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)-1 β , 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 down-regulation of IL-2 production. For IL-1 β production, infection with *S. typhimurium* had little effect, whilst infection with *S. gallinarum* or *S. enteritidis* caused a reduction in IL-1 β 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 patho-

gen 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

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Abbreviations: CKC, chick kidney cells; CM, conditioned medium; C_t, threshold cycle value; DMEM, Dulbecco's modified Eagle's medium; FAM, 5-carboxyfluorescein; FBS, foetal bovine serum; IFN, interferon; IL, interleukin; PMN, polymorphonuclear cell; P/S, penicillin and streptomycin; rm, recombinant murine; TAMRA, *N,N,N,N'*-tetramethyl-6-carboxyrhodamine.

The GenBank accession numbers for the sequences reported in this paper are AI982185 for chicken IL-6 cDNA and AJ250838 for the partial chicken IL-6 genomic sequence, respectively.

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 30 000 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 the faecal-oral route and to contamination of meat 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 (Digby & Lowenthal, 1995; Sundick & Gill-Dixon, 1997), as have the pro-inflammatory cytokines IL-1 β (Weining *et al.*, 1998) and IL-6 (accession no. A1982185). 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-1 β (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-1 β , 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

RNA target		Probe/primer sequence (5'–3')	Exon boundary	Accession no.*
28S	Probe	(VIC)-AGGACCGCTACGGACCTCCACCA-(TAMRA)		X59733
	F	GGCGAAGCCAGAGGAAACT		
	R	GACGACCGATTTGCACGTC		
IFN- γ	Probe	(FAM)-TGGCCAAGCTCCCGATGAACGA-(TAMRA)	3/4	Y07922
	F	GTGAAGAAGGTGAAAGATATCATGGA		
	R	GCTTTGCGCTGGATTCTCA		
IL-1 β	Probe	(FAM)-CCACACTGCAGCTGGAGGAAGCC-(TAMRA)	5/6	AJ245728
	F	GCTCTACATGTCGTGTGTGATGAG		
	R	TGTCGATGTCCCGCATGA		
IL-2	Probe	(FAM)-ACTGAGACCCAGGAGTGCACCCAGC-(TAMRA)	2/3	AJ009800
	F	TTGGAAAATATCAAGAACAAGATTTCATC		
	R	TCCCAGGTAACACTGCAGAGTTT		
IL-6	Probe	(FAM)-AGGAGAAATGCCTGACGAAGCTCTCCA-(TAMRA)	3/4	AJ250838
	F	GCTCGCCGGCTTCGA		
	R	GGTAGGTCTGAAAGGCGAACAG		

* 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 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 × 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) tryptose 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 × 10⁵ 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% tryptose phosphate broth and P/S. 7TD1 (an IL-6-dependent mouse plasmacytoma cell line) cells (van Snick *et al.*, 1986) were seeded at 2 × 10⁵ 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 × 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 *N,N,N,N'*-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 × EZ RT-PCR buffer [including 60 mM 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-

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

Target	ΔR_n^* significance threshold	Log dilutions	C_t values†	$R^2‡$	Slope
28S	0.05	10^{-1} – 10^{-5}	8–22	0.9719	3.138
IFN- γ	0.01	10^{-1} – 10^{-5}	16–31	0.9933	3.503
IL-1 β	0.02	10^{-1} – 10^{-5}	22–35	0.9903	3.124
IL-2	0.01	10^{-1} – 10^{-4}	24–34	0.9980	3.122
IL-6	0.02	10^{-1} – 10^{-4}	23–36	0.9832	4.333

* ΔR_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 ΔR_n .

‡ R^2 = coefficient of regression.

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 (ΔR_n) passes a significance threshold. In this work, the threshold values of ΔR_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 log₁₀ dilution series. Each experiment was performed in triplicate, with replicates performed on different days. Regression analysis of the mean values of six replicate RT-PCRs for the log₁₀ 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 5×10^4 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, naphthylethylenediamine dihydrochloride (Sigma), 1% (w/v) sulphanilamide (Sigma) in 2.5% H_3PO_4 (BDH)] and incubating for 10 min at room temperature. The absorbance of the reaction product was

measured in a Titretek 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 $\mu\text{M NO}_2^-$ (5×10^4 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 1E12, an anti-chicken IFN- γ neutralizing mAb (Lambrecht *et al.*, 2000), or the LPS inhibitor polymyxin B.

7TD1 bioassay. The 7TD1 bioassay was based on that described by van Snick *et al.* (1986). Cells were plated at 2×10^3 cells per well in 96-well plates and then cultured at 37 °C, 5% CO_2 for 72 h in the presence of a variety of stimuli. The presence of IL-6-like activity was indicated by proliferation, as measured by the incorporation of [^3H]thymidine (0.5 μCi [^3H]thymidine per well) during the final 6 h incubation. IL-6-like factor activity of the test samples was determined using log-linear regression analysis against rmIL-6 standards. The dynamic range of the rmIL-6 standard was determined from the linear part of the curve and plotted on the same dilution scale as the samples. This range was then used to regress the curve of the test sample back to the level of half maximal proliferation seen in the rmIL-6 standard curve. The dilution of test sample required to bring about this level of proliferation was referred to as one dilution unit. Taking into account the dilution factor, the number of dilution units in each test sample was determined. The number of dilution units in samples run in separate assays was compared by regressing each sample using the standard curve run within the same assay.

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

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

Values are means $\pm$ 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} .

Bacterial strain	Log ₁₀ c.f.u. invading bacteria (ml CKC) $^{-1}$ *
<i>S. typhimurium</i> F98	4.81 ± 0.08^a
<i>S. gallinarum</i> 9	3.50 ± 0.18^c
<i>S. dublin</i> 2229	3.40 ± 0.24^c
<i>S. enteritidis</i> 125589	3.31 ± 0.15^c
<i>E. coli</i> K-12	1.70 ± 0.03^b

* Where mean values for the groups differ significantly ($P < 0.05$), the values carry different superscript letters.

Fig. 1. (a) Quantification of cytokine mRNA in RNA extracted from uninfected CKC, or from CKC infected with *Salmonella* spp. or *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 as 1. *** = $P < 0.01$, compared to levels in *E. coli*-infected CKC. S.t., *S. typhimurium*; E.c., *E. coli*; S.g., *S. gallinarum*; S.d., *S. dublin*; S.e., *S. enteritidis*. Error bars show SEM for triplicate samples from three separate experiments.

caused little damage to the cell monolayer, with any damage being most pronounced following invasion with *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)

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 ($10 \mu\text{g ml}^{-1}$; black bars). All assays were carried out in triplicate. Error bars show SEM. Abbreviations are as in Fig. 1.

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.

Bacterial strain	Dilution units $\pm$ SEM of IL-6-like activity*
<i>S. typhimurium</i> F98	77.18 ± 3.32^a
<i>S. gallinarum</i> 9	9.02 ± 0.48^b
<i>S. dublin</i> 2229	77.37 ± 3.90^a
<i>S. enteritidis</i> 125589	91.14 ± 4.40^a
<i>E. coli</i> K-12	20.82 ± 0.65^c
Uninfected	17.31 ± 0.53^d

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

shows the effect of standardizing cytokine-specific C_t 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-1 β mRNA expression, infection of CKC with *E. coli* caused a reduction in IL-1 β 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-1 β 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-1 β 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 $\mu\text{M NO}_2^- (5 \times 10^4 \text{ cells})^{-1} (48 \text{ h})^{-1}$, 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 NO_2^- produced titred out with increasing dilution of the CM down to background levels. CM from CKC infected with *S. typhimurium* contained most NO_2^- , followed by that from *S. enteritidis*-infected CKC, with that from *S. gallinarum*-infected CKC containing least NO_2^- . CM from CKC infected with *S. dublin* or *E. coli* gave intermediate levels of NO_2^- .

Treatment with the anti-IFN- γ neutralizing mAb, 1E12 (Lambrecht *et al.*, 2000) removed some, but by no means all, of the NO_2^- -inducing activity from the CM (Fig. 2), and neutralized most of the NO_2^- -inducing activity of the recombinant chicken IFN- γ . Treatment with polymyxin B, an inhibitor of LPS, removed all significant NO_2^- -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_2^- -inducing activity of the recombinant chicken IFN- γ .

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 ($P < 0.01$), than CM from CKC infected with *E. coli*. CM from *S. gallinarum*-infected CKC contained least IL-6-like activity, significantly less ($P < 0.01$) 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- γ and IL-2, and the pro-inflammatory cytokines IL-1 β 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 β . *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 β 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- γ -like and IL-6-like activity in the chicken are available, allowing quantification of IFN- γ - 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- γ mRNA expression in infected CKC compared with uninfected controls. However, the bioassay showed significant IFN- γ -like activity in the CM from all infected CKC. This activity was not removed by pre-treating the CM with an anti-chicken

IFN- γ 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_2^-), especially as the IFN- γ -like activity in the CM of uninfected CKC was very low, as were the IFN- γ 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* invasion (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 septicaemia 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-1 β during *Salmonella* invasion. Little is known regarding the production of IL-1 β by non-phagocytic cells invaded by *Salmonella*. IL-1 β 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-1 β 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-1 β is also produced by murine macrophages and dendritic cells when invaded by *Salmonella in vitro* (Marriott *et al.*, 1999). The production of IL-1 β 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-1 β 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 & Groisman,

1996)], it is becoming more apparent that even closely related pathogens may have a different repertoire of secreted protein effectors (Hardt & Galán, 1997; Miold *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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REFERENCES

- Baird, G. D., Manning, E. J. & Jones, P. W. (1985). Evidence for related virulence sequences in plasmids of *Salmonella dublin* and *Salmonella typhimurium*. *J Gen Microbiol* **131**, 1815–1823.
- Barrow, P. A. (1996). Immunity to *Salmonella* and other bacteria. In *Poultry Immunology: Poultry Science Symposium Series*, vol. 24, pp. 243–263. Edited by T. F. Davison, T. R. Morris and L. N. Payne. Abingdon, UK: Carfax Publishing.
- Barrow, P. A. & Lovell, M. A. (1989). Invasion of Vero cells by *Salmonella* species. *J Med Microbiol* **28**, 59–67.
- Barrow, P. A. & Lovell, M. A. (1990). Experimental infection of egg-laying hens with *Salmonella enteritidis* phage type 4. *Avian Pathol* **20**, 335–348.
- Barrow, P. A., Huggins, M. B., Lovell, M. A. & Simpson, J. M. (1987). Observations on the pathogenesis of experimental *Salmonella typhimurium* infection in chickens. *Res Vet Sci* **42**, 194–199.
- Barrow, P. A., Huggins, M. B. & Lovell, M. A. (1994). Host specificity of *Salmonella* infection in chickens and mice is expressed *in vivo* primarily at the level of the reticuloendothelial system. *Infect Immun* **62**, 4602–4610.
- Beug, H., von Kirchbach, A., Doderlein, G., Conscience, J.-F. & Graf, T. (1979). Chicken hematopoietic cells transformed by seven strains of defective avian leukemia viruses display three distinct phenotypes of differentiation. *Cell* **18**, 375–390.
- Darwin, K. H. & Miller, V. L. (1999). Molecular basis of the interaction of *Salmonella* with the intestinal mucosa. *Clin Microbiol Rev* **12**, 405–428.
- Digby, M. R. & Lowenthal, J. W. (1995). Cloning and expression of the chicken interferon- γ gene. *J Interferon Cytokine Res* **15**, 939–945.
- Ding, A. H., Nathan, C. F. & Stuehr, D. J. (1988). Release of reactive nitrogen intermediates from mouse peritoneal macrophages: comparison of activating cytokines and evidence for independent production. *J Immunol* **141**, 2407–2412.
- Eckman, L., Kagnoff, M. & Fierer, J. (1993). Epithelial cells secrete the chemokine interleukin-8 in response to bacterial entry. *Infect Immun* **61**, 4569–4574.
- Galán, J. E. & Sansonetti, P. J. (1996). Molecular and cellular bases of *Salmonella* and *Shigella* interactions with hosts. In *Escherichia coli and Salmonella: Cellular and Molecular Biology*, pp. 2757–2773. Edited by F. C. Neidhardt and others. Washington DC: American Society for Microbiology.
- Hardt, W.-D. & Galán, J. E. (1997). A secreted *Salmonella* protein with homology to an avirulence determinant of plant-pathogenic bacteria. *Proc Natl Acad Sci USA* **94**, 9887–9892.
- Henderson, S. C., Bounous, D. I. & Lee, M. D. (1999). Early events in the pathogenesis of avian salmonellosis. *Infect Immun* **67**, 3580–3586.
- Hirana, T. (1994). Interleukin-6. In *The Cytokine Handbook*, 2nd edn, pp. 145–168. Edited by A. Thomson. London: Academic Press.
- Humphrey, T. J. (1999). Contamination of meat and eggs with *Salmonella enterica* serotype Enteritidis. In *Salmonella enterica Serotype Enteritidis in Humans and Animals*, pp. 183–192. Edited by A. M. Saeed. Ames, IA: Iowa State University Press.
- Kaiser, P. & Mariani, P. (1999). Promoter sequence, exon:intron structure, and synteny of genetic location show that a chicken cytokine with T-cell proliferative activity is IL2 and not IL15. *Immunogenetics* **49**, 26–35.
- Kaiser, P., Wain, H. M. & Rothwell, L. (1998). Structure of the chicken interferon- γ gene, and comparison to mammalian homologues. *Gene* **207**, 25–32.
- Kogut, M. H., McGruder, E. D., Hargis, B. M., Corrier, D. E. & DeLoach, J. R. (1994a). Dynamics of avian inflammatory response to *Salmonella*-immune lymphokines: changes in avian blood leukocyte populations. *Inflammation* **18**, 373–388.
- Kogut, M. H., McGruder, E. D., Hargis, B. M., Corrier, D. E. & DeLoach, J. R. (1994b). Characterization of the pattern of inflammatory cell influx in chicks following the intraperitoneal administration of live *Salmonella enteritidis* and *Salmonella enteritidis*-immune lymphokines. *Poult Sci* **74**, 8–17.
- Kogut, M. H., Tellez, G. I., McGruder, E. D., Hargis, B. M., Williams, J. D., Corrier, D. E. & DeLoach, J. D. (1994c). Heterophils are decisive components in early responses of chickens to *Salmonella enteritidis* infections. *Microb Pathog* **16**, 141–151.
- Kogut, M. H., McGruder, E. D., Hargis, B. M., Corrier, D. E. & DeLoach, J. R. (1995). *In vivo* activation of heterophil function in chickens following injection with *Salmonella enteritidis*-immune lymphokines. *J Leukoc Biol* **57**, 56–62.
- Lambrecht, B., Gonze, M., Meulemans, G. & van den Berg, T. P. (2000). Production of antibodies against chicken interferon-gamma: demonstration of neutralizing activity and development of a quantitative ELISA. *Vet Immunol Immunopathol* **74**, 137–144.
- Lowenthal, J. W., Digby, M. R. & York, J. J. (1995). Production of interferon- γ by chicken T cells. *J Interferon Cytokine Res* **15**, 933–938.
- Lynagh, G. R. (1998). *The role of IL-6 in the immune response to coccidia*. PhD thesis, University of Bristol.
- McCormick, B. A., Miller, S. I., Carnes, D. & Madara, J. L. (1995). Transepithelial signalling to neutrophils by salmonellae: a novel virulence mechanism for gastroenteritis. *Infect Immun* **63**, 2302–2309.
- McCormick, B. A., Parkos, C. A., Colgan, S. P., Carnes, D. K. & Madara, J. L. (1998). Apical secretion of a pathogen-elicited epithelial chemoattractant activity in response to surface colonization of intestinal epithelia by *Salmonella typhimurium*. *J Immunol* **160**, 455–466.
- Marriott, I., Hammond, T. G., Thomas, E. K. & Bost, K. L. (1999). *Salmonella* efficiently enter and survive within cultured CD11c⁺ dendritic cells initiating cytokine expression. *Eur J Immunol* **29**, 1107–1115.

- Mirolid, S., Rabach, W., Rohde, M., Stender, S., Tschäpe, H., Rüssmann, H., Igwe, E. & Hardt, W.-D. (1999). Isolation of a temperate bacteriophage encoding the type III effector protein SopE from an epidemic *Salmonella typhimurium* strain. *Proc Natl Acad Sci USA* **96**, 9845–9850.
- Moody, A., Sellers, S. & Bumstead, N. (2000). Measuring infectious bursal disease virus RNA in blood by multiplex real-time quantitative RT-PCR. *J Virol Methods* **85**, 55–64.
- Nagaraja, K. V., Pomeroy, B. S. & Williams, J. E. (1991). Paratyphoid infections. In *Diseases of Poultry*, 9th edn, pp. 99–130. Edited by B. W. Calnek. Ames, IA: University of Iowa Press.
- Nakamura, K., Mitarai, Y., Yoshioka, M., Koizumi, N., Shibahara, T. & Nakajima, Y. (1998). Serum levels of interleukin-6, alpha1-acid glycoprotein, and corticosterone in two-week-old chickens inoculated with *Escherichia coli* lipopolysaccharides. *Poult Sci* **77**, 908–911.
- Ochman, O. & Groisman, E. A. (1996). Distribution of pathogenicity islands in *Salmonella* spp. *Infect Immun* **64**, 5410–5412.
- Pomeroy, B. S. & Nagaraja, K. V. (1991). Fowl typhoid. In *Diseases of Poultry*, 9th edn, pp. 87–98. Edited by B. W. Calnek. Ames, IA: University of Iowa Press.
- Smith, H. W. (1955). Observations on experimental fowl typhoid. *J Comp Pathol* **65**, 37–54.
- Smith, H. W. (1956). The susceptibility of different chicken breeds to *Salmonella gallinarum* infection. *Poult Sci* **35**, 701–705.
- Smith, H. W. (1978). Transmissible pathogenic characteristics of invasive strains of *Escherichia coli*. *J Am Vet Med Assoc* **172**, 601–607.
- Smith, H. W. & Tucker, J. F. (1975). The effect of antibiotic therapy on the excretion of *Salmonella typhimurium* by experimentally infected chickens. *J Hyg* **75**, 272–292.
- van Snick, J., Cayphas, S., Vink, A., Uyttenhove, C., Coulie, P. G., Rubira, M. R. & Simpson, R. J. (1986). Purification and NH₂-terminal amino acid sequence of a T-cell-derived lymphokine with growth factor activity for B-cell hybridomas. *Proc Natl Acad Sci USA* **83**, 9679–9683.
- Snoeyenbos, G. H. (1991). Pullorum disease. In *Diseases of Poultry*, 9th edn, pp. 73–86. Edited by B. W. Calnek. Ames, IA: University of Iowa Press.
- Sundick, R. S. & Gill-Dixon, C. (1997). A cloned chicken lymphokine homologous to both mammalian IL-2 and IL-15. *J Immunol* **159**, 720–725.
- Sung, Y.-J., Hotchkiss, J. H., Austic, R. E. & Dietert, R. R. (1991). L-Arginine dependent production of a reactive nitrogen intermediate by macrophages of a uricotelic species. *J Leukoc Biol* **50**, 49–56.
- Wall, P. G. & Ward, L. R. (1999). Epidemiology of *Salmonella enterica* serotype Enteritidis Phage Type 4 in England and Wales. In *Salmonella enterica Serotype Enteritidis in Humans and Animals*, pp. 19–26. Edited by A. M. Saeed. Ames, IA: Iowa State University Press.
- Weining, K. C., Sick, C., Kaspers, B. & Staeheli, P. (1998). A chicken homologue of mammalian interleukin-1 β : cDNA cloning and purification of active recombinant protein. *Eur J Biochem* **258**, 994–1000.
- Weinstein, D. L., O'Neil, B. L., Hone, D. M. & Metcalf, E. S. (1998). Differential early interactions between *Salmonella enterica* serotype Typhi and two other pathogenic *Salmonella* serotypes with intestinal epithelial cells. *Infect Immun* **66**, 2310–2318.

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