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Resistance to Salmonellosis in the Chicken Is Linked to *NRAMP1* and *TNC*

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Natural resistance to infection with *Salmonella typhimurium* in mice is controlled by two major loci, *Bcg* and *Lps*, located on mouse chromosomes 1 and 4, respectively. Both *Bcg* and *Lps* exert pleiotropic effects and contribute to cytostatic/cytocidal activities of the macrophage. *Bcg* encodes for a membrane phosphoglycoprotein designated *Nrampl* (natural resistance-associated macrophage protein 1), which belongs to an ancient family of membrane proteins. *Lps* has not been cloned yet, but its location on mouse chromosome 4 has been refined for positional cloning. As in mice, chicken inbred lines differ in their susceptibility to infection with *Salmonella typhimurium*. We have tested the candidacy of the chicken homologs of *Nrampl* and *Tnc* (a locus closely linked to *Lps*), in the differential resistance of chicken inbred lines to infection with *S. typhimurium*. We have first analyzed six inbred chicken lines of *Salmonella*-resistant or *Salmonella*-susceptible phenotypes for the presence of nucleotide sequence variations within the coding portion of *NRAMP1*. We have identified 11 sequence variations within *NRAMP1* in the chicken inbred lines tested: 10 of these represented either silent mutations or conservative changes. However, one G → A substitution at nucleotide 696 resulted in the nonconservative replacement of Arg²²³ to Gln²²³ within the predicted TM5-6 region. This allelic variant was specific to the susceptible line C and not observed in any of the resistant strains. To investigate the effect of *NRAMP1* and *TNC* on resistance to infection with *S. typhimurium*, 425 (W1 × C)_{F1} × C chicken progeny were examined during a period of 15 days postinfection. Together, *NRAMP1* and *TNC* explain 33% of the early differential resistance to infection with *S. typhimurium* of parental lines C and W1. Our data established that resistance to infection with *S. typhimurium* in chickens is inherited as a complex trait and that comparative mapping has proven to be useful to identify *Salmonella*-resistance genes in the chicken.

Numerous studies in humans and other animal species have suggested a strong effect of genetic factors on the ability of the host to respond to invasion by infectious pathogens (Malo and Skamene 1994; Hill 1996). Resistance to infectious diseases is usually a complex genetic trait and recent data suggest that many resistance/susceptibility genes affect more than one infectious disease (Vidal et al. 1995). In industrialized countries, nontyphoidal *Salmonella* spp. from contaminated poultry products is the most commonly reported pathogen causing food-borne disease in humans. The determination of the role of genes involved in host resistance to salmonellosis in the chicken may have an impact on future efforts toward the control of salmonellosis in chickens and subsequently the prevention of human salmonellosis.

Early exposure to invasive serotypes such as *Sal-*

monella typhimurium is associated with subsequent development of clinical disease in young chickens, leading to high mortality and persistence of infection in the survivors. As the chicks mature, their resistance to infection with *Salmonella* increases; however, most infected adult birds excrete *Salmonellae* for a variable period of time, and some become healthy carriers of the microorganism (Gast and Beard 1989). Many factors, including age, *Salmonella* strain, inoculation dose, route of infection, and environmental stress, affect the susceptibility of chickens to *Salmonella* infection. The host genetic background also appears to play a crucial role in resistance and susceptibility of chickens to infection with *Salmonella* (Bumstead and Barrow 1988). Genetically controlled variation in the host response to infection with different serotypes of *Salmonellae* has been reported among different lines of chicken (Pevzner et al. 1981; Bumstead and Barrow 1988, 1993; Guillot et al. 1995; Protais et al. 1996). Large differences in mortality after oral or intramuscular

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challenge with *Salmonella typhimurium*, *S. gallinarum*, *S. pullorum*, and *S. enteritidis* have been reported in six different inbred chicken lines: Lines 6₁, W1, and N were resistant to all four serotypes, whereas line 15I, 7₂ and C were relatively susceptible (Bumstead and Barrow 1993). Susceptible chickens appeared to have unrestricted bacterial growth in their reticuloendothelial system (RES) early after infection, suggesting a function of the phagocytic cells of the spleen and liver in the expression of the susceptibility phenotype. Crosses between *Salmonella*-resistant and *Salmonella*-susceptible lines indicated that resistance to infection is fully dominant, not sex-linked or associated with the major histocompatibility complex (MHC) (Bumstead and Barrow 1988).

Variation in susceptibility to salmonellosis has long been known to occur in laboratory strains of mice (Mastroeni et al. 1994). When mice of different inbred strains are inoculated with *S. typhimurium*, they show a dose-dependent susceptibility: the A/J strain is resistant compared with the BALB/cJ and C3H/HeJ strains, which appear very susceptible. DBA/2J and CBA/N mice have an intermediate phenotype (Mastroeni et al. 1994). Crosses between resistant and susceptible strains led to the identification of several loci involved in natural resistance and acquired immunity to *Salmonella* infection. Two of these loci, *Nramp1* and *Lps*, are involved in the early nonimmune phase of the host response. The *Nramp1* gene is involved in the differential resistance/susceptibility of inbred strains of mice to infection with taxonomically and antigenically unrelated intracellular pathogens, including several *Mycobacterium* species, *Leishmania donovani* and *S. typhimurium* (Vidal et al. 1993, 1995). The major effect of the *Nramp1* gene is the modulation of the growth rate of these pathogens in cells of the RES of the mouse early during infection through an unknown mechanism. Nramp1 is an integral membrane phosphoglycoprotein of 90,000–100,000 daltons, which shares structural characteristics with ion channels and transporters (Cellier et al. 1996; Vidal et al. 1996). Susceptibility to infection in inbred laboratory mice is caused by a null allele associated with a single nonconservative glycine-to-aspartic acid substitution at position 169 within transmembrane domain 4 of *Nramp1* (Malo et al. 1994; Vidal et al. 1996). Nramp1 is expressed exclusively in professional phagocytes and modulates the cytostatic/cytocidal activity of these cells through an unknown mechanism (Vidal et al. 1993; Cellier et al. 1994). Homologs of *Nramp* have been identified in several phylogenically distant organisms, in-

cluding mammals, birds, insects, plants, yeast, and bacteria (Cellier et al. 1995; Hu et al. 1995). The chicken NRAMP1 sequence is 68% identical to the mouse and human sequences (Hu et al. 1996). The overall structure and particularly the position and sequences of the transmembrane (TM) domains and the consensus transport motif are highly conserved (Hu et al. 1996). Several promoter consensus elements, such as the myeloid-specific PU.1/Spi-1, γ -interferon (IFN- γ)-responsive elements and binding sites for NF-IL6 and NF- κ B are present in chicken, mouse, and human promoters (Hu et al. 1996; Cellier et al. 1997). In the chicken, NRAMP1 is also expressed in RES (spleen and liver). The high degree of sequence and structure conservation between chicken and mouse NRAMP1/Nramp1, the presence of similar regulatory elements within the promoter regions of the two homologous genes, and similar tissue expression support the concept that the NRAMP1 proteins have similar roles in vivo in both mice and birds.

In mice, the *Lps* mutation controls the responsiveness to lipopolysaccharide (LPS). The genetic basis of the host response to LPS was initially defined with the discovery of the C3H/HeJ mutant mouse in the late 1960s (Sultzter 1968). As a result of a spontaneous mutation, the C3H/HeJ mouse strain exhibits a profound state of hyporesponsiveness to the immunostimulatory and pathophysiological effects of the lipid A component of LPS (Watson and Riblet 1974) and is highly susceptible to *S. typhimurium* (O'Brien et al. 1980; Qureshi et al. 1996). The death of C3H/HeJ mice following infection with *S. typhimurium* appears to result from uncontrolled multiplication of the bacteria in the RES of these animals and reduced recruitment of inflammatory cells into the RES (O'Brien et al. 1980). The defective response of C3H/HeJ to LPS is controlled by a single codominant gene designated *Lps*, which has two alleles in inbred mouse strains: the endotoxin low responder, *Lps^d* (LPS defective) and the endotoxin responder, *Lpsⁿ* (normal) alleles. Mice of the C3H/HeJ inbred strain are homozygous for the *Lps^d* allele, which renders them low responders to several bacterial LPS. *Lps* is located on mouse chromosome 4 within a 1.1-cM interval, flanked proximally by three known genes, among which is the gene tenascin C (*Tnc*), and distally by two microsatellite markers (Qureshi et al. 1996). The phenotypic expression of the *Lps* gene is pleiotropic and affects the response of several cell types, including macrophages, B cells, T cells, and fibroblasts; however, the macrophage is central in the clinical and cellular manifestations mediated by LPS (Vogel 1992). In macrophages, *Lps*

affects LPS-induced cytotoxicity and production of immunoregulatory factors such as interleukin-1 (IL-1), IL-6, colony-stimulating factor (CSF), prostaglandin E₂ (PGE₂) factor, tumor necrosis factor α (TNF α), etc. (Vogel 1992).

To investigate the possible roles of *NRAMP1* and the chromosomal region surrounding *LPS* in the differential resistance/susceptibility of chickens to infection with *S. typhimurium*, we have (1) determined *NRAMP1* nucleotide sequences from chickens that are either resistant or susceptible to infection, (2) followed the segregation of *NRAMP1* alleles in a panel of 425 (W1 \times C)F₁ \times C backcross chicken progeny generated from resistant W1 and susceptible C chickens, and (3) examined the association of alleles at *TNC* (which is closely linked to *Lps* in the mouse genome) with the differential resistance or susceptibility of chickens to infection with *S. typhimurium* in the same backcross panel.

RESULTS

NRAMP1 Sequence Variation in Inbred Chicken Lines

The chicken lines used in these experiments were derived from different ancestral stocks: Lines 6₁, 7₂, N, and 15I were developed at the Regional Poultry Research Laboratory at East Lansing, MI, established in Houghton in 1972 (lines 6₁, 7₂, and 15I) or 1982 (line N) and transferred to Compton in 1992. These inbred lines were initially selected for high and low incidence of visceral or neural leukosis. Line C was initially developed in 1938 at the Northern Poultry Breeding Station (Reaseheath, Cheshire, UK). Line W1 was originally obtained from Dr. J. Ivanyi (Wellcome Laboratories, Beckenham, UK).

Resistance or susceptibility to lethal infection with different serotypes of *Salmonella* was previously established by us in chicken lines N, 6₁, W1, 7₂, 15I, and C (Bumstead and Barrow 1988, 1993). The LD₅₀ of the different chicken lines following intramuscular challenge was determined: lines N, 6₁, and W1 were classified as resistant to infection with *S. typhimurium*, whereas lines 7₂, 15I, and C were very susceptible (LD₅₀ values varied by at least 100-fold between resistant and susceptible lines). Lines resistant to *S. typhimurium* were also resistant to *S. gallinarum*, and lines susceptible to *S. typhimurium* were also more susceptible to the other *Salmonella* serotype.

To investigate the possible role of *NRAMP1* in the differential resistance/susceptibility of chickens to infection with *S. typhimurium*, we first analyzed *NRAMP1* mRNA levels in the spleen of *Salmonella*-

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resistant (lines N, 6₁, and W1) and *Salmonella*-susceptible (7₂, 15I, and C) chicken lines. This analysis revealed similar levels of *NRAMP1* gene expression in resistant and susceptible chickens (Fig. 1). Therefore, we analyzed *NRAMP1* transcripts from lines N, 6₁, W1, 7₂, 15I, and C for the presence of sequence variations within the coding portion of *NRAMP1*. *NRAMP1* cDNA was obtained by RT-PCR using total spleen RNA, and each sequence was verified in three independent clones derived from the same cDNA. All PCR-amplified cDNA fragments from N, 6₁, W1, 7₂, 15I, and C cDNA samples had sizes expected from the original cDNA sequence (Hu et al. 1996). Nucleotide sequence analyses of the coding portion of *NRAMP1* in lines N, 6₁, W1, 7₂, 15I, and C revealed 11 sequence variants (Table 1). Among all of the coding sequence variants detected in these six lines, 8 of 11 were silent mutations (ACC \rightarrow ACT, Thr⁶⁷, TM1; ACT \rightarrow ACC, Thr²⁰⁹, TM5; TCA \rightarrow TCC, Ser²⁶⁷, TM6; CTC \rightarrow CTG, Leu²⁹⁷, TM7; TTC \rightarrow TTT, Phe³¹¹, TM7-8 interval; GGC \rightarrow GGT, Gly³¹², TM7-8 interval; ATT \rightarrow ATC, Ile³⁵⁶, TM8; and AGC \rightarrow AGT, Ser³⁵⁹, consensus transport sequence motif). Three nonsilent mutations were identified at nucleotide positions 191 (Thr⁵⁵ \rightarrow Ala⁵⁵), 696 (Arg²²³ \rightarrow Gln²²³), and 946 (Val³⁰⁷ \rightarrow Ile³⁰⁷). The first conservative replacement (Thr⁵⁵ \rightarrow Ala⁵⁵) is located in the amino-terminal region of the NRAMP1 protein, a region that is heterogeneous in length and very variable in sequence among Nramp-related polypeptides; the second

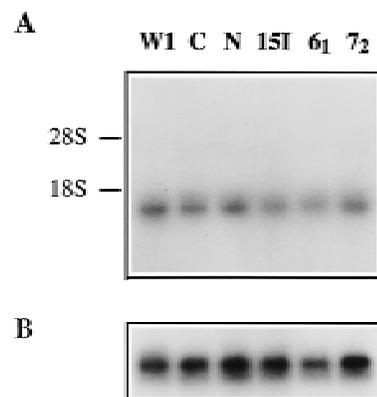


Figure 1 Northern blot analysis of *NRAMP1* expression in inbred chicken lines resistant (W1, N, 6₁) and susceptible (C, 15I, 7₂) to infection with *S. typhimurium*. The blot contained 10 μ g of spleen total RNA. The filters were hybridized with a full-length chicken *NRAMP1* cDNA clone (A) and with a partial chicken *Actin* cDNA probe (B) as a control. The positions of the 28S and 18S rRNA subunits are shown (left).

