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The full expression of the *Ity* phenotype in *Ity*^{r} mice requires C3 activation by *Salmonella* lipopolysaccharide

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SUMMARY

Our previous study has shown that the rapid and sufficient activation of complement by *Salmonella* lipopolysaccharide occurs in genetically resistant (*Ity*^{r}) A/J mice. To assess whether the level of complement activation by a virulent strain of *Salmonella typhimurium* regulates the level of murine natural resistance, we compared levels of serum complement activation by *S. typhimurium* and kinetics of serum-opsonized *S. typhimurium* grown in macrophages using several strains of resistant (*Ity*^{r}) and susceptible (*Ity*^{s}) mice. *Ity*^{s} macrophages killed intracellular *S. typhimurium* to the same extent as did *Ity*^{r} macrophages when the pathogen was opsonized with *Ity*^{r} serum. Opsonization of *S. typhimurium* with *Ity*^{s} serum reduced intracellular killing activity in *Ity*^{r} macrophages to the same level as seen with *Ity*^{s} macrophages. Incubation of *S. typhimurium* with 25% Mg^{2+} EGTA (5 mM $MgCl_2$–3 mM ethylene glycol-bis (β-aminotheyl either)-*N,N,N′,N′*-tetraacetic acid)-chelated *Ity*^{r} serum resulted in higher levels of C3 deposition onto the surface of this bacteria, C3b generation and also C3 consumption, compared with that with Mg^{2+} EGTA-chelated *Ity*^{s} serum. Opsonization of *S. typhimurium* with A/J serum prior to infection increased early resistance in *Ity*^{s} mice. Infection with a virulent strain of *S. typhimurium* induced the expression of interleukin-10 (IL-10) mRNA at higher levels in C57BL/6 mice than in A/J mice. However, opsonization of *S. typhimurium* with A/J serum decreased bacterial growth in the spleen of C57BL/6 mice to the same level as observed for A/J mice in association with decreased expression levels of IL-10 mRNA. Moreover, administration of anti-C3 antibodies reduced the resistance of A/J mice in association with a decrease in serum levels of C3. These results indicate that the high level of complement activation via the alternative pathway in *Ity*^{r} serum by a virulent strain of *S. typhimurium* reduces the virulence of this pathogen, which may contribute to the full expression of *Ity* phenotype in *Ity*^{r} mice.

INTRODUCTION

Salmonella typhimurium is a facultative intracellular pathogen for mice. An intravenous challenge with a virulent strain of *S. typhimurium* results in the exponential growth of the bacteria in the spleen and liver of susceptible mice; the net growth rate is considered to be regulated mainly by the *Ity* gene.[1–4] Mice homozygous or heterozygous for the dominant resistance allele (*Ity*^{r}) are usually 100- or 1000-fold more resistant to challenge with virulent *S. typhimurium* on the basis of the number of bacteria to achieve the lethal dose 100% (LD_{100}), compared with mice homozygous for the susceptible allele (*Ity*^{s}).[2]

The precise mechanism of action of the *Ity* gene product is not known, but the natural resistance-associated macrophage protein 1 (Nramp 1) has been identified by positional cloning as a candidate product.[5–7] Nramp 1 is a type II integral membrane protein, and is expressed in macrophage cell lines and splenic macrophages. The protein has significant homology with known transmembrane transport proteins, including a nitrate/nitrite concentrator of *Aspergillus nidulans*.[8] Thus, it has been postulated that the function of Nramp 1 may be related to the transport of simple nitrogen compounds required for the generation of reactive nitrogen intermediates which are microbicidal against intracellular pathogens.[9]

Several investigators have shown that endogenous production of interferon-γ (IFN-γ), together with tumour necrosis factor-α (TNF-α), plays an important role in the early phase of resistance to *S. typhimurium*.[10–12] Above all, IFN-γ activates the microbicidal activity of macrophages by the induction of nitric oxide synthesis;[13] so that, Nramp 1 seems to play a role for the transportation of reactive nitrogen intermediates in IFN-γ-activated macrophages from the cytoplasm to the phagosomes in which *S. typhimurium* is multiplying.

