Infection of the Reproductive Tract and Eggs with *Salmonella enterica* Serovar Pullorum in the Chicken Is Associated with Suppression of Cellular Immunity at Sexual Maturity

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*Salmonella enterica* serovar Pullorum causes persistent infections in laying hens. Splenic macrophages are the main site of persistence. At sexual maturity, numbers of bacteria increase and spread to the reproductive tract, which may result in vertical transmission to eggs or chicks. In this study we demonstrate that both male and female chickens may develop a carrier state following infection but that the increases in bacterial numbers and spread to the reproductive tract are phenomena restricted to hens, indicating that such changes are likely to be related to the onset of egg laying. The immunological responses during the carrier state and through the onset of laying in hens were determined. These indicate that chickens produce both humoral and T-cell responses to infection, but at the onset of laying both the T-cell response to *Salmonella* and nonspecific responses to mitogenic stimulation fall sharply in both infected and noninfected birds. The fall in T-cell responsiveness coincided with the increase in numbers of *Salmonella* serovar Pullorum and its spread to the reproductive tract. Three weeks after the onset of egg laying, T-cell responsiveness began to increase and bacterial numbers declined. Specific antibody levels changed little at the onset of laying but increased following the rise in bacterial numbers in a manner reminiscent of a secondary antibody response to rechallenge. These findings indicate that a nonspecific suppression of cellular responses occurs at the onset of laying and plays a major role the ability of *Salmonella* serovar Pullorum to infect the reproductive tract, leading to transmission to eggs. The loss of T-cell activity at the point of laying also has implications for *Salmonella enterica* serovar Enteritidis infection and transmission to eggs, along with its control by vaccination offering a “window of opportunity” in which infection may occur.

The development of persistent infection is a feature of a small number of *Salmonella enterica* serovars that cause systemic disease in a restricted range of host species. These include *Salmonella enterica* serovar Typhi in humans, both of which are believed to localize in the gall bladder and spleen and may lead to the development of carriers that may continue to shed *Salmonella* for years in the absence of clinical disease (10, 12, 19, 28). In the chicken, *Salmonella enterica* serovar Pullorum may persist for a number of months in the spleen, leading to infection of the reproductive tract and, consequently, to vertical transmission of the infection to eggs or to progeny (22). Recently, persistent infection of mice with *Salmonella enterica* serovar Typhimurium has been increasingly investigated. A number of studies have used mutant strains of *Salmonella* serovar Typhimurium to achieve persistence, including mutations in aromatic amino acid synthesis (18, 23), ClpXP protease (29), and polynucleotide phosphorylase (8). More recently, a persistent model of infection has been developed using a virulent *Salmonella* serovar Typhimurium strain (17).

In common with *Salmonella enterica* serovar Pullorum infection of chickens, infection of *N. ramp1*<sup>−/−</sup> (*Slc11a1*<sup>−/−</sup>) *Salmonella*-resistant 129Sv mice results in persistence of *Salmonella* within macrophages despite the presence of high titers of circulating antibodies (17, 25). The main site of *Salmonella* serovar Typhimurium persistence in mice appears to be within the mesenteric lymph nodes, an organ absent in the chicken. Treatment of chronically infected mice with a gamma interferon (IFN-γ)-neutralizing antibody results in a breakdown of the immune response (17). A similar phenomenon is observed in chickens persistently infected with *Salmonella* serovar Pullorum as they become sexually mature and commence egg laying (25). At this point the numbers of *Salmonella* in the spleen increase dramatically, and *Salmonella* infects the reproductive tract. This suggests that some suppression of the chicken immune system may play a role in the increase in numbers of *Salmonella* organisms and the spread of infection at this time point.

The mechanisms by which *Salmonella* serovar Pullorum persists in the chicken are poorly understood. While it is clear that intramacrophage survival is a crucial part of persistent infection (25) and that the *Salmonella* pathogenicity island 2 type III secretion system is required for persistence (26), the roles of other virulence factors in infection have yet to be described. At the genomic level, *Salmonella* serovar Pullorum is most closely related to *Salmonella enterica* serovar Gallinarum and is closely related to serovars Dublin and Enteritidis (7), though there is evidence of considerable rearrangement within the genome (15). However, the biology of *Salmonella* serovar Pullorum infection is markedly different even from that of *Salmonella* serovar Gallinarum, which causes a severe systemic infection in...
chickens, with a high mortality rate and distinctively different pathology (22). Although a number of older reports indicate that *Salmonella* serovar Gallinarum is also transmitted vertically (22), more-recent studies suggest that infection of the oviduct and transmission to eggs occur only rarely with this serovar (6).

The aim of this study is to investigate the cellular and humoral immune responses during persistent infection with *Salmonella* serovar Pullorum in the chicken, in particular any changes associated with the onset of sexual maturity and the subsequent increase in bacterial numbers and spread of infection to the reproductive tract. Additionally, we have investigated whether the carrier state is restricted to hens and whether the increase in bacterial numbers at sexual maturity is related to the onset of laying.

### MATERIALS AND METHODS

**Bacterial strains.** A spontaneous nalidixic acid-resistant mutant of *Salmonella* serovar Pullorum 449/87 (25) was maintained as a glycerol stock at −70°C and grown for 18 h in Luria-Bertani broth at 37°C in an orbital shaking incubator at 150 rpm.

**Experimental animals.** Commercial brown-egg-laying hens (experiments 1 and 2) and cockerels (experiment 1) were obtained as 1-day-old chicks from a commercial poultry supplier from non-*Salmonella*-vaccinated parent stocks. Chicks were raised in a dedicated poultry facility in wire cages at a temperature of 30°C, which was reduced to 20°C at 2 weeks of age. Birds were given ad libitum access to water and a vegetable protein-based diet (SDS, Witham, Essex, United Kingdom). Prior to experiments, birds were checked for the presence of *Salmonella* by cloacal swabbing as described previously (2). All experiments were described previously (4, 5). Briefly, single-cell suspensions were prepared by passing splenic tissue through 40-μm-pore-size Falcon cell strainers (BD Biosciences, Oxford, United Kingdom) in RPMI 1640 supplemented as described above.

**Erythrocytes.** Chicken erythrocytes are large nucleated cells, this low-speed centrifugation leads to the sedimentation of the vast majority of these cells. The supernatant cell suspension of mononuclear cells was retained and adjusted to 10^7^ cells per ml. Cells were added at 10^6^ per well in U-bottom microtiter plates and cocultured with either 10 μg/ml of a soluble *Salmonella* serovar Pullorum lysate antigen produced as described previously (4, 5, 25). 20 μg/ml phytohemagglutinin (PHA) as a positive mitogen control, or supplemented RPMI alone in a final volume of 200 μl per well. Cells were incubated at 41°C under 5% CO_2_ for 24 h, at which point 1 μCi/well tritiated thymidine (Amersham, Little Chalfont, United Kingdom) in RPMI 1640 supplemented with 10% fetal calf serum (Life Technologies, Paisley, Scotland) for 18 h. Cells were harvested on a Tomtec Mach IIIM cell harvester (Receptor Technologies, Banbury, United Kingdom) and incorporation of tritiated thymidine determined on a 1450 MicroBeta Trilux scintillation counter (Perkin-Elmer, Beconsfield, United Kingdom). The aim of this study is to investigate the cellular and humoral immune responses during persistent infection with *Salmonella* serovar Pullorum in the chicken, in particular any changes associated with the onset of sexual maturity and the subsequent increase in bacterial numbers and spread of infection to the reproductive tract. Additionally, we have investigated whether the carrier state is restricted to hens and whether the increase in bacterial numbers at sexual maturity is related to the onset of laying.

### RESULTS

**Experiment 1. *Salmonella* serovar Pullorum infection and carriage in male and female chickens.** Thirty-five 1-week-old male and female commercial brown-egg-laying chickens were housed separately and infected orally with 10^8^ CFU of *Salmonella* serovar Pullorum 449/87. At 3 days and 1, 2, 5, 10, 15, and 18 weeks postinfection, five birds from each group were killed for postmortem analysis. Birds were bled by cardiac puncture to obtain serum to determine antibody responses. Spleen, liver, and, at later stages, ovary and oviduct tissue samples were removed aseptically from female birds and processed for bacteriological analysis as described previously (25). Samples were plated onto selective Brilliant Green agar (Difco) containing 20 μg/ml sodium nalidixate and 1 μg/ml novobiocin (Sigma, Poole, Dorset, United Kingdom) and incubated at 37°C for 48 h to determine bacterial counts. Developing colonies were removed from the oviduct, placed in glass jars containing selenite broth, and processed as described previously (25). Spleen and liver samples from male birds were processed as described above. Testicular tissue was removed aseptically and homogenized with a sterile mortar and pestle with the addition of a little sterile sand to improve grinding. Samples were then diluted with phosphate-buffered saline and plated as described above.