Table 1. Distribution of Nucleotide Sequence Variation in the Coding Portion of *NRAMP1* in *Salmonella*-Resistant and *Salmonella*-Susceptible Chicken Lines

Line ^a	Thr/Ala ⁵⁵	Thr ⁶⁷	Thr ²⁰⁹	Arg/Gln ²²³	Ser ²⁶⁷	Leu ²⁹⁷	Val/Ile ³⁰⁷	Phe ³¹¹	Gly ³¹²	Ile ³⁵⁶	Ser ³⁷⁹
N	<u>GCG</u>	ACC	ACT	CGG	TCA	CTC	<u>ATC</u>	TTC	GGC	<u>ATT</u>	<u>AGC</u>
6 ₁	<u>GCG</u>	ACC	ACT	CGG	TCA	CTC	<u>ATC</u>	TTC	GGC	<u>ATT</u>	<u>AGC</u>
W1	<u>ACG</u>	<u>ACT</u>	ACT	CGG	TCA	CTG	<u>GTC</u>	TTT	GGC	<u>ATC</u>	<u>AGT</u>
7 ₂	ACG	<u>ACT</u>	<u>ACC</u>	CGG	TCA	CTG	GTC	<u>TTT</u>	GGC	ATC	AGT
15I	ACG	ACC	<u>ACC</u>	CGG	TCA	CTG	GTC	<u>TTC</u>	GGT	ATC	AGT
C	ACG	ACC	ACT	<u>CAG</u>	<u>TCC</u>	GTC	GTC	TTC	GGC	ATC	AGT

^aChickens were classified *Salmonella*-resistant (lines N, 6₁, and W1) or *Salmonella*-susceptible (lines 7₂, 15I, and C) according to LD₅₀ values following inoculation with different serotypes of *Salmonella* (Bumstead and Barrow 1993).

conservative replacement (Val³⁰⁷ → Ile³⁰⁷) is located between predicted TM7 and TM8. A Val or Ile residue is present at the equivalent positions of mouse Nramp1 and Nramp2, human NRAMP1, rat Nramp1, *Drosophila* Mvl, rice OsNramp1, and yeast Smf1 and Smf2 (Cellier et al. 1995; Gruenheid et al. 1995). Resistant lines N and 6₁ presented a Thr residue at position 55 and a Val residue at position 307, whereas conservative replacements (Ala⁵⁵ and Ile³⁰⁷) at both positions were observed in the resistant line W1 and the three susceptible lines C, 15I, and 7₂. Taken together, these data suggest that the two conservative changes, Thr⁵⁵ → Ala⁵⁵ and Val³⁰⁷ → Ile³⁰⁷, are probably without functional consequences for the biochemical properties of NRAMP1 and are not associated with susceptibility to infection. The third nonsilent mutation involved a G → A transition at nucleotide position 696 that resulted in a nonconservative Arg → Gln substitution at position 223 within predicted TM5–6 interval. This mutation was specific to the susceptible C line and was not present in any of the resistant lines tested. Alignment of the deduced amino acid se-

quences of TM5 and adjacent regions of the NRAMP1 protein from different species is presented in Figure 2. TM5 is one of the most hydrophobic and best delineated segments (Cellier et al. 1995) and is highly conserved among species including mammals (Fig. 2), *Drosophila*, yeast, and plants (Cellier et al. 1995). In chicken, predicted TM5 is flanked by charged amino acids (Arg¹⁹⁸, Lys¹⁹⁹, and Glu²⁰¹ residues on the intracytoplasmic side and Arg²²³, Lys²³¹, and Glu²⁴⁵ on the predicted extracytoplasmic loop) that are highly conserved in all species analyzed. Arg²²³ is present in chicken, human, rat, and rabbit NRAMP1 and is replaced by another basic amino acid in mouse Nramp1 (His²²³), mouse Nramp2 (Lys²²³), rice OsNramp1 (Lys²²³), and yeast, Smf2 (Lys²²³) (Fig. 2; Cellier et al. 1995). These results suggest that TM5 and flanking charge residues may play an important functional role and that nonconservative substitution in codon 223 has not been tolerated during evolution to preserve function.

Five different *NRAMP1* haplotypes were observed in the chicken lines studied. We performed pairwise comparison of nucleotide differences among the six inbred chicken line haplotypes of *NRAMP1*. The smallest number of pairwise differences was observed between resistant lines 6₁ and N (100% allele similarity) and the susceptible 7₂ and resistant W1 lines (91% allele similarity). The haplotype shared by resistant lines N and 6₁ is very different from those exhibited by susceptible lines 7₂ and 15 that have been

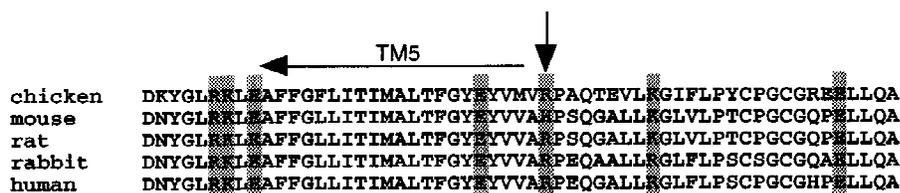


Figure 2. Alignment of the TM5 region of chicken NRAMP1 protein and related mammalian sequences. Shaded residues indicate conserved charged residues; the horizontal arrow indicates the position of predicted TM5 (Cellier et al. 1995); the vertical arrow indicates the position of the Arg²²³ → Gln²²³ mutation. Mouse Nramp1 (Genbank accession no. L13732), human NRAMP1 (Genbank accession no. L32185), chicken NRAMP1 (Genbank accession no. U40598), rat, and rabbit Nramp1 (D. Malo, unpubl.).

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derived from the same commercial flocks. Susceptible line C showed the largest number of nucleotide sequence differences from all other chicken lines.

NRAMP1 and *TNC* Are Associated with Susceptibility to Infection with *S. typhimurium* in Chickens

To examine further the possibility that *NRAMP1* is associated with resistance or susceptibility to infection with *S. typhimurium* in the chicken, we compared the degree of resistance to infection with *S.*

typhimurium in resistant line W1 (Arg²²³), susceptible line C (Gln²²³), and 425 (W1 × C)_{F1} × C segregating backcross progeny. Survival to infection was assessed in parental lines W1 and C and in the 425 backcross progeny after intramuscular challenge with 10³ CFU *S. typhimurium*, strain F98. Results from Figure 3 (experiment A) show that almost all W1 chickens survived infection, in agreement with previous data (Bumstead and Barrow 1988, 1993). In contrast, only 11% of chickens from line C survived >15 days postinfection. Mortality rate in line C occurred in two phases: a first phase (day 1–7)

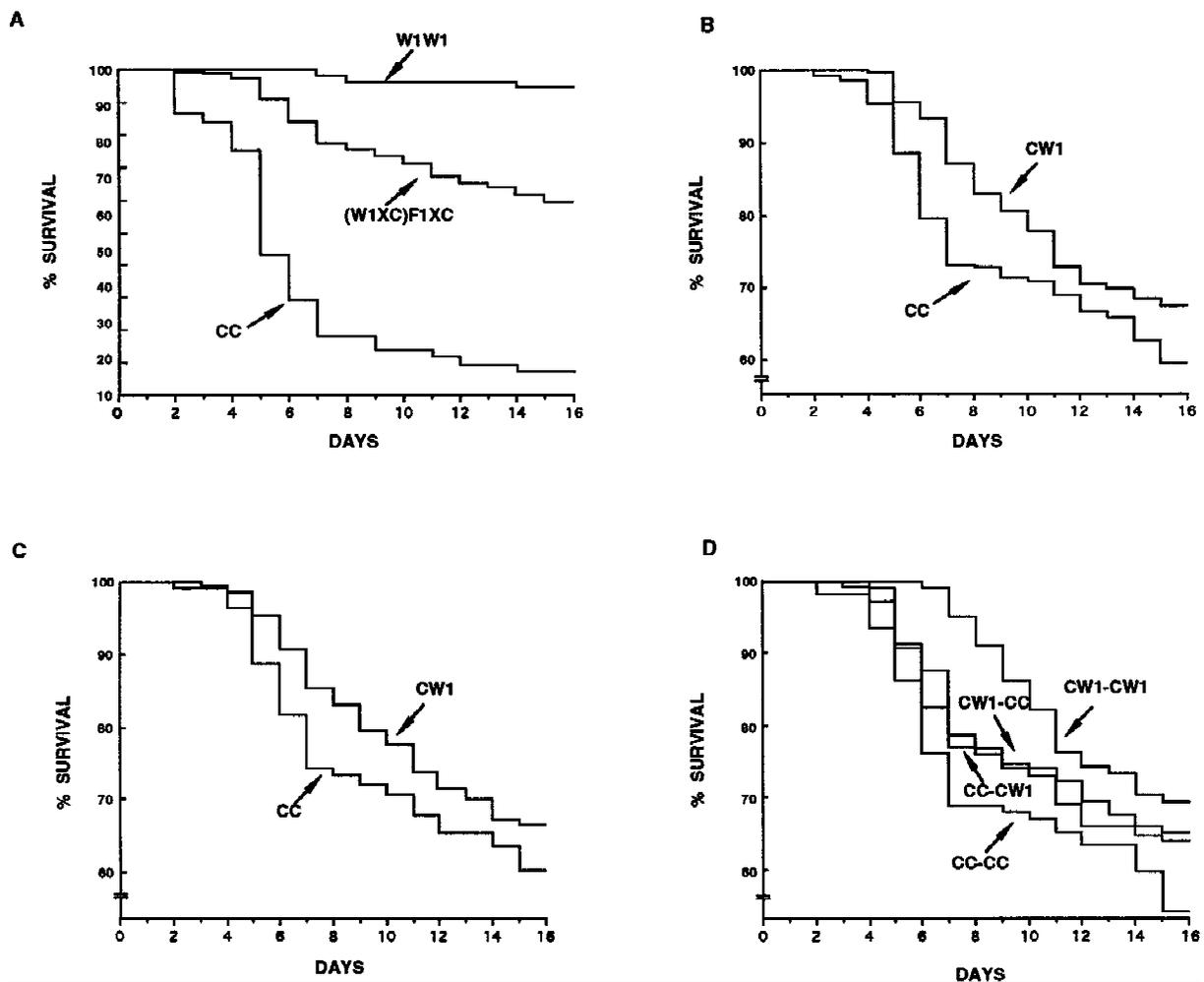


Figure 3 *S. typhimurium* infection in resistant line W1 ($n = 60$), susceptible line C ($n = 49$), and 425 (W1 × C)_{F1} × C backcross chicken progeny (A). Chickens were infected intramuscularly with 10³ CFU *S. typhimurium*, and survival to infection was monitored for a period of 15 days. The data are expressed as the proportion of animals surviving the infection at each time point and are shown as the percentage of survival. Survival curves are shown for (W1 × C)_{F1} × C progeny with genotype CW1 or CC at *NRAMP1* (B); genotype CW1 or CC at *TNC* (C). (D) Survival curves are presented for backcross chicken progeny that were grouped according to their genotype at *NRAMP1* and *TNC* jointly: 110 chickens were *NRAMP1* and *TNC* homozygous (CC–CC), 102 were heterozygous CW1 at both loci (CW1–CW1), 109 were homozygous at *NRAMP1* and heterozygous at *TNC* (CC–CW1), and 104 were heterozygous at *NRAMP1* and homozygous at *TNC* (CW1–CC).