IFN-γ is produced during the early growth phase of *S. typhimurium*, which is the time when the *Ity* phenotype is expressed.[10] Some data have shown that *Ity*^{s} mice can produce

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IFN-γ after infection with virulent *S. typhimurium* to the same extent as do *Ity*^{r} mice.[14] Based on the fact that the degree of phagocytosis by macrophages is not substantially different between *Ity*^{r} and *Ity*^{s} mice,[15] the expression of the *Ity* phenotype seems to play a role in controlling multiplication of *S. typhimurium* in IFN-γ-activated macrophages by transporting reactive nitrogen intermediates effectively. However, our previous study[16] demonstrated that IFN-γ-activated macrophages from C57BL/6 (*Ity*^{s}) mice exhibited the same level of intracellular killing activity against virulent *S. typhimurium* opsonized with A/J (*Ity*^{r}) serum as did IFN-γ-activated A/J macrophages, and that A/J macrophages were less microbicidal against C57BL/6 serum-opsonized *S. typhimurium*. Moreover, opsonization of virulent *S. typhimurium* with A/J serum was found to increase significantly the median survival time of infected C57BL/6 mice,[16] suggesting that *Ity*^{r} serum can either enhance anti-*Salmonella* activity in macrophages or reduce the virulence of *S. typhimurium*.

We report here that *S. typhimurium* activates complement of *Ity*^{r} mice to a greater extent than that of *Ity*^{s} mice, and that this difference is closely associated with the difference in the expression of interleukin-10 (IL-10) mRNA between *Ity*^{r} and *Ity*^{s} mice in the early phase of infection. Results presented in this study may suggest that the rapid activation of the third complement component (C3) by *S. typhimurium* occurs in *Ity*^{r} mice to an extent sufficient for attenuation of virulent *S. typhimurium*, which might be required for the full expression of the *Ity* phenotype in *Ity*^{r} mice.

MATERIALS AND METHODS

Mice

Four-week-old, specific-pathogen-free (SPF), female A/J, DBA/1, DBA/2, CBA, C57BL/6, BALB/c, C3H/HeN, and B10 mice were purchased from SLC Japan (Hamamatsu, Japan). They were maintained under strict SPF conditions at the animal center of Nara Medical University, and used at the age of 5–6 weeks.

Bacteria

Salmonella typhimurium LT2 was stored at −80° in a gelatin-disk form.[17] At each experiment, a disk was thawed and grown overnight on tryptic soy agar (Difco Laboratories, Detroit, MI) at 37°. Bacteria grown on tryptic soy agar were regrown in tryptic soy broth to log phase at 37°, washed in Ca^{2+}-, Mg^{2+}-free balanced salt solution (CMFS), pH 7·2, and adjusted to the desired concentrations before use. The number of viable organisms was determined by plate count.

Serum preparation

Blood was extracted from the retro-orbital venous plexus, allowed to clot for 30 min at 37°, and for a further 60 min on ice. Serum was separated by centrifugation at 10 000*g* for 20 min, and stored at −80° until use. When each serum was assayed, it was used without prior treatment or was treated with 5 mM $MgCl_2$–3 mM ethylene glycol-bis (β-aminoethyl ether)-*N,N,N′,N′*,-tetraacetic acid (Mg^{2+} EGTA) to limit complement activation to the alternative pathway. For controls, mouse serum was inactivated at 56° for 30 min or treated with 5 mM ethylene diaminetetraacetic acid (EDTA) to block both complement activation pathways.

Intracellular killing assay

Resident peritoneal cells were harvested and suspended at a concentration of 10^6 cells/ml in Earle's minimal essential medium (MEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Hyclone, Logan, UT), 10 mM HEPES, and 2 mM L-glutamine (defined as basal medium). One-millilitre cell suspensions were distributed into the wells of 24-well culture plates and were incubated to allow the cells to adhere to the plastic wells for 4–6 hr at 37° in 5% CO_2. Then, non-adherent cells were removed by washing with warm CMFS. This procedure usually yielded $2·5 \times 10^5$–$3·6 \times 10^5$ adherent cells per well, which was independent of mouse strains. A cytotoxic test using anti-murine macrophage antibody and complement as described before[18] demonstrated that more than 92% of adherent cells were macrophages. The viability of each cell preparation was determined by exclusion of trypan blue.

Assay of the intracellular killing of *S. typhimurium* by macrophages was carried out at a 10:1 ratio of infecting bacteria to macrophages following the method of Lissner *et al.*[15] with a slight modification. Adherent macrophages in wells were washed once with warm CMFS containing 10% heat-inactivated FBS, and then exposed to 500 μl of basal medium containing *S. typhimurium* which had been opsonized with fresh mouse serum or heat-inactivated mouse serum (control) as described below. Culture plates were horizontally rotated at 35 r.p.m. at 37° for 10 min, and then kept without rotation at 37° for a further 40 min in a 5% CO_2 incubator to permit phagocytosis by the macrophages. The actual number of infecting bacteria opsonized with fresh mouse serum was verified by plate count. After the 50-min phagocytosis period, macrophages in all wells were washed three times with 500 μl of warm CMFS, and the last wash was stored for enumeration of bacteria. For determination of viable numbers of *S. typhimurium* which was phagocytosed by macrophages during this 50-min phagocytosis period, the macrophages were lysed with 500 μl of 0·1% (v/v) Triton-X in CMFS, and the numbers of *S. typhimurium* present in the last wash and in macrophage lysate [colony-forming units (CFU) time 0] were determined by plate count. Those wells that contained macrophages to be sampled later were overlaid with 500 μl of fresh basal medium containing 5 μg/ml of gentamicin, and were incubated for 4 hr at 37° in 5% CO_2. After a 4-hr incubation, these wells were treated in the same way as at the end of a 50-min phagocytosis. The numbers of *S. typhimurium* in the last wash and in macrophages (CFU time 4 hr) were determined by plate count.

To determine the number of viable macrophages present in wells at each sample time, macrophages infected with *S. typhimurium* in wells, which were prepared separately from the wells used for CFU determination, were washed with warm CMFS and then detached from the surface of each well with 10 units/ml of collagenase S-1 (Nitta Gelatine, Osaka, Japan). The number of viable macrophages released after a 10-min treatment of collagenase S-1 was determined by the trypan blue exclusion test.

The phagocytic ability and the intracellular killing activity of macrophages were expressed by the mean values of t_0 (CFU time 0/viable macrophage numbers) and t_{4hr} (CFU time 4 hr/viable macrophage numbers), respectively.

In this assay, a 30-min incubation of the bacteria in 0·1% Triton-X in CMFS did not affect the viability of *S. typhimurium*

LT2.[16] The minimum inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC) of gentamicin for strain LT2 were 4·5 μg/ml and 5·0 μg/ml, respectively.[19] The gentamicin concentration of 5 μg/ml in the basal medium used during a 4-hr incubation was the lowest concentration that would consistently prevent extracellular replication of strain LT2 in infected macrophage culture.

Opsonization of S. typhimurium *with fresh mouse serum*

Salmonella typhimurium LT2 (5×10^6 CFU) was suspended in 500 μl of fresh serum obtained from mice of each strain, and the mixture in a test tube was rotated in a gyrorotary shaker at 45 r.p.m. overnight at 4°. Thereafter, the opsonized organisms were harvested by centrifugation, and washed twice with cold CMFS. To verify that this opsonizing procedure did not affect the viability of *S. typhimurium*, the viable number of strain LT2 at the end of opsonization was determined by plate count. Decrease in the number of CFU by serum opsonization was less than 0·001% of the number of CFU in the original bacterial suspension, which was independent of the source of mouse serum.

Measurement of C3 consumption and C3b

Salmonella typhimurium (5×10^7 heat-killed organisms) was incubated with 500 μl of 25% Mg^{2+} EGTA-chelated mouse serum at 37° for 30 min, and centrifuged at 13 000 ***g*** for 10 min. Supernatant serum was heat-inactivated at 56° for 30 min, and C3 in the inactivated serum was quantified by single radial immunodiffusion (SRID) using goat anti-mouse C3 antibody (Organo Teknika Corporation, Durham, NC) as described before.[16] A standard curve was constructed with purified mouse C3. C3 was prepared from pooled Institute of Cancer Research (ICR) mouse plasma following the method of Al Salihi *et al.*,[20] and further purified by affinity chromatography using anti-mouse C3-conjugated Sepharose 4B. Consumption of C3 was expressed as (1 − C3 in supernatant serum/C3 in original chelated serum) × 100 (%).

C3b in sera of infected and uninfected mice was quantified by the immune adherence haemagglutination (IAHA) assay, exactly following the methods as described before.[16] The degree of haemagglutination was determined under an inverted microscope. IAHA titres were expressed as the highest dilution ($\log_2$) yielding strong haemagglutination.

Measurement of C3 deposition on S. typhimurium

C3 deposition on the surface of *S. typhimurium* was quantified by enzyme-linked immunosorbent assay (ELISA). Heat-killed *S. typhimurium* LT2 was suspended in CMFS at concentrations of 5×10^9–5×10^6 per ml, and 100 μl aliquots of bacterial suspensions were dispensed into wells of 96-well vinyl microtitre plates. Bacterial suspensions in wells were allowed to evaporate to dryness at 37° overnight. All wells were treated with 10% H_2O_2 at room temperature for 30 min to inactivate remaining endogenous peroxidase. After several washings with CMFS, all wells were blocked with 3% (w/v) bovine serum albumin (BSA) in 20 mM sodium phosphate buffer, pH 7·4, for 1 hr. After several washings with CMFS containing 0·01% Tween (wash buffer), 100 μl of each mouse serum diluted to 25% with GVB^{2+} (Veronal buffer, pH 7·6 containing 0·1% gelatin, 3 mM Ca^{2+} and 1 mM Mg^{2+}) was added to wells, and incubated at 37° for 30 min. Then, the plate was incubated at 56° for 30 min in order to inactivate further complement reaction. All wells were washed three times with wash buffer containing 5 mM di-isopropyl-fluorophosphate (to minimize further proteolytic cleavage of C3) and 0·01% Tween. The plate was incubated with 100 μl of 10 μg/ml goat anti-mouse C3 antibody in wash buffer containing 1% BSA for 2 hr at 37°, and then overnight at 4°. Unabsorbed antibodies were removed by successive washing with wash buffer. Then, 100 μl of 2 μg/ml biotinylated rabbit anti-goat IgG antibody in wash buffer containing 1% BSA was added to each well, and incubated at 37° for 1 hr. The plate was washed three times with wash buffer, and incubated for 1 hr with peroxidase-labelled streptavidine (Sigma, St Louis, MO) at a final dilution of 1/200 (100 μl per well) in wash buffer containing 1% BSA. The plate was washed five times with CMFS, rinsed once with distilled water and carefully dried. The reaction was then developed using the enzyme substrate solution. Optical density of each well was measured at 480 nm with a Micro ELISA reader (Bio-Rad Laboratories, Melville, NY).

Treatment of mouse C3 antibody

Goat anti-mouse C3 serum was partially purified by 50% ammonium sulphate precipitation, and the IgG fractions were collected by passage over a protein G–Sepharose 4 Fast Flow (Pharmacia Finne Chemicals, Tokyo, Japan) column, concentrated and dialysed against CMFS. A/J mice were injected intraperitoneally with various doses of the purified goat IgG antibody to mouse C3, and 24 hr later C3b generation in the serum was quantified by IAHA assay after 500 μl of 25% diluted serum was incubated with 5×10^7 heat-killed *S. typhimurium* LT2. Over the dose range of 1·0–1·4 mg per mouse of the antibody administered, C3b yield in the serum of treated A/J mice was reduced to the same levels as seen in the serum of untreated C57BL/6 mice. Thus, A/J mice were treated intraperitoneally with 1·2 mg of the purified anti-mouse C3 IgG antibody 1 hr before and 48 hr after infection. The animals receiving the antibody were infected intravenously with 5000 CFU of strain LT2. Control A/J mice were treated with the same amount of normal goat IgG.

Competitive quantitative polymerase chain reaction

RNA extraction from spleens was carried out using the TRIZOL reagent (Gibco BRL Life Technologies, Inc., Tokyo, Japan). Complementary DNA was synthesized from 1 μg of total RNA in a volume of 20 μl with the Preamplification system (Gibco BRL) using Molony murine leukaemia virus reverse transcriptase (Gibco BRL) according to the protocol supplied by the manufacturer. After reverse transcription, IFN-γ, IL-10, IL-12 p40 and β-actin mRNA levels were quantified using competitive quantitative polymerase chain reaction (PCR) employing an internal standard (plasmid control pUT-M template) according to the method reported elsewhere.[21,22] The primer pairs used in these experiments were as follows: for β-actin, 5′-ctg aag tac ccc att gaa cat ggc-3′ (upstream) and 5′-cag agc agt aat ctc ctt ctg cat-3′ (downstream); for IFN-γ, 5′-act gcc acg gca cag tca-3′ (upstream) and 5′-gcg act cct ttt ccg ctt-3′ (downstream); for IL-10, 5′-ctc tta ctg act ggc atg agg atc-3′ (upstream) and 5′-cta tgc agt tga aga tgt caa att-3′ (downstream); and for IL-12 p40, 5′-aac tgg cgt tgg aag cac gg-3′ (upstream) and 5′-gaa cac atg ccc act tgc tg-3′ (downstream).

Five microlitres of diluted cDNA samples were mixed with decreasing concentrations of competitive DNAs and amplified simultaneously using a specific set of primers of IFN-γ, IL-10, IL-12 p40 and β-actin. The PCR products were fractionated on a 1·5% agarose gel. After staining with ethidium bromide and photography, the intensities of the target bands and competitor bands were measured using densitometric scanning (Argus-50, Hamamatsu Photogenics, Hamamatsu, Japan), and were adjusted for the number of base pairs. The levels of the targets were calculated from the plot using the intensity ratio of target and competitor as described previously.[22] In order to account for the variations in RNA isolation and reverse transcription efficiencies, the levels of these cytokine messages were expressed as ratio of cytokine to β-actin.

Statistical analysis

Levels of significance for the observed frequencies were determined by Student's *t*-test or Fisher's test for 2 × 2 tables. Differences were considered significant when *P* was $\leqslant 0{\cdot}05$.

RESULTS

Activation of serum C3 by *S. typhimurium*

The degree of C3 deposition on the surface of *S. typhimurium* LT2 was quantified by ELISA using antiserum to mouse C3. In preliminary experiments, the optimal amount of bacteria for this assay was determined by dispensing 100 μl of two-fold serial dilutions from mouse serum-treated strain LT2 per well. At bacterial concentrations below 5×10^7 per well, the optical absorbance of bound C3 varied in a dose-dependent manner. The optical density reached a plateau at concentrations up to 25% of mouse serum, regardless of mouse strains. Consequently, a concentration of about 5×10^7 organisms per well and a dilution of 25% of mouse serum were selected in subsequent experiments.

Higher degrees of C3 deposition on the bacterial surface occurred in untreated Ity^r mouse sera after 30 min of incubation, compared with untreated Ity^s mouse sera (Table 1). Among Ity^r mouse sera, A/J serum yielded the highest C3 deposition and C3H/He serum resulted in the lowest one: however, this difference was not statistically significant. When each serum was pretreated with Mg^{2+} EGTA to prevent potential activation of the classical pathway by chelating Ca^{2+}, similar levels of C3 deposition occurred in each serum. In contrast, EDTA-treated serum of each mouse strain supported only background binding. These results indicate that Ity^r serum generates higher amounts of C3 deposition onto the surface of *S. typhimurium*, and that C3 deposition results from the alternative pathway activation.

Table 1. C3 deposition on *S. typhimurium* as determined by ELISA

Source of mouse serum	C3 deposition (A_{480} values)* treated with: None	EDTA	Mg^{2+} EGTA
Ity^r mice			
A/J	2·652±0·314	0·147±0·028	2·546±0·145
DBA/2	2·312±0·289	0·132±0·033	2·038±0·216
CBA	2·284±0·267	0·106±0·045	1·986±0·173
C3H/He	2·065±0·119	0·127±0·036	2·005±0·202
Ity^s mice			
C57BL/6	1·092±0·132	0·112±0·016	0·964±0·136
BALB/c	0·984±0·254	0·109±0·022	0·887±0·127
DBA/1	1·126±0·148	0·120±0·037	1·043±0·264
B10	1·134±0·236	0·115±0·025	1·102±0·212
Control			
CMFS	0·022±0·008	0·021±0·012	0·022±0·014

*Results are the mean ± SD for five different mouse sera per group.

The degree of C3b yield was determined by IAHA titres. When untreated serum of each mouse was incubated with 5×10^7 heat-killed *S. typhimurium* LT2 for 30 min, IAHA titres were at least eight-fold higher (maximal fold-difference; 30) in sera of Ity^r mice than in those of Ity^s mice (Table 2). A similar difference was obtained by chelating of each serum with Mg^{2+} EGTA. These results indicate that higher C3 deposition on the bacterial surface results in higher C3b generation via the alternative pathway activation in Ity^r serum, and that *S. typhimurium* activates Ity^r serum C3 to a greater extent than Ity^s serum C3.

Serum opsonization and intracellular killing

The average number of viable *S. typhimurium* per macrophage was determined at the end of a 50-min phagocytosis period (Fig. 1). Both Ity^r and Ity^s macrophages exhibited the similar level of phagocytic activity (t_0), regardless of serum used for opsonization; the mean t_0 values ranged from 0·764 (Ity^r macrophages and Ity^r homologous serum) to 0·820 (Ity^s macrophages and A/J serum), with the mean t_0 value of 0·798. There were no statistically significant differences in the mean t_0 value between Ity^r and Ity^s macrophages, whichever serum was used for opsonization. Against non-opsonized *S. typhimurium*, macrophages from both Ity^r and Ity^s mice phagocytosed

Table 2. IAHA activity in mouse serum after incubation with heat-killed *S. typhimurium*

Source of mouse serum	IAHA titres ($\log_2$)* treated with: None	EDTA	Mg^{2+} EGTA
Ity^r mice			
A/J	6	<1	5
DBA/2	5	<1	4
CBA	5	<1	5
C3H/He	4	<1	3
Ity^s mice			
C57BL/6	2	<1	1
BALB/c	1	<1	1
DBA/1	2	<1	1
B10	1	<1	1

*Serum was treated with EDTA or Mg^{2+} EGTA, or untreated, and adjusted to 25% with GVB^{2+} before incubating with 5×10^7 heat-killed *S. typhimurium*. IAHA titres were expressed as the highest dilution ($\log_2$) of each serum resulting in strong haemagglutination. Results are the mean of three experiments in which three mice per group were used.

bacteria to a lesser extent; the mean t_0 values were 0·25 for *Ity*^{r} cells and 0·23 for *Ity*^{s} cells, respectively (data not shown).

Over the next 4 hr, *Ity*^{r} macrophages showed a decline in CFU time 4 hr compared with CFU time 0, when *S. typhimurium* was opsonized with each of the homologous sera or A/J serum. The mean $t_{4\ hr}$ values of infected *Ity*^{r} macrophages were reduced by at least 90%, compared with the mean t_0 values (Fig. 1). Opsonization with A/J serum allowed *Ity*^{s} macrophages to reduce the mean $t_{4\ hr}$ values to the same levels as those obtained from *Ity*^{r} macrophages against this pathogen opsonized with either A/J serum or each of *Ity*^{r} homologous sera. When *S. typhimurium* was opsonized with A/J serum, *Ity*^{s} macrophages reduced the mean $t_{4\ hr}$ values by more than 93% compared with the t_0 values. In contrast, opsonization of *S. typhimurium* with C57BL/6 serum resulted in at most 66·5% reduction in the mean $t_{4\ hr}$ values for *Ity*^{r} macrophages and 67·2% reduction for *Ity*^{s} macrophages, respectively. Moreover, *Ity*^{s} macrophages decreased the mean $t_{4\ hr}$ values by 65·0–67·2% compared with the t_0 values, when opsonization was done with each *Ity*^{s} homologous serum. There was a significant difference ($P<0·01$) in the mean $t_{4\ hr}$ value of macrophages between opsonization with A/J serum and that with C57BL/6 serum, irrespective of the *Ity* phenotype of the cells.

These results indicate that opsonization with *Ity*^{r} serum decreases bacterