Differential cytokine expression in avian cells in response to invasion by Salmonella typhimurium, Salmonella enteritidis and Salmonella gallinarum

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Salmonella enterica is a facultative intracellular pathogen that is capable of causing disease in a range of hosts. Although human salmonellosis is frequently associated with consumption of contaminated poultry and eggs, and the serotypes Salmonella gallinarum and Salmonella pullorum are important world-wide pathogens of poultry, little is understood of the mechanisms of pathogenesis of Salmonella in the chicken. Type III secretion systems play a key role in host cell invasiveness and trigger the production of pro-inflammatory cytokines during invasion of mammalian hosts. This results in a polymorphonuclear cell influx that contributes to the resulting enteritis. In this study, a chicken primary cell culture model was used to investigate the cytokine responses to entry by the broad host range serotypes S. enteritidis and S. typhimurium, and the host specific serotype S. gallinarum, which rarely causes disease outside its main host, the chicken. The cytokines interleukin (IL)-1β, IL-2, IL-6 and interferon (IFN)-γ were measured by quantitative RT-PCR, and production of IL-6 and IFN-γ was also determined through bioassays. All serotypes were invasive and had little effect on the production of IFN-γ compared with non-infected cells; S. enteritidis invasion caused a slight downregulation of IL-2 production. For IL-1β production, infection with S. typhimurium had little effect, whilst infection with S. gallinarum or S. enteritidis caused a reduction in IL-1β mRNA levels. Invasion of S. typhimurium and S. enteritidis caused an eight- to tenfold increase in production of the pro-inflammatory cytokine IL-6, whilst invasion by S. gallinarum caused no increase. These findings correlate with the pathogenesis of Salmonella in poultry. S. typhimurium and S. enteritidis invasion produces a strong inflammatory response, that may limit the spread of Salmonella largely to the gut, whilst S. gallinarum does not induce an inflammatory response and may not be limited by the immune system, leading to the severe systemic disease fowl typhoid.

Keywords: Salmonella, cytokines, interleukin-6, chicken, inflammatory response

INTRODUCTION

Salmonella enterica is a facultative intracellular pathogen capable of causing disease in a wide range of host species. S. enterica serotypes may be divided into two groups on the basis of disease caused and their host
species range (Barrow, 1996). Broad host range serotypes including *Salmonella typhimurium* and *Salmonella enteritidis* can cause enteritis in a wide range of host species, these two serotypes being responsible for the majority of *Salmonella* food-borne enteritis in man. Around 30000 cases of human salmonellosis are reported in the United Kingdom each year (Wall & Ward, 1999) and are often associated with consumption of contaminated poultry meat or eggs (Humphrey, 1999). However, with the exception of very young chicks, *S. enteritidis* and *S. typhimurium* rarely cause clinical disease, but can colonize the gut of poultry (Barrow et al., 1987; Barrow & Lovell, 1990). Salmonellae may then be shed in the faeces and can lead to horizontal transmission to other birds in the flock by faeces at the time of slaughter. *S. enteritidis* may also colonize the reproductive tract, leading to the contamination of eggs (reviewed by Humphrey, 1999). Restricted host range serotypes rarely cause disease outside their natural host, in which they cause systemic typhoid-like disease. Examples of these serotypes include *Salmonella typhi* in man, and *Salmonella pullorum* and *Salmonella gallinarum* in the chicken, which cause pullorum disease and fowl typhoid respectively (Snoeyenbos, 1991; Pomeroy & Nagaraja, 1991). These diseases have been largely controlled in Europe and North America, but still cause substantial losses of poultry in South America and Asia where intensification of the poultry industry is in its infancy.

Recent reports suggest that differences in the disease caused by ‘typhoid-like’ restricted host range serotypes and enteritis caused by broad host range serotypes may be the results of differences in the early stages of pathogenesis (Weinstein et al., 1998; Henderson et al., 1999). Following oral infection by *S. typhimurium* in mammals, *Salmonella* penetrates through the intestinal epithelium (Galán & Sansonetti, 1996). Entry into epithelial cells is mediated by a type III secretion system encoded on *Salmonella* pathogenicity island 1 (Darwin & Miller, 1999). The proteins secreted by this system interact with the epithelial cells, triggering a number of responses including the production of pro-inflammatory cytokines (Galán & Sansonetti, 1996; Darwin & Miller, 1999), leading to an influx of polymorphonuclear cells (PMNs) accompanied by increased fluid secretion. The interaction of *Salmonella* with epithelial cells results in a number of responses including damage to the intestinal epithelium and diarrhoea. *S. typhimurium* is also capable of causing gastroenteritis in young chicks and causes intestinal lesions, but not clinical disease, in older birds (Barrow et al., 1987). Relatively little is known about the molecular mechanisms of *Salmonella* entry in the chicken gut, though the basic mechanisms of pathogenesis of *S. typhimurium* appear to be similar to those in mammals (Henderson et al., 1999). Invasion causes rapid inflammation of the intestinal mucosa and infiltration of large numbers of heterophils, the avian equivalent of neutrophils, followed by macrophages, resulting in intestinal lesions. In contrast, following infection with *S. pullorum*, rapid inflammation does not occur, and only small numbers of heterophils are found associated with the intestinal epithelium (Henderson et al., 1999). *S. pullorum* is less invasive than *S. typhimurium* in a range of avian and mammalian primary and continuous cell lines. *S. gallinarum* has also previously been shown to be less invasive than *S. typhimurium* in both avian and mammalian cells (Barrow & Lovell, 1989).

Greater understanding of how *Salmonella* interacts with the host is needed to understand the disease and colonization processes in the chicken both in terms of animal and public health. Recent progress in the cloning of avian cytokines has led to the development of reagents with which to measure cytokine production in response to infection in the chicken, and this may allow a greater insight into interactions between the host and salmonellae at a cellular and molecular level. The avian orthologues of the Th1 cytokines interferon-γ (IFN-γ) and interleukin-2 (IL-2) have recently been cloned (Dgby & Lowenthal, 1995; Sundick & Gill-Dixon, 1997), as have the pro-inflammatory cytokines IL-1β (Weining et al., 1998) and IL-6 (accession no. AJ982185). Reproducible, sensitive bioassays exist to measure chicken IFN-γ-like (Lowenthal et al., 1995) and IL-6-like (Lynagh, 1998; Nakamura et al., 1998) activities. The genomic sequences and gene structure for IFN-γ (Kaiser et al., 1998), IL-2 (Kaiser & Mariani, 1999) and IL-1β (accession no. AJ245728) have been fully determined. A partial genomic sequence for IL-6 has also recently been isolated (accession no. AJ250838). Gene structure information makes possible the design of probes and primers to specifically quantify cytokine mRNA levels using real-time quantitative RT-PCR.

We aimed to determine the levels of cytokines, particularly the pro-inflammatory cytokines IL-6 and IL-1β, produced following the invasion of the broad host range serotypes *S. typhimurium* and *S. enteritidis*, and the host specific serotype *S. gallinarum*, into chicken cells *in vitro*. Primary chick kidney cells (CKC) were chosen as the host cell for the invasion model; there is no current suitable chicken intestinal epithelium model. CKC contain a high proportion of epithelial cells, few phagocytic cells and have previously been shown to be invaded by a range of *Salmonella* serotypes (Barrow & Lovell, 1989). Different levels of cytokine production following invasion with the broad host range serotypes (*S. typhimurium* and *S. enteritidis*), compared to the host specific serotype (*S. gallinarum*), would provide insight into the mechanisms of immunopathogenesis of salmonellosis in the chicken.

**METHODS**

**Bacterial strains.** The following well-characterized strains were selected as representative of their serotype for their *in vitro* behaviour in the chicken: *Salmonella gallinarum* 9 causes severe systemic disease in adults and chicks (Smith, 1955), *Salmonella typhimurium* Phage Type (PT)14 strain F98...
Table 1. Real-time quantitative RT-PCR probes and primers

<table>
<thead>
<tr>
<th>RNA target</th>
<th>Probe/primer sequence (5‘-3‘)</th>
<th>Exon boundary</th>
<th>Accession no.*</th>
</tr>
</thead>
<tbody>
<tr>
<td>28S</td>
<td>Probe (VIC)-AGGACCGCTACGGACCTCCACCA-(TAMRA)</td>
<td>3/4</td>
<td>X59733</td>
</tr>
<tr>
<td></td>
<td>F GGCAGAAAGCGAAGGAACCT</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>R GACGGTCATTTGAGCTGTC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IFN-β</td>
<td>Probe (FAM)-TGGCCGAACCGTCCGATGAAGCAGA-(TAMRA)</td>
<td>5/6</td>
<td>Y07922</td>
</tr>
<tr>
<td></td>
<td>F GTCAGAGAGGTTGAAGATATCTAGGA</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>R GCTTGGACCGTCTGACTCA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-1β</td>
<td>Probe (FAM)-CCACACTGCAGCTGGAGAAGCAGC-(TAMRA)</td>
<td>2/3</td>
<td>AJ245728</td>
</tr>
<tr>
<td></td>
<td>F GCTCTACATGGTCGTGTGTGATGAG</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>R TGTGATGTCCCGCAGTGA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-2</td>
<td>Probe (FAM)-ACTGAGACCGAGGGTGACCCACG-(TAMRA)</td>
<td>3/4</td>
<td>AJ009800</td>
</tr>
<tr>
<td></td>
<td>F TTGAAATATACAAAGACAAGATTTAC</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>R TCCAGGTAACACTGGAGATTGTTTT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-6</td>
<td>Probe (FAM)-AGGAGAAATGCGTGAGAAGCTGCTTCA-(TAMRA)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>F GCTGCGGCGCTCTGA</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>R GGTAGGTCGAAAGGCGACAG</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Refers to the genomic DNA sequence.

(Smith & Tucker, 1975) and Salmonella enteritidis PT4 strain 125589 (Barrow & Lovell, 1990) cause disease in young chicks but colonize older birds without disease. Salmonella dublin 2229 (Baird et al., 1985) and Escherichia coli K-12 (Smith, 1978) were also tested. With the exception of S. dublin 2229, the invasiveness of all strains in primary chicken cell lines has been previously determined (Barrow & Lovell, 1989). Bacteria were cultured in Luria–Bertani (LB) broth (Difco) at 37 °C and 22 h at 37 °C in an orbital shaking incubator at 150 r.p.m. (Barrow & Lovell, 1989). Bacteria were cultured in Luria–Bertani (LB) broth (Difco) at 37 °C in an orbital shaking incubator at 150 r.p.m.

Cell culture. Primary CKC were prepared from the kidneys of 1–2 week old Rhode Island Red chicks as previously described (Barrow & Lovell, 1989). Briefly, kidneys were removed aseptically, teased apart and trypsinized with versene (0.8% NaCl, 0.02% KH2PO4, 0.15% Na2HPO4, 0.02% KCl and 0.02% EDTA). Cell concentrations were adjusted to 1 x 106 ml−1 in complete DMEM (Dulbecco’s modified Eagle’s medium) supplemented with 12.5% (v/v) heat-inactivated foetal bovine serum (FBS), 10% (v/v) trypsin–phosphate broth (Difco), 25 U nystatin ml−1, 100 U penicillin ml−1 and 1 µg streptomycin ml−1 (P/S), at pH 7.0 and grown in 1 ml per well in 24-well Nunclon plates (Nunc) for 72 h at 37 °C, 5% CO2. Two hours prior to use in invasion assays, the media was replaced with DMEM without antibiotics.

HD11 (a chicken macrophage cell line) cells (Beug et al., 1979) were seeded at 4 x 106 ml−1 and grown at 41 °C, 5% CO2 in RPMI 1640 medium (Life Technologies) containing 20 mM L-glutamine (Life Technologies), 2.5% FBS, 2.5% chicken serum, 10% trypsin–phosphate broth and P/S. 7TD1 (an IL-6-dependent mouse plasmacytoma cell line) cells (van Snick et al., 1986) were seeded at 2 x 106 ml−1 and grown at 37 °C, 5% CO2 in RPMI 1640 medium containing 20 mM L-glutamine, 10% FBS, 0.05 mM 2-mercaptoethanol and P/S. During routine culture, recombinant murine (rm) IL-6 was added at 10 pg ml−1.

Invasion of cells. Bacterial cultures were diluted in LB to 1 x 108 ml−1 and 100 µl added to the CKC to give a m.o.i. of 10 bacteria per chicken cell. Cells were incubated for either 2 or 4 h at 37 °C, 5% CO2. After incubation, cell supernatants were removed, filtered through a 0.22 µm filter and stored at −20 °C prior to determination of cytokine production. Extracellular bacteria were then killed by incubating cells for 1 h at 37 °C in DMEM containing 100 µg gentamicin ml−1 (Sigma). Cells were washed three times in Hank’s buffered saline solution (Life Technologies) and lysed with 1 ml 1% (v/v) Triton X-100 (Sigma) for 30 min at 37 °C. Viable counts of the intracellular bacteria in the lysate were made on LB agar. Each assay was performed in triplicate for each serotype. Statistical analysis of variance between species and serotypes was made using the Minitab for Windows v12.21 statistical program (Minitab). The probability level for significance was taken as P < 0.05.

Real-time quantitative RT-PCR. Cytokine mRNA levels in infected and control CKC cultures were quantified using a method based on that of Moody et al. (2000).

Total RNA was prepared from CKC cultures using the RNeasy mini kit (Qiagen) following the manufacturer’s instructions. Purified RNA was eluted in 50 µl RNase-free water and stored at −70 °C.

For both cytokine and 28S rRNA-specific amplification, primers and probes were designed using the Primer Express software program (PE Applied Biosystems). Details of the probes and primers are given in Table 1. All cytokine probes were designed, from the sequence of the relevant genes, to lie across intron–exon boundaries. Cytokine probes were labelled with the fluorescent reporter dye 5-carboxyfluorescein (FAM) at the 5′ end and the quencher N3,N3′-tetramethyl-6-carboxyrhodamine (TAMRA) at the 3′ end. The 28S probe was labelled with the fluorescent reporter dye VIC (PE Applied Biosystems) at the 5′ end and TAMRA at the 3′ end.

RT-PCR was performed using the TaqMan EZ RT-PCR kit (PE Applied Biosystems). The RT-PCR mixture consisted of 1 x EZ RT-PCR buffer (including 60 nM 6-carboxy-x-rhodamine (a fluorescent reference dye)), 3 mM manganese acetate, 300 µM dATP, dCTP and dGTP, 600 µM dUTP, 600 nM each primer, 100 nM probe, 0.1 U rTth polymerase, 0.01 U AmpErase UNG (uracil-N-glycosylase), 5 μl total RNA, made up to 25 μl with water. Amplification and detection of specific products were performed using the ABI PRISM 7700 Sequence Detection System (PE Applied Bio-
systems) with the following cycle profile: 1 cycle of 50 °C for 2 min, 96 °C for 5 min, 60 °C for 30 min and 95 °C for 5 min, and 40 cycles of 94 °C for 20 s, 59 °C for 1 min.

Quantification was based on the increased fluorescence detected by the ABI PRISM 7700 Sequence Detection System due to hydrolysis of the target-specific probes by the 5′ nuclease activity of the rTth DNA polymerase during PCR amplification. The passive reference dye 6-carboxy-x-rhodamine, which is not involved in amplification, was used to correct for fluorescent fluctuations resulting from changes in the reaction conditions, for normalization of the reporter signal. Results are expressed in terms of the threshold cycle value (C_t), the cycle at which the change in the reporter dye (∆R_n) passes a significance threshold. In this work, the threshold values of ∆R_n are as shown in Table 2, for all reactions described.

To generate standard curves for the cytokine and 28S rRNA-specific reactions, total RNA extracted from stimulated splenocytes was serially diluted in sterile RNase-free water and dilutions made from 10^{-1} to 10^{-5}. Each RT-PCR experiment contained three no-template controls, test samples and a log_{10} dilution series. Each experiment was performed in triplicate, with replicates performed on different days. Regression analysis of the mean values of six replicate RT-PCR for the log_{10} diluted RNA was used to generate standard curves.

### Macrophage activation factor assay

Macrophage activation factor activity, typically used in the chicken as a measure of IFN-γ activity (Lowenthal et al., 1995), was assayed by the production of nitric oxide by stimulated HD11 cells (Beug et al., 1979), quantitated by the accumulation of nitrite (NO\_2) in the culture medium (Ding et al., 1988; Sung et al., 1991). HD11 cells were seeded at a density of 5 × 10^5 cells per well in flat-bottomed 96-well microtitre plates (Corning) in growth medium (as described above). Subsequently, triplicate 100 µl samples of twofold serial dilutions of CKC conditioned medium (CM) were added and the cells cultured for 48 h at 41 °C, 5% CO\_2. The nitrite concentration was assayed by mixing 100 µl cell-free culture supernatant with 100 µl Griess reagent [0.3%, w/v, naphthylethenediamine dihydrochloride (Sigma), 1% (w/v) sulphanilamide (Sigma) in 2.5% H_2PO_4 (BDH)] and incubating for 10 min at room temperature. The absorbance of the reaction product was measured in a Titertek Multiscan MCC/340 ELISA reader (ICN) at 543 nm. Serial dilutions of sodium nitrite (Sigma) were used to determine a standard curve. Data are expressed as µM NO\_2 (5 × 10^3 cells)^{-1} (48 h)^{-1}. IFN-γ-like activity of the test samples was determined using log-linear regression analysis against the NO\_2 standard curve.

To confirm the specificity of the NO\_2-inducing activity, the CM were also pre-incubated for 1 h, prior to their addition to the HD11 cells, with either IE12, an anti-chicken IFN-γ neutralizing mAb (Lambrecht et al., 2000), or the LPS inhibitor polymyxin B.

### RESULTS

#### Invasion of CKC cells by Salmonella and E. coli

All four Salmonella serotypes invaded CKC at levels higher than the non-invasive E. coli control (P < 0.05). *S. typhimurium* was significantly more invasive than *S. gallinarum, S. enteritidis or S. dublin* (P < 0.001). No significant difference in invasion was found between *S. gallinarum, S. enteritidis or S. dublin* (P > 0.2). Results for a 2 h contact time are shown in Table 3. Invasion

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**Table 2. Standard curve data from real-time quantitative RT-PCRs on total RNA extracted from stimulated splenocytes**

<table>
<thead>
<tr>
<th>Target</th>
<th>∆R_n* significance threshold</th>
<th>Log dilutions</th>
<th>C_t values†</th>
<th>R^2‡</th>
<th>Slope</th>
</tr>
</thead>
<tbody>
<tr>
<td>28S</td>
<td>0.05</td>
<td>10^{-1}–10^{-3}</td>
<td>8–22</td>
<td>0.9719</td>
<td>3.138</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>0.01</td>
<td>10^{-1}–10^{-3}</td>
<td>16–31</td>
<td>0.9933</td>
<td>3.503</td>
</tr>
<tr>
<td>IL-1β</td>
<td>0.02</td>
<td>10^{-1}–10^{-3}</td>
<td>22–35</td>
<td>0.9903</td>
<td>3.124</td>
</tr>
<tr>
<td>IL-2</td>
<td>0.01</td>
<td>10^{-1}–10^{-4}</td>
<td>24–34</td>
<td>0.9980</td>
<td>3.122</td>
</tr>
<tr>
<td>IL-6</td>
<td>0.02</td>
<td>10^{-1}–10^{-4}</td>
<td>23–36</td>
<td>0.9832</td>
<td>4.333</td>
</tr>
</tbody>
</table>

* ∆R_n = change in the reporter dye.
† C_t = threshold cycle value, the cycle at which the change in the reporter dye levels detected passes the ∆R_n.
‡ R^2 = coefficient of regression.

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**Table 3. Invasion of CKC with Salmonella serotypes and E. coli**

Values are means ± SEM of three repeats of the experiment. Bacteria had a contact time of 2 h with the CKC prior to killing of extracellular bacteria with 100 µg gentamicin ml^{-1}.

<table>
<thead>
<tr>
<th>Bacterial strain</th>
<th>Log_{10} c.f.u. invading bacteria (ml CKC)^{-1}</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>S. typhimurium</em> F98</td>
<td>4.81 ± 0.08a†</td>
</tr>
<tr>
<td><em>S. gallinarum</em> 9</td>
<td>3.50 ± 0.18b†</td>
</tr>
<tr>
<td><em>S. dublin</em> 2229</td>
<td>3.40 ± 0.24b†</td>
</tr>
<tr>
<td><em>S. enteritidis</em> 125589</td>
<td>3.31 ± 0.15c†</td>
</tr>
<tr>
<td><em>E. coli</em> K-12</td>
<td>1.70 ± 0.03a†</td>
</tr>
</tbody>
</table>

* Where mean values for the groups differ significantly (P < 0.05), the values carry different superscript letters.
caused little damage to the cell monolayer, with any damage being most pronounced following invasion with \textit{S. enteritidis}. There was only a slight increase in bacterial invasion with increased contact time of 4 h (data not shown) with no observable increase in cell monolayer damage.

**Cytokine levels by real-time quantitative RT-PCR**

Replicate measurements on different days were highly repeatable, with a coefficient of variation for six replicate RT-PCRs of log\(_{10}\) serially diluted RNA for the different reactions as shown in Table 2. There was a linear relationship between the amount of input RNA and the \(C_t\) values for the various reactions, as shown in Table 2. Regression analyses of the \(C_t\) values generated by the log\(_{10}\) dilution series gave R\(^2\) values for all reactions in excess of 0.97 (see Table 2 for details). The increase in cycles per log\(_{10}\) decrease in input RNA for each specific reaction, as calculated from the slope of the respective regression line, is given in Table 2.

Attempts were made to set up multiplex RT-PCRs, in which a cytokine-specific and 28S rRNA-specific reaction were carried out on the same sample in the same tube. However, there were significant differences in the cytokine-specific \(C_t\) values from the multiplex RT-PCRs compared to the single RT-PCRs. Also, the gradients generated from the regression analyses of the log\(_{10}\) dilution series following multiplex and single RT-PCRs were quite different. Multiplex RT-PCR therefore seemed to significantly affect the quantification of cytokine RNA. It was therefore decided to continue with single RT-PCRs, but to run both cytokine-specific and 28S rRNA-specific reactions on the same samples, in triplicate, in the same experiment, with replicate experiments.

To control for variation in sampling and RNA preparation, the \(C_t\) values for cytokine-specific product for each sample were standardized using the \(C_t\) value of 28S rRNA product for the same sample from the reaction run simultaneously. The \(C_t\) values for 28S rRNA did not alter significantly from sample to sample; the mean 28S rRNA \(C_t\) values for uninfected or infected CKC ranged from 8.58 to 8.94. Cytokine-specific \(C_t\) values varied from sample to sample, and from cytokine to cytokine. The \(C_t\) values for 28S rRNA thus appeared to be independent of cytokine production and infection. They were therefore taken to be representative of the level of RNA extracted from the CKC cultures. To normalize RNA levels between samples within an experiment, the mean \(C_t\) value for 28S rRNA-specific product was calculated by pooling values from all samples in that experiment. Tube to tube variations in 28S rRNA \(C_t\) values about the experimental mean were calculated. The slope of the 28S rRNA log\(_{10}\) dilution series regression line was used to calculate differences in input total RNA. Using the slopes of the respective cytokine log\(_{10}\) dilution series regression lines, the difference in input total RNA, as represented by the 28S rRNA, was then used to adjust cytokine-specific \(C_t\) values. Fig. 1(a)

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**Fig. 1.** (a) Quantification of cytokine mRNA in RNA extracted from uninfected CKC, or from CKC infected with \textit{Salmonella} spp. or \textit{E. coli} for 4 h. Cycle threshold values are expressed subtracted from 40 (the negative end point). Therefore, higher values represent higher levels of cytokine mRNA. White bars represent cytokine mRNA specific \(C_t\) values before standardization for input RNA. Black bars represent standardized values for cytokine mRNA corrected for variation in input RNA measured by 28S rRNA levels. (b) The above corrected data from (a) expressed as fold change in cytokine mRNA levels, when compared to those from uninfected CKC. Cytokine mRNA levels in uninfected CKC are set at 1. *** = \(P < 0.001\), compared to levels in \textit{E. coli}-infected CKC S.t., \textit{S. typhimurium} E.c., \textit{S. gallinarum} S.g., \textit{S. dublin} S.d., \textit{S. enteritidis} S.e. Error bars show SEM for triplicate samples from three separate experiments.
Fig. 2. IFN-γ-like activity in the CM of infected or uninfected CKC. Data shown as calculated from the LPS standard curve run in each assay. HD11 cells were cultured in the presence of sample only (CM or recombinant chicken IFN-γ); white bars), or sample which had been pre-treated with an anti-chicken neutralizing mAb (1E12, 1:100 dilution; hatched bars), or the LPS inhibitor polymyxin B (10 µg ml⁻¹; black bars). All assays were carried out in triplicate. Error bars show SEM. Abbreviations are as in Fig. 1.

IL-6-like activity in the CM of infected or uninfected CKC

Table 4. IL-6-like activity in the CM of infected or uninfected CKC

Dilution units were calculated from the dilution curve of each sample, with one dilution unit corresponding to the dilution of serum required to bring about half maximal proliferation of 7TD1 cells. Half maximal proliferation was determined from the recombinant murine IL-6 standard curve run in each assay. Values represent the mean of triplicate samples from a representative experiment. All experiments were performed at least three times.

<table>
<thead>
<tr>
<th>Bacterial strain</th>
<th>Dilution units ± SEM of IL-6-like activity*</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>S. typhimurium</em> F98</td>
<td>77.18 ± 3.32a</td>
</tr>
<tr>
<td>S. gallinarum 9</td>
<td>9.02 ± 0.48b</td>
</tr>
<tr>
<td><em>S. dublin</em> 2229</td>
<td>77.37 ± 3.90b</td>
</tr>
<tr>
<td><em>S. enteritidis</em> 125589</td>
<td>91.14 ± 4.40b</td>
</tr>
<tr>
<td><em>E. coli</em> K-12</td>
<td>20.82 ± 0.65b</td>
</tr>
<tr>
<td>Uninfected</td>
<td>17.31 ± 0.53b</td>
</tr>
</tbody>
</table>

*Where mean values for the groups differ significantly (P < 0.01), the values carry different superscript letters.

shows the effect of standardizing cytokine-specific C₅ values to correct for tube to tube variation in RNA levels. Standardization does not dramatically alter the distribution of the results as a whole. Fig. 1(b) shows the standardized data expressed as fold changes in mRNA levels in samples from infected CKC compared to those from uninfected CKC, which was set at a basal level of

1. For statistical comparisons, cytokine mRNA changes following *Salmonella* infection were compared to those following *E. coli* infection. In terms of levels of IFN-γ and IL-2 mRNA expression, there was very little effect following infection, except that, in the case of *S. enteritidis*, there was an approximately fivefold decrease in IL-2 mRNA (P < 0.01). For IL-1β mRNA expression, infection of CKC with *E. coli* caused a reduction in IL-1β mRNA levels compared to uninfected CKC. Infection with *S. gallinarum* or *S. enteritidis* had similar effects to the *E. coli* infection. However, compared to the *E. coli* infection, infection with *S. typhimurium* or *S. dublin* gave significant levels of IL-1β mRNA expression (P < 0.01), similar to the uninfected controls. The most striking results were seen with regard to IL-6 mRNA expression levels. Infection of CKC with *S. typhimurium*, *S. dublin* or *S. enteritidis* caused a seven- to tenfold increase in IL-6 mRNA expression (P < 0.01). However, following *S. gallinarum* infection there was a threefold decrease in IL-6 mRNA expression (P < 0.01).

Cytokine levels by bioassay

There are few well-characterized, reproducible, specific bioassays for avian cytokines. Bioassays to reliably measure IL-1β and IL-2 content of samples were not available for use. However, there are reliable bioassays to measure IFN-γ-like and IL-6-like activity in the chicken.

CM from all CKC cultures were harvested after 4 h culture and assayed for IFN-γ-like activity using the macrophage activation factor assay. Fig. 2 shows the amount of nitrite, expressed as µM NO₂⁻ (5 × 10⁴ cells⁻¹ (48 h)⁻¹), produced by macrophages grown in the presence of CM from uninfected CKC, and CKC infected either with one of the four species of *Salmonella*, or with *E. coli*. As a positive control, macrophages were also grown in the presence of a 1:1000 dilution of recombinant chicken IFN-γ. Only very low (background) levels of nitrite were produced by HD11 cells cultured in the presence of CM from uninfected CKC, regardless of the dilution of the CM. Nitrite was produced by HD11 cells grown in the presence of CM from all the infected CKC. The quantity of NO₂⁻ produced titrated out with increasing dilution of the CM down to background levels. CM from CKC infected with *S. typhimurium* contained most NO₂⁻, followed by that from *S. enteritidis*-infected CKC, with that from *S. gallinarum*-infected CKC containing least NO₂⁻. CM from CKC infected with *S. dublin* or *E. coli* gave intermediate levels of NO₂⁻.

Treatment with the anti-IFN-γ neutralizing mAb, 1E12 (Lambrecht et al., 2000) removed some, but by no means all, of the NO₂⁻-inducing activity from the CM (Fig. 2), and neutralized most of the NO₂⁻-inducing activity of the recombinant chicken IFN-γ. Treatment with polymyxin B, an inhibitor of LPS, removed all significant NO₂⁻-inducing activity from the CM. However, at the concentration used, polymyxin B may be toxic to the
HD11 cells, as it also ablated some of the NO$\gamma$-inducing activity of the recombinant chicken IFN-$\gamma$.

Levels of IL-6-like factor activity in the CM from the same CKC cultures were measured using the 7TD1 bioassay (van Snick et al., 1986). 7TD1 cells are dependent on the presence of IL-6 for growth and proliferation, and the assay can detect IL-6 levels as low as 0·1 pg ml$^{-1}$. This assay has been used to measure the presence of chicken IL-6-like activity in the CM from LPS-stimulated fibroblasts and HD11 cells (Lynagh, 1998), and in serum (Nakamura et al., 1998; Lynagh, 1998). Table 4 shows IL-6-like activity in the CM analysed by the 7TD1 assay, expressed as dilution units.

CM from CKC infected with the broad host range serotypes ($S$. enteritidis, $S$. dublin or $S$. typhimurium) contained significantly more IL-6-like activity ($P < 0·001$), than CM from CKC infected with $E$. coli. CM from $S$. gallinarum-infected CKC contained least IL-6-like activity, significantly less ($P < 0·001$) than CM from CKC infected with $E$. coli.