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Table 2. Association of *NRAMP1* and *TNC* Alleles with Susceptibility to *S. typhimurium* Infection in Line C, Line W1, and in Informative (W1 × C)_{F1} × C Backcross Progeny

Chicken	N ₁	N ₇	N ₇ /N ₁	N ₁₅	N ₁₅ /N ₁	N ₁₅ /N ₇
Line C	46	10	0.22	5	0.11	0.50
Line W1	56	55	0.98	53	0.95	0.96
(W1 × C) _{F1} × C	425	339	0.80	270	0.64	0.80
<i>NRAMP1</i> C	219	160	0.73	131	0.60	0.82
<i>NRAMP1</i> W1	206	179	0.87	139	0.67	0.78
<i>TNC</i> C	214	159	0.74	128	0.60	0.81
<i>TNC</i> W1	211	180	0.85	142	0.67	0.79
CC–CC	110	77	0.70	61	0.55	0.79
CC–CW1	109	83	0.76	70	0.64	0.84
CW1–CC	104	82	0.79	67	0.64	0.82
CW1–CW1	102	97	0.95	72	0.71	0.74

(N₁) Number of birds in the experiment; (N₇) Number of birds alive after day 7; (N₁₅) Number of birds surviving the entire experiment. The ratios N₇/N₁ and N₁₅/N₁ are the proportion of birds alive after day 7 and day 15, respectively. The ratio N₁₅/N₇ is the proportion of birds alive after day 7 who survive to the end of the experiment.

where most C animals died from infection, and a second phase (day 8–15) where the mortality rate is much lower and similar to that observed in resistant line W1 (Table 2). (W1 × C)_{F1} progeny were as resistant as the W1 parent indicating full dominance of resistance to infection with *S. typhimurium* (data not shown). The overall mortality rate in the 425 (W1 × C)_{F1} × C backcross progeny was intermediate between those of resistant W1 and susceptible C lines (Table 2).

We then analyzed the genotype frequencies of *NRAMP1* in the backcross progeny using two different DNA polymorphisms: The first one was detected using the restriction enzyme *Pst*I and a genomic DNA probe, MCG2, corresponding to the 5' end of chicken *NRAMP1* (Fig. 4A). We also had the opportunity to type directly for the Arg²²³ → Gln²²³ mutation, as the G → A transition at nucleotide position 696 disrupts the *Eag*I restriction site present in *NRAMP1* cDNA of the resistant W1 line. The two DNA markers gave identical genotyping for each individual progeny. Survival curves for chickens harboring CC or CW1 genotype at *NRAMP1* are presented in Figure 3 (experiment B). As expected using this type of cross, approximately half of the progeny were homozygous CC at *NRAMP1* and the other half were heterozygous CW1.

Observation of the survival rate in the susceptible parental strain (C) led us to believe that the survival may not be constant over the entire duration of the experiment. Therefore, in addition to

testing for differences in survival over the entire 15-day period, we tested separately for two time periods, day 1–7, and day 8–15 (Table 3). The effect of *NRAMP1* on survival to infection was highly significant during the first time period ($P = 0.0004$; Table 3). The Cox proportional hazards model shows that the chickens carrying two susceptible alleles (CC homozygotes) at *NRAMP1* have a significantly higher risk of death than the ones carrying one resistant and one susceptible allele (CW1 heterozygous) (Table 3). The greatest difference in cumulative mortality rate was observed at day 7, at which time *NRAMP1* CC homozygote chickens had a mortality rate two times higher than *NRAMP1* CW1 heterozygous chickens (27% vs. 13%) (Table 2). The data show that *NRAMP1* explains part of the genetic variation observed between parental chicken lines W1 and C with respect to resistance to *Salmonella* infection.

We also tested another chromosomal region carrying a *Salmonella*-resistance locus in the mouse, *Lps*, for additional contribution to genetic resistance of the chicken W1 line. Because the *Lps* gene has not been cloned yet, we have used a cDNA probe, tenascin C (*TNC*), that has been shown by high-resolution linkage mapping to be located 0.7 cM proximal to the *Lps* gene in the mouse genome (Qureshi et al. 1996). Under stringent hybridization and washing conditions, probe *TNC* hybridizes to only one *Stu*I-digested genomic fragment that is 10.5 kb long in line W1 and 18.0 kb long in line C

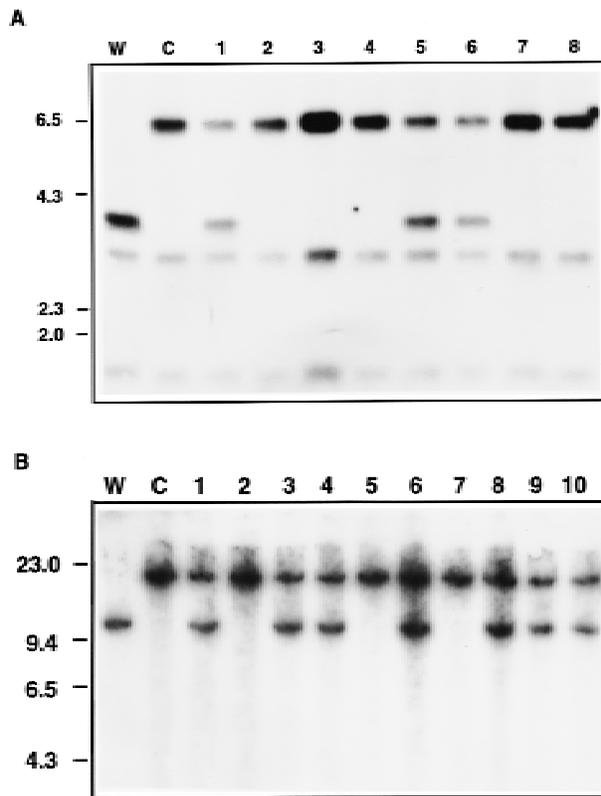


Figure 4 Example of Southern blot analysis of genomic DNA from the $(W1 \times C)F_1 \times C$ progeny. DNA samples from W1, C, and backcross progeny were digested with *PstI* and hybridized to full-length chicken *NRAMP1* cDNA (A) or with *Stul* and hybridized to a partial human *TNC* cDNA (B).

(Fig. 4B). We first compared the allele distribution pattern of the 425 backcross progeny for *NRAMP1* and *TNC* to determine whether the two genes mapped to the same subchromosomal region. In fact we observed a recombination fraction of 50% between *NRAMP1* and *TNC*, arguing against linkage (data not shown). We then analyzed the genotype frequencies of *TNC* in all backcross progeny with respect to survival to infection (Fig. 3C). Survival curves discriminating between homozygous CC and heterozygous CW1 at *TNC* resemble those observed with *NRAMP1*. In this case, also the major effect of the gene is seen early after infection and a smaller proportion of chicken progeny that were homozygous CC survived infection compared with the CW1 heterozygote ($P = 0.005$; Table 3).

Both *NRAMP1* and *TNC* have a highly significant impact on resistance to infection during the first 7 days, but neither gene has a significant impact on resistance to infection during the second

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time period (Table 3). To test the interaction between *NRAMP1* and *TNC*, we divided the 425 backcross progeny into four two-locus genotypes of *NRAMP1* and *TNC* (Table 2). All two-locus genotypes were approximately equal in numbers: 110 chickens were *NRAMP1* and *TNC* homozygous (CC-CC), 102 were heterozygous CW1 at both loci (CW1-CW1), 109 were homozygous at *NRAMP1* and heterozygous at *TNC* (CC-CW1), and 104 were heterozygous at *NRAMP1* and homozygous at *TNC* (CW1-CC). Survival curves are presented in Figure 3D. During the first week, postchallenge, groups of chickens with CC genotypes at *NRAMP1*, *TNC*, or both loci had comparable survival curves. However, the survival curve of CW1-CW1 genotype was shifted toward greater survival. This group presented the highest survival rate at day 7 (95% compared with 70% in the CC-CC group) whereas the survival rates from infection in the CC-CW and CW-CC groups were intermediate (Table 2). A model including interaction between *NRAMP1* and *TNC* was fit over the entire period but was not significant (data not shown). For the first time period, the model with the interaction term ($N + T + N \cdot T$) fits the data significantly better than the model without the interaction term (Likelihood-ratio test, $\chi^2 = 5.9$, $df = 1$, $P = 0.015$) (Table 3). The sign of the interaction coefficient is negative, indicating that the increase survival for an *NRAMP1* CW1 heterozygote compared to that for an *NRAMP1* CC homozygote is greater if the birds are also CW1 heterozygous at *TNC*. Similarly, the relative benefit in survival of being *TNC* heterozygous compared to being *TNC* homozygous is greater if the bird is also heterozygous at *NRAMP1*. From Table 2, we observe that the difference between the double heterozygotes and double homozygotes for *NRAMP1* and *TNC* in proportion of birds alive at day 7 is 0.25, which accounts for 33% of the difference between the W1 and C parental lines in the proportion of birds alive at that time.

DISCUSSION

S. typhimurium is a facultative intracellular pathogen of both phagocytes (macrophages and polymorphonuclear cells) and nonphagocytes, which causes in susceptible mice and young chickens a systemic disease of the RES resulting in overwhelming infection and death. In susceptible animals of both species, unrestricted growth of the bacteria is detected in spleen and liver, and tissue damage is secondary to an excessive inflammatory host response to the bacteria. Experimental infection of inbred strains of

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Table 3. Cox Proportional Hazard Models

Model ^a	<i>N + T</i>	<i>N + T</i>	<i>N + T + N · T</i>	<i>N + T</i>
Time period (day)	1–15	1–7	1–7	8–15
<i>NRAMP1</i> (<i>N</i>)				
coefficient	−0.155	−0.410	−0.535	0.126
S.E.	0.081	0.116	0.14	0.121
<i>P</i> value	0.057	0.0004	0.0001	0.30
<i>TNC</i> (<i>T</i>)				
coefficient	−0.144	−0.315	−0.461	0.073
S.E.	0.081	0.112	0.14	0.120
<i>P</i> value	0.074	0.005	0.001	0.54
Interaction (<i>N · T</i>)				
coefficient	N.A.	N.A.	−0.317	N.A.
S.E.			0.14	
<i>P</i> value			0.023	
Likelihood ratio statistic ^b	6.94	21.8	27.7	1.47
df	2	2	3	2
<i>P</i> value	0.031	2×10^{-5}	4×10^{-6}	0.479

^aIn each model, *N* represents the coefficient of the *NRAMP1* covariate, *T* represents the coefficient of the *TNC* covariate, *N · T* represents the coefficient of the interaction between *NRAMP1* and *TNC*.

^bThe likelihood ratio statistic is $2\log(L_1/L_0)$ where L_1 is the likelihood of the specified model and L_0 is the likelihood of the null model that includes no covariates.

mice with a sublethal inoculum of *S. typhimurium* has led to the identification of four distinct phases of infection (Mastroeni et al. 1994). The first phase occurs within 2 hr of infection and involves complement-mediated clearance of the organism from the blood by resident macrophages. A small fraction of the initial inoculum will survive in cells of the reticuloendothelial system (liver and spleen). Bacteria that survive within this safe site will then divide exponentially for the first week after infection (phase 2). In the mouse, there are two well-characterized genetic loci (*Nramp1* and *Lps*) that control the rate of exponential bacterial growth in reticuloendothelial organs during phase 2 of infection (O'Brein et al. 1980; Vidal et al. 1993). Phase 3 occurs 7 days after the initial inoculation and is characterized by the arrest of bacterial replication, resulting in a plateau. Finally in the late phase, humoral and cell-mediated immune responses mediate clearance of bacteria from the RES. Three other mouse loci, *nu* (nude), *xid* (X-linked immunodeficiency), and certain *H2* (MCH) haplotypes act during the late phase of infection through their effects on the acquired immune response (Mastroeni et al. 1994).

Study of experimental primary infection of inbred lines of chicken with different species of *Salmonellae* has shown that both innate and acquired immune responses are involved in the control of *Salmonellae* infection. Chickens are highly susceptible to infection with *Salmonella* during the first days post-hatching, after which they build up their resistance to infection (Gast and Beard 1989). During this early period of life, protection against *Salmonella* infection involves activated heterophils, the avian equivalents to mammalian neutrophils, which migrate rapidly to the inflammatory site where they phagocyte and destroy opsonized bacteria through an oxygen-independent mechanism (Kogut et al. 1995). Resistance of older chickens (4-week-old) to *Salmonella* infection involves both cellular and humoral immune functions as measured by B and T cell proliferation, antibody responses, and cytotoxic activity of NK cells (Lessard et al. 1995). We have used a candidate

gene approach toward the identification of host genetic loci involved in the differential resistance/susceptibility of inbred chicken lines to infection with *S. typhimurium*. We have tested the candidacy of two homologs of mouse resistance genes, *Nramp1* and *Lps*, in resistance of young chicks to infection with *S. typhimurium*. The chicken homolog of *Nramp1* has been cloned (Hu et al. 1996) and was tested directly. For *Lps*, we have used mapping information in the mouse to screen the homologous region in the chicken genome (comparative gene mapping) and to determine whether it is linked to resistance of chickens to infection with *Salmonella*.

Our first objective was to analyze *NRAMP1* mRNA transcripts from chickens that are either resistant or susceptible to infection with *S. typhimurium* to identify amino acid sequence variants that could be associated with the disease phenotype. Sequence analysis revealed the presence of 11 sequence variants in *NRAMP1* mRNA obtained from 3 *Salmonella*-resistant and 3 *Salmonella*-susceptible chicken lines. Almost all of these sequence variants (10 of 11) resulted in silent mutations or conservative changes that were detected both in resistant and susceptible chicken lines. Only one sequence

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variant corresponding to a G → A transition at position 696, resulting in a nonconservative substitution of a positively charged residue (Arg²²³) by a polar residue (Gln²²³), was specific to the susceptible line C. According to NRAMP consensus topology, Arg²²³ is located at the extracellular junction between predicted TM5 and TM5–6 interval. TM5 is one of the most hydrophobic and best delineated segments and is highly conserved between birds and mammals (18/20 amino acid residues are identical between the two species). Helical wheel projection of the putative TM5 identifies a biphasic helix that is composed of a nonpolar hydrophobic face and a polar face, enriched for charged/polar residues. The uncharged face is expected to be in contact with the lipid bilayer, whereas the polar face could contact other helices or participate in the formation of a permeation pathway (Cellier et al. 1995, 1996). Arginine side chains are amphipathic and can be located near the end of TM helices, with their charged moieties exposed to solvent (Reithmeier 1995). Arg²²³ → Gln²²³ could be expected to alter the physical properties of the proposed TM domain and subsequently affect the overall membrane-associated structure and/or membrane insertion of the protein in the susceptible chicken line. To obtain some evidence on the functional importance of this amino acid residue and the region surrounding it, we compared this chicken NRAMP1 domain to Nramp homologs of distantly related species. Arg²²³ has been conserved in protein sequences from human, rat, and rabbits, and a positively charged amino acid is present at equivalent position in mouse Nramp1 (His), mouse Nramp2 (Lys), rice OsNramp1 (Lys), yeast Smf2 (Lys), and *Caenorhabditis elegans* Ce2 (Lys). This high degree of conservation supports the notion that Arg²²³ → Gln²²³ may be the cause of the disease rather than a neutral polymorphism associated with the susceptible phenotype. A nonconservative mutation within mouse Nramp1 polypeptide having a functional importance has been reported by us previously: a Gly¹⁶⁹ → Asp¹⁶⁹ substitution, which introduces a charged residue into predicted lipid face of TM4, is present in all susceptible inbred mouse strains that have been tested (Vidal et al. 1993; Malo et al. 1994). Nramp1 polypeptide is completely absent in macrophages from inbred mice homozygous for *Nramp1*^{Asp169}, which implies that the mutation causes structural alteration that somehow prevents accumulation of the mature Nramp1 protein in macrophages (Vidal et al. 1996). Mice homozygous for *Nramp1*^{Asp169} are as susceptible to infection with *S. typhimurium* as the knockout *Nramp1*^{-/-} (Vidal et

al. 1996). Because Arg²²³ → Gln²²³ does not constitute an obvious loss of function, additional experimental data are needed to establish that Arg²²³ → Gln²²³ causes loss of NRAMP1 function in chickens and is the cause of susceptibility to infection in line C.

To analyze further the functional importance of NRAMP1 in resistance of chicken to infection with *S. typhimurium*, we performed a linkage analysis using a backcross chicken panel derived from resistant line W1 (Arg²²³) and susceptible line C (Gln²²³). In our experimental model, survival to infection is very high in the *Salmonella*-resistant line W (95%) compared to the *Salmonella*-susceptible line C (11%). Survival to infection in *Salmonella*-susceptible line C is biphasic, with an early phase (0–1 week) where most of the birds died from infection and a second phase (1–2 weeks) that is characterized by a low mortality rate, similar to that observed in *Salmonella*-resistant W1 chickens. NRAMP1 has a significant effect on survival during the first phase of infection and accounts for 18% of the differential resistance to infection of chicken lines C and W1. We have also tested the function of NRAMP1 on different genetic backgrounds: it appears that NRAMP1 has no effect in crosses made between resistant line N or 6₁ and susceptible line 15 (N. Bumstead and D. Malo, unpubl.). The fact that we were unable to link NRAMP1 to susceptibility to infection in these two crosses is consistent with our sequencing studies. The observation that only a proportion of early survival following challenge with *S. typhimurium* is accounted by NRAMP1 suggests that there are additional genes involved in the genetic control of *Salmonella* resistance in the chicken. We then tested a second candidate locus, TNC, in the same backcross panel. Survival analysis indicates that TNC is also involved in early resistance of chickens to infection with *S. typhimurium*. The magnitude of the TNC effect in the differential resistance/susceptibility of chicken lines C and W1 is similar (17%) to that observed with NRAMP1. Taken together, these two genetic loci combined contribute to 33% of the differential survival to infection of parental lines C and W1. The effect of these two loci is seen early after infection: At day 7 postinfection mortality rate is 6.3 times higher in NRAMP1 and TNC homozygous CC–CC chickens compared to heterozygous CW–CW chickens. The fact that chickens homozygous for susceptible alleles at NRAMP1 and TNC survive far better than the parental susceptible line indicates that other genetic factors exist that affect survival in the early time period. The mechanisms responsible for the differences in resistance to infection with *S. typhimurium*

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observed in our experimental model appear to be attributable to a better ability of the phagocytic systems to contain the infection during the initial phase of infection (Bumstead and Barrow 1993; N. Bumstead and P. Barrow, unpubl.). In the mouse, *Nramp1* and *Lps* mutations cause an uncontrolled intracellular replication of *S. typhimurium* in phagocytes of the RES during the early phase of infection through unknown mechanisms (O'Brein et al. 1980; Vidal et al. 1993). In the mouse, *Nramp1* is localized to compartments (late endosomal/early lysosomal compartment of resting macrophages and phagosomes) that are essential effectors in the intracellular killing of *Salmonellae* by phagocytes (Gruenheid et al. 1997).

During the course of infection, we observe that chickens surviving the first period and presenting different genotype at *NRAMP1* and *TNC* exhibit similar survival during the second period. However, parental resistant line W1 experiences better survival than the backcross population during the second period, suggesting again the presence of other genes that affect survival during the second time period. It is clear that several genetic and nongenetic factors affect both the early and late phases of infection in this chicken experimental model. Additional mouse *Salmonella* resistance genes involved in innate or acquired immunity need to be tested in the chicken. With the progress in the development of the genetic map of the chicken genome based on polymorphic microsatellite markers, identification of regions of the chicken genome that control resistance to infection with *Salmonella* is now possible using a genome scanning approach. Once chromosomal segments are identified, the identification of candidate gene(s) that modify the host resistance phenotype may be facilitated using comparative mapping with well developed mouse and human genetic maps (Hudson et al. 1995; Dietrich et al. 1996).

METHODS

Birds

Inbred white leghorn chicken lines W1, C, N, 6₁, 7₂, and 15I used in these experiments were maintained at the Institute for Animal Health (Compton, Newbury, Berkshire, UK) in specific pathogen-free facilities. Lines N, 15I, 6₁, and 7₂ were derived from the flocks held at the Avian Diseases and Oncology Laboratory (East Lansing, MI), line C was originally developed at the Northern Poultry Breeding Station (Reaseheath, Cheshire, UK); and line W1 was acquired from Dr. J. Ivanyi (Wellcome Laboratories, Beckenham, UK). Resistant W1 and susceptible C lines were used to produce 425 (W1 × C)_{F1} × C segregating backcross progeny.

Determination of Susceptibility to Infection

Chicks were inoculated intramuscularly with a 0.1-ml inoculum containing 10³ CFU of the highly virulent *S. typhimurium* strain F98 on the day of hatch within 18 hours of hatching. The infectious inoculum of *S. typhimurium* was prepared as described previously (Bumstead and Barrow 1988). Birds were examined daily for a period of 2 weeks; deaths were recorded and moribund animals were sacrificed.

Molecular Probes and Genetic Typing

Probe MCG2 is a repeat-free 2.7-kb *Bam*HI genomic DNA fragment containing the 5' coding portion of chicken *NRAMP1*, derived from cosmid clones originally isolated with a mouse *Nramp1* cDNA probe (Hu et al. 1995). The *TNC* probe was a 2.1-kb partial human cDNA (gift of Dr. L. Zardi, Istituto Nazionale per la Ricerca sul Cancro, Genoa, Italy).

High-molecular-weight DNA was prepared from red blood cells as described previously (Bumstead et al. 1987). To identify restriction fragment length polymorphisms (RFLPs) among inbred lines, 2 μg of DNA was digested to completion with several restriction enzymes. Restricted fragments were separated by electrophoresis, transferred to nylon membranes (Amersham), and hybridized to MCG2 and *TNC* probes labeled to high specific activity with [α -³²P]dATP under stringent hybridization conditions (0.1 × SSC and 0.1% SDS for 20 min at 65°C).

PCR amplifications of genomic DNA were carried out in 50-μl reaction volumes containing 10 ng of template DNA as described previously (Malo et al. 1994). Cycling conditions were as follows: incubation for 2 min at 94°C, followed by 35 cycles of 1 min at 94°C, 1 min at 52°C, 1 min at 72°C, and a final extension step of 10 min at 72°C. The primers used to amplify the region of *NRAMP1* showing an altered *Eag*I restriction site between line C and W were 5'-CAAGCTGGAGGCGCTTCTTCGGCTT-3' (positions 622-645) and 5'-GAGGAGTGCAGGAAGATGTTATGG-3' (positions 805-828). Restricted PCR products were detected by ethidium bromide staining on 2% agarose gels.

RT-PCR and Nucleotide Sequencing

Total spleen RNA (2 μg) samples from chicken lines W1, C, N, 15I, 6₁, and 7₂ were used as templates for RT-PCR (Hu et al. 1995). Line-specific *NRAMP1* cDNA clones were obtained by PCR amplification of three overlapping fragments (A, 791 bp; B, 779 bp; and C, 416 bp) covering the entire coding region of chicken *NRAMP1*. Sequence-specific oligonucleotide primers 5'-CACCCAGAGGTAGAGGTCC3' (positions 1-20) and 5'-CACCAACGATGCCACA-3' (positions 775-791) were used to amplify fragment A, primers 5'-CAAGCTGGAGCGCTTCTTCGGCTT-3' (positions 622-645) and 5'-GCAGGCTGGTGAAGGTG-3' (positions 1384-1400) for segment B, and primers 5'-ACGTGAGCCAGCTGACG-3' (positions 1302-1318) and 5'-CCGTGTGCGGGACGGAAC-3' (positions 1700-1717) for segment C. PCR amplification was performed for 35 three-step cycles at 94°C for 1 min, 60°C for 1 min, and 72°C for 1 min. Amplified products were gel purified and cloned into a dT-tailed pBluescript II KS(+) vector. For each ligation, at least three clones were sequenced in both sense and antisense orientation. Nucleotide sequencing was performed by the dideoxy chain termination method of

Sanger et al. (1977), using modified T7 DNA polymerase (Pharmacia) and denatured double-stranded DNA templates. Oligonucleotide primers were derived from the known sequence of the *NRAMP1* cDNA (Hu et al. 1996) or from plasmid sequence flanking the cloning sites.

Northern Blot

Total RNA was isolated from W1, C, N, 15I, 6₁, and 7₂ chicken spleens as described previously (Hu et al. 1996). Total RNA (10 µg) was fractionated by electrophoresis in 1% agarose gels containing 0.66 M formaldehyde and MOPS buffer [1 × MOPS = 40 mM morpholinopropanesulfonic acid, 10 mM sodium acetate, 10 mM EDTA (pH 7.2)] and blotted onto nylon membranes (GeneScreen Plus, New England Nuclear) in 20× SSC. Hybridizations were performed in a solution containing 6× SSC, 50% formamide, 1% SDS, 5× Denhardt's solution, 10% dextran sulfate, 100 µg/ml of denatured sheared salmon sperm DNA and ³²P-radiolabeled full-length *NRAMP1* cDNA probe (1 × 10⁶ cpm/ml) for 16 hr at 42°C. Membranes were then washed to a final stringency of 0.2× SSC, 0.5% SDS at 65°C for 60 min.

Statistical Analysis

(W1 × C)F₁ × C chickens were grouped, separately by their *NRAMP1* and *TNC* genotypes for analysis and also classified into the four groups defined by the genotypes jointly at both loci. The effect of *NRAMP1* and *TNC* on survival was tested using a Cox proportional hazards model (Kalbfleisch and Prentice 1980; Matthews and Farewell 1996) as implemented in S-PLUS (Statistical Sciences, S-PLUS v. 3.3 supplement, Seattle, WA. StatSci, a division of MathSoft Inc., 1995). The hazard rate is defined as the probability of dying on a specific day given that the individual bird did not die before that day. The model assumes that the hazard function is a product of a baseline hazard that is dependent on time but not the covariates, multiplied by a term that is dependent on covariates but not time. The Cox model is semiparametric in the sense that the magnitude of the effect will be estimated for each each locus, but there is no explicit estimation of the baseline hazard (Matthews and Farewell 1996). Models are tested against the null model, which includes no covariates, using the likelihood ratio statistic test. We subsequently tested for the presence of an interaction between *NRAMP1* and *TNC*. The existence of an interaction term may indicate that the loci exhibit epistasis, or a nonadditive effect. Although *NRAMP1* and *TNC* are not linked, it is not possible to distinguish the effect due to variation at the typed locus from effects due variation at other tightly linked loci.

Observation of the susceptible parental strain (C) led us to believe that the hazard ratios may not be constant over the entire duration of the experiment. Therefore, in addition to testing for differences in survival over the entire 15-day period, we tested separately for two time periods, day 1–7, and day 8–15.

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Resistance to Salmonellosis in the Chicken Is Linked to *NRAMP1* and *TNC*

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