**Experiment 2. Immunological responses in the carrier state and at the onset of egg laying.** A total of 110 1-week-old commercial brown-egg-laying hens were infected orally with 10^8^ CFU *Salmonella* serovar Pullorum 449/87. A group of equal size was housed separately as uninfected controls. At 1, 5, 9, 11, 13, 15, 16, 17, 18, and 22 weeks postinfection, groups of between four and eight birds were killed for postmortem analysis. Birds were sampled weekly between 15 to 18 weeks postinfection, as commercial laying hens typically commence egg laying (point of lay) at around 17 or 18 weeks of age, thereby allowing detailed sampling just prior to and at the point of lay. A later sample was taken to determine the immune responses of birds fully in lay. Spleen, liver, oviduct, and oviduct-elevating egg samples were processed as described above and blood samples taken for determination of antibody responses. Samples of splenic tissue were also taken into RPMI 1640 supplemented with 100 U/ml penicillin, 1 μg/ml streptomycin, and 5% fetal calf serum (Life Technologies, Paisley, Scotland) for T-cell proliferation assays.

**T-cell proliferation assays.** T-cell proliferation assays were performed as described previously (4, 5). Briefly, single-cell suspensions were prepared by passing splenic tissue through 40-μm-pore-size Falcon cell strainers (BD Biosciences, Oxford, United Kingdom) in RPMI 1640 supplemented as described above. Erythrocytes were removed by centrifugation at 35 × g for 10 min. Because chicken erythrocytes are large nucleated cells, this low-speed centrifugation leads to the sedimentation of the vast majority of these cells. The supernatant cell suspension of mononuclear cells was retained and adjusted to 10^7^ cells per ml. Cells were added at 10^6^ per well in U-bottom microtiter plates and cocultured with either 10 μg/ml of a soluble *Salmonella* serovar Pullorum lysate antigen produced as described previously (4, 5, 25). 20 μg/ml phytohemagglutinin (PHA) as a positive mitogen control, or supplemented RPMI alone in a final volume of 200 μl per well. Cells were incubated at 41°C under 5% CO_2_ for 24 h, at which point 1 μCi/well tritiated thymidine (Amersham, Little Chalfont, United Kingdom) was added per well and cells were then incubated for a further 18 h. Cells were harvested on a Tomtec Mach IIIM cell harvester (Receptor Technologies, Banbury, United Kingdom) and incorporation of thymidine determined on a 1450 MicroBeta Trilux scintillation counter (Perkin-Elmer, Beconsfield, United Kingdom).

**Determination of anti-*Salmonella* antibody responses by ELISA.** Anti-*Salmonella* immunoglobulin G (IgG) responses were determined by enzyme-linked immunosorbent assay (ELISA) on plates coated with *Salmonella* serovar Pullorum lysate antigen as described previously (1, 4, 5, 25).

**Statistical analysis.** Statistical analysis was performed using Microsoft Excel 2002 SP3. Differences in bacterial numbers and T-cell proliferation were analyzed by analysis of variance. Significance was taken as a P value of <0.05.
ever, at the onset of sexual maturity at 18 weeks of age, an increase in bacterial numbers was found in female but not male birds. The numbers of *Salmonella* organisms recovered from infected carriers were significantly greater in female birds (*P* < 0.05).

*Salmonella* serovar Pullorum was recovered from the reproductive tracts of four out of six female chickens, but no *Salmonella* was found in the testes of any of the male birds. These findings suggest that although both sexes develop a carrier state when infected with *Salmonella* serovar Pullorum, infection of the reproductive tract and increases in bacterial numbers at sexual maturity are restricted to female birds only. No differences were found in antibody responses between groups throughout the course of the experiment (data not shown).

### Experiment 2. Immunological responses in the carrier state and at the onset of egg laying.

The infection of laying hens with *Salmonella* serovar Pullorum progressed as expected, with an initial acute systemic infection and development of a carrier state in around 60% of the infected birds (Table 2). At 16 to 17 weeks postinfection, birds in the control group commenced egg laying, while commencement of laying was delayed until 17 to 18 weeks postinfection for the infected group, consistent with previous studies (25). An antigen-specific T-cell response to *Salmonella* antigen was found in infected birds at 5 and 9 weeks postinfection (Fig. 1A). The response at 1 week postinfection could not be determined, because insufficient cells could be isolated from the spleens of chicks. The response of infected birds was significantly greater at 5 and 9 weeks postinfection (*P* < 0.05). However, responses against *Salmonella* were raised in control birds by 13 weeks postinfection, though they remained lower than those in infected animals. This rise is consistent with previous studies of *Salmonella* serovar Typhimurium infection, where birds become increasingly responsive with age (4, 5), and probably reflects a degree of cross-reactivity to other enterobacteriaceae such as *Escherichia coli* in the gut flora that are likely to share common antigens within the crude antigen preparation used.

At 16 weeks postinfection, the T-cell response to *Salmonella* antigen fell significantly in control birds, dropping to negligible levels. In the infected birds the T-cell response began to decline at the same point but dropped to negligible levels a week later, at 17 weeks postinfection. The fall in T-cell responsiveness coincided with the onset of laying in both groups. The response to the mitogen PHA also declined in a similar fashion in both groups, indicating that the loss of T-cell proliferation activity is not *Salmonella* specific (Fig. 1B). At 18 weeks postinfection, immediately following the fall in T-cell proliferation activity, the numbers of *Salmonella* serovar Pullorum bacteria recovered from the spleen and liver increased, and salmonellae

### TABLE 2. Tissue distribution of *Salmonella* serovar Pullorum 449/87 following oral infection of 1-week-old commercial brown-egg-laying hens with 10⁸ CFU

<table>
<thead>
<tr>
<th>Wk postinfection</th>
<th>Spleen</th>
<th>Liver</th>
<th>Oviduct</th>
<th>Ovary</th>
<th>Developing eggs</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean log₁₀ CFU/g (SEM)</td>
<td>No. positive/total no.</td>
<td>Mean log₁₀ CFU/g (SEM)</td>
<td>No. positive/total no.</td>
<td>Mean log₁₀ CFU/g (SEM)</td>
</tr>
<tr>
<td>1</td>
<td>4.17 (0.19)</td>
<td>5/5</td>
<td>3.09 (0.36)</td>
<td>5/5</td>
<td>2.87 (0.66)</td>
</tr>
<tr>
<td>5</td>
<td>1.70 (0.75)</td>
<td>3/5</td>
<td>1.70 (0.75)</td>
<td>3/5</td>
<td>&lt;1</td>
</tr>
<tr>
<td>9</td>
<td>1.64 (0.67)</td>
<td>3/5</td>
<td>&lt;1</td>
<td>2/5</td>
<td>1.64 (0.67)</td>
</tr>
<tr>
<td>13</td>
<td>&lt;1</td>
<td>2/4</td>
<td>&lt;1</td>
<td>2/4*</td>
<td>&lt;1</td>
</tr>
<tr>
<td>15</td>
<td>&lt;1</td>
<td>1/4*</td>
<td>&lt;1</td>
<td>0/4</td>
<td>&lt;1</td>
</tr>
<tr>
<td>16</td>
<td>&lt;1</td>
<td>0/4</td>
<td>&lt;1</td>
<td>1/4*</td>
<td>&lt;1</td>
</tr>
<tr>
<td>17</td>
<td>&lt;1</td>
<td>1/4</td>
<td>&lt;1</td>
<td>1/4*</td>
<td>&lt;1</td>
</tr>
<tr>
<td>18</td>
<td>2.87 (0.66)</td>
<td>4/5</td>
<td>1.74 (0.75)</td>
<td>3/5</td>
<td>2.01 (0.71)</td>
</tr>
</tbody>
</table>

* Positive following enrichment with selenite broth only.
were recovered from the reproductive tracts and developing eggs of the infected group (Table 2). As T-cell proliferation began to increase, at 22 weeks postinfection (Fig. 1), the numbers of salmonellae began to decline from the oviduct, spleen, and liver.

In contrast to T-cell responses, antibody responses did not decline at the point of lay (Fig. 2). A strong anti-Salmonella IgG response was detected at 5 weeks onward in the infected group and remained high. Interestingly, following the rise in bacterial numbers at the point of lay, the levels of antibody also rose significantly by 22 weeks postinfection, in a manner reminiscent of secondary antibody responses following rechallenge.

**DISCUSSION**

In this study we have demonstrated that although development of the carrier state in Salmonella serovar Pullorum-infected chickens is unrelated to sex, infection of the reproductive tract along with an increase of systemic Salmonella numbers is specific to hens and is associated with nonspecific suppression of cellular immunity. A substantial reduction in the ability of T cells to proliferate in response to either Salmonella antigen or mitogenic stimulation was found at the point of lay. Infection of eggs and greatly increased numbers of salmonellae in the reproductive tracts of carrier birds were found immediately following this decline. Numbers of salmonellae also increased significantly in the spleen and liver at this point. Infection of the reproductive tract and subsequent vertical transmission to eggs are important biological features of avian salmonellosis (14, 25). In the case of Salmonella serovar Enteritidis, egg infection has potential consequences for public health, with many cases of gastroenteritis associated with consumption of infected eggs (13). The basis of reproductive tract infection by Salmonella serovar Enteritidis has been reviewed recently (9); however, most studies have focused on the role of bacterial factors such as fimbiae and lipopolysaccharide structure. The data presented here indicate that, in the case of Salmonella serovar Pullorum at least, suppression of cellular immunity in the chicken is closely associated with egg infection. The drop in cellular immunity may offer an opportunity for both primary reproductive tract infection and the spread of persistently infecting bacteria, as appears to be the case in this study. Suppression of cellular responses, particularly Th1 responses, may present problems in clearing both primary and secondary Salmonella infections, because T-cell responses appear to play a crucial role in clearance of Salmonella serovar Typhimurium infections in chickens (4, 5). There are also implications for the use of vaccination, particularly live attenuated vaccines, to control Salmonella serovar Enteritidis in that a drop in immunity in vaccinated birds at the point of lay may allow infection to progress. Because the suppression of cellular immunity is not Salmonella specific, a wide range of pathogens may show increased ability to infect or cause disease at this time.

The initiation of egg production in birds has a number of parallels with pregnancy in mammals in that it is a period of considerable physiological and hormonal stress. Pregnancy and parturition in mammals are generally regarded as a period of immunosuppression, largely mediated by hormonal changes. The effects of pregnancy and parturition on systemic salmonellosis are well illustrated by Salmonella infections of cattle, most notably by increases in fecal shedding around the time of calving in pregnant animals (11, 21). The effects of the onset of laying, and particularly hormonal changes, on immunity in the chicken are not clear (16). Although the onset of sexual maturity leads to an influx of macrophages and lymphocytes into the oviducts of hens (3, 27), there is little description of changes to immune function. It is clear from the loss of T-cell proliferation in response to both Salmonella antigen and mitogenic stimulation in infected and uninfected birds that the onset of laying results in pronounced immunosuppression. As a consequence, numbers of Salmonella serovar Pullorum bacteria increase dramatically in carrier birds. This has parallels to the reactivation of bacterial replication in persistent Salmonella serovar Typhimurium infection of Nramp1<sup>−/−</sup> mice following treatment with IFN-γ-neutralizing antibody (17). Survival within macrophages in low numbers appears to be a crucial factor in both systems (17, 25). It seems that the production of IFN-γ, presumably by activated T cells, is required to develop and maintain a latent Salmonella serovar Typhimurium infection in mice through regulating macrophage activation (17, 29). It appears T-cell responses are required to maintain a chronic low-level Salmonella serovar Pullorum infection in the chicken, as loss of T-cell function leads to reemergence of an acute systemic infection.

We propose the hypothesis that the activation of T cells following infection with Salmonella serovar Pullorum leads to the production of IFN-γ, which in turn activates macrophages, leading to a reduction in the initial acute systemic infection and the development of a carrier state with low numbers of bacteria persisting within macrophages. The carrier state is maintained by production of IFN-γ by T cells, and the loss of T-cell activity at the point of lay leads to a drop in IFN-γ production. This in turn leads to a decrease in the activation state of infected macrophages, allowing bacteria to replicate, increase in number, and spread to the reproductive tract. As T-cell function increases, bacterial numbers decline once more, presumably as macrophage activation increases. It is also intriguing that during the latent stage of infection, the T-cell responses to mitogenic stimulation are significantly lower in infected birds (Fig. 1). It is tempting to speculate that Salmonella serovar Pullorum may also be modulating immune activity in carrier birds, for
example through inducing interleukin-10 production by Salmonella-infected macrophages (24), and that persistence is a result of “equilibrium” between activation through production of IFN-γ and modulation by the persistent Salmonella bacteria. Such mechanisms have been suggested for persistent Salmonella serovar Typhimurium infection of mice (17, 20). With recent progress in cloning and characterization of avian cytokines, future studies may further elucidate these mechanisms.

As described previously, Salmonella serovar Pullorum infection results in a strong anti-Salmonella IgG antibody response (25), in common with Salmonella serovar Typhimurium infection of mice, though such responses are ineffective at clearing intracellular bacteria. Although antibody responses appear to be largely unaffected by the onset of sexual maturity in chickens, there is a considerable rise in antibody responses following the increase in bacterial numbers (Fig. 2). It is not yet clear whether this secondary-like antibody response plays a role in reducing numbers of salmonellae at this stage or is merely a consequence of the increased bacterial load.

The data presented here show that there is a close association between the loss of T-cell activity and the onset of egg production in the chicken. The increase in systemic Salmonella serovar Pullorum numbers at sexual maturity is restricted to production in the chicken. The increase in systemic infection results in a strong anti-Salmonella IgG antibody response, presumably a consequence of the increased bacterial load.

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