**DISCUSSION**

Although the role of cytokines in the immune response to *Salmonella* infection/invasion and their role in pathogenesis have been well characterized in mammals, it is poorly understood in the chicken. *S*. enteritidis-immune lymphokines have been partially characterized and shown to influence an inflammatory response and to activate heterophils (Kogut et al., 1994a, b, 1995). However, until the recent progress in cloning avian cytokine genes, it had not been possible to fully characterize these lymphokines due to the lack of avian cytokine reagents. It is now possible to specifically quantify, amongst others, the avian orthologues of the Th1 cytokines IFN-$\gamma$ and IL-2, and the pro-inflammatory cytokines IL-1$\beta$ and IL-6. The real-time quantitative RT-PCR results suggest that, with the exception of *S*. typhimurium, in *vitro* infection of CKC with *Salmonella* or *E*. coli results in the down-regulation of expression of mRNA for the pro-inflammatory cytokine IL-1$\beta$. In *vivo*, such down-regulation might lead to a reduced rapid inflammatory response in the gut and allow initial entry of the bacteria into epithelial cells. Infection seems to have little effect on IL-2 production, with the exception of *S*. enteritidis, which down-regulates IL-2 mRNA expression. With the lack of reliable IL-1$\beta$ or IL-2 bioassays, these levels of mRNA expression cannot be correlated with the presence of cytokine in the CM, or actual biological activity.

By contrast, assays which measure IFN-$\gamma$-like and IL-6-like activity in the chicken are available, allowing quantification of IFN-$\gamma$- and IL-6-like activities in the CM from *Salmonella*- and *E*. coli-infected CKC. The real-time quantitative RT-PCR results showed little or no induction of IFN-$\gamma$ mRNA expression in infected CKC compared with uninfected controls. However, the bioassay showed significant IFN-$\gamma$-like activity in the CM from all infected CKC. This activity was not removed by pre-treating the CM with an anti-chicken IFN-$\gamma$ neutralizing antibody (Lambrecht et al., 2000), but was removed by pre-treatment with the LPS inhibitor polymyxin B (see Fig. 2). The most likely explanation for these results is contamination of the CM from infected CKC with bacterial LPS (LPS stimulates HD11 cells to produce NO$\gamma$), especially as the IFN-$\gamma$-like activity in the CM of uninfected CKC was very low, as were the IFN-$\gamma$ mRNA levels in the uninfected CKC themselves.

**Invasion of *S*. typhimurium and *S*. enteritidis into avian cells produces an inflammatory acute phase response**

Infection with *S*. typhimurium, *S*. dublin and *S*. enteritidis gave both high levels of IL-6-like activity in the CM and induced high levels of IL-6 mRNA expression in the CKC. *E*. coli infection gave slightly higher levels of both IL-6 mRNA and IL-6-like activity, compared to those from uninfected CKC. Finally, *S*. gallinarum infection seems to result in down-regulation, or non-induction, of IL-6 expression. Both IL-6 mRNA levels, and levels of IL-6-like activity, were lower in CKC infected with *S*. gallinarum than in uninfected CKC. There is good correlation between IL-6 mRNA levels in the CKC and IL-6-like activity in the CM from the CKC, following infection.

It would appear that production of IL-6 is not simply due to invasion. Higher levels of IL-6 were produced by CKC incubated with the non-invasive *E*. coli K-12. It appears that the down-regulation of IL-6 is a specific effect of *S*. gallinarum. IL-6 is a multifunctional cytokine that has pro-inflammatory activity via the induction of acute phase protein synthesis, and is important in the development of adaptive immune responses leading to the differentiation of B lymphocytes, cytotoxic T cells and the growth of T cells (Hirana, 1994). The role of IL-6 in the pathogenesis of *S*. typhi and *S*. typhimurium has been investigated in human and murine epithelial cell lines (Weinstein et al., 1998). The invasion of *S*. typhi into human or murine epithelial cells results in the production of high levels of IL-6. In contrast, invasion of *S*. typhimurium into mice and humans results in only low levels of IL-6 production. The data presented here indicate that the invasion of *S*. gallinarum into non-phagocytic chicken cells results in a low level of IL-6 production, and suggests that early pathogenesis of fowl typhoid, at least in terms of IL-6 production, may more closely resemble the typhoid-like disease found in *S*. typhimurium-infected mice than human typhoid fever.

The high levels of IL-6 production following invasion by *S*. typhimurium, *S*. enteritidis and *S*. dublin also suggest differences in the early interactions and pathogenesis of these broad host range serotypes compared with the chicken-specific serotype *S*. gallinarum. *S*. typhimurium does not frequently cause clinical disease except in very young chicks (Barrow et al., 1987), where it has been shown to cause damage to the intestinal mucosa, particularly flattening of the microvilli structure (Nagaraja et al., 1991). Although older birds rarely
show clinical disease following *S. typhimurium* infection, 1-d-old birds infected orally with *S. typhimurium* develop intestinal lesions and flattened microvilli 2–3 d following infection (Henderson et al., 1999). Increased numbers of heterophils and mononuclear cells in the gut accompany the intestinal damage, suggesting that the mechanisms of disease resemble those found in mammals (Darwin & Miller, 1999). In contrast, *S. gallinarum* does not cause intestinal damage in the early stages of infection but causes an acute and virulent systemic disease with lesions of the spleen, liver and heart often accompanied by bacteraemia (Smith, 1956; Pomeroy & Nagaraja, 1991). The differences in IL-6 production found in this study indicate that invasion by *S. typhimurium*, *S. enteritidis* or *S. dublin* induces a strong acute phase inflammatory response and activation of innate and adaptive immune responses. This may contribute to both the damage of the intestinal epithelium observed following in vivo infection (Henderson et al., 1999) and also to an effective immune response that prevents systemic disease. The increased levels of heterophils found following *S. typhimurium* infection are also likely to play a major role in disease pathogenesis and immune protection. Heterophils have been demonstrated to play an important role in protection against *S. enteritidis* infection in the chicken (Kogut et al., 1994c). In mammals it has been shown that IL-8 is produced in response to *Salmonella* infection (Eckman et al., 1993), along with a pathogen-elicited epithelial chemoattractant (PEEC) (McCormick et al., 1998). These act to induce an influx of PMNs that are involved in the pathogenesis of *Salmonella* gastroenteritis (Darwin & Miller, 1999). However, no equivalent of PEEC has yet been found in the chicken, and there are as yet no specific assays to measure IL-8 in the chicken. However, it seems likely that invasion by *S. typhimurium* or *S. enteritidis* may induce IL-8 production, and hence heterophil influx in the chicken gut.

