy in chickens (data not shown) and in humans [11–13]. In fact, the interaction between the vaccine introduced intramuscularly and cells of the immune system must be very short because of the limited duration of stress (2–4 h after injection) (Table 4). Reduction of stress by incorporation of ascorbic acid in the diet of the chickens [23] increased the protective effect of vaccination. The regimen leading to highest protection by the M. avium vaccines consists of vaccination during short-term stress followed by reduction of stress.

The results here demonstrate that any program of vaccine development must include the regulation of stress levels in any experimental animal. Stress levels can be modulated inexpensively and easily in chickens by inclusion of corticosteroids in feed, because chickens feed regularly and feed can be changed. The influence of stress on the efficacy of M. avium vaccine preparations suggests one possible explanation for the variable efficacy of BCG vaccination in different human populations [15, 24]. Published reports of the reduced vaccine efficacy in individuals subject to long-term, chronic stress [11–13] and the results reported here would predict that BCG vaccination would be less protective amongst individuals subjected to long-term stress.

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