**Invasion by *S. gallinarum* does not induce an inflammatory response**

In contrast, invasion by *S. gallinarum* results in little or no production of IL-6. This suggests that the entry of *S. gallinarum* does not trigger a strong immune or inflammatory response. Such a mechanism would allow entry without intestinal damage, and may fail to trigger an effective host response allowing the development of systemic disease. The closely related *S. pullorum* appears to enter through chicken epithelium without causing an inflammatory response, intestinal damage or heterophil infiltration, though the main route of entry appears to be through lymphoid tissue (Henderson et al., 1999). In addition, in a human cell-culture system *S. pullorum* is capable of both attachment and entry into epithelial cells, but does not induce transepithelial migration of PMNs (McCormick et al., 1995). Depletion of PMNs in chickens results in systemic septicemia following *S. enteritidis* infection (Kogut et al., 1994c), resulting in disease akin to that caused by *S. gallinarum*. It appears that inflammatory responses and PMNs in the gut are important in protection against the development of systemic typhoid-like disease, though they may result in tissue damage in birds or gastroenteritis in mammals. The failure of *S. gallinarum* to induce an inflammatory response in invasion in the chicken may be an adaptation that contributes to its host specific nature. However, it is likely to be of secondary importance compared to the interaction with the host’s reticuloendothelial system, which has been shown to be of prime importance in host specificity in vivo in both the chicken and mouse (Barrow et al., 1994).

It is interesting that there is a down-regulation of IL-1β during *Salmonella* invasion. Little is known regarding the production of IL-1β by non-phagocytic cells invaded by *Salmonella*. IL-1β is a potent pro-inflammatory cytokine and high levels were produced in the early stages of invasion, which may act to inhibit *Salmonella* crossing the intestinal epithelium. This is in contrast to the production of IL-8 and PMN influx triggered by *Salmonella* invasion leading to damage of the gut mucosa, allowing bacterial entry (Galán & Sansonetti, 1996; Darwin & Miller, 1999). In the mouse, IL-1β is produced by macrophages following invasion across the gut epithelium and contributes to both the subsequent inflammation of the gut and pyrexia during *Salmonella* infection (Galán & Sansonetti, 1996). IL-1β is also produced by murine macrophages and dendritic cells when invaded by *Salmonella in vitro* (Marriott et al., 1999). The production of IL-1β by chicken macrophages in response to *Salmonella* is as yet undefined.

In this work we have demonstrated methods for investigating cytokine production by cultured chicken cells in response to challenge with bacterial pathogens, and, though limited by the current availability of assays in the chicken, have shown differential production of cytokines in response to host specific and broad host range *Salmonella*. These differences suggest that pathogenesis and host specificity of *S. gallinarum* infection in the chicken may be related to some extent to the lack of inflammatory response in the early stages of infection in the gut. Production of pro-inflammatory cytokines, including IL-6 and probably IL-8, may limit serotypes such as *S. typhimurium* and *S. enteritidis* to the gut by induction of a strong immune response, but as a result produce lesions and flatten intestinal microvilli, the same mechanism that produces gastroenteritis in mammals (Darwin & Miller, 1999). It appears that all serotypes down-regulate IL-1β production in chicken cells, possibly to facilitate entry in vivo by inhibiting inflammation of the gut epithelium during invasion. It is anticipated that the quantitative RT-PCR techniques will be utilized to determine cytokine production from cells and tissues obtained from *in vivo* *Salmonella* infections of poultry. The interaction of *Salmonella* with eukaryotic cells is largely dependent on Type III secretion systems (Darwin & Miller, 1999), and their secreted proteins. Although Type III secretion systems are conserved in different bacteria [for example, the inv/spa SPI1 system is present and conserved in all *Salmonella* serotypes tested (Ochman & Grosman,
1996), it is becoming more apparent that even closely related pathogens may have a different repertoire of secreted protein effectors (Hardt & Galán, 1997; Mirotl et al., 1999). It is tempting to speculate that this may play a role in the differences in the early pathogenic behaviour of different Salmonella serotypes. Further work with the host-specific S. gallinarum and broad host range salmonellae in the chicken will concentrate on studies of this matter. In addition, the application of the techniques described here will help investigation of pathological mechanisms of avian salmonellosis and other diseases at a molecular level in the chicken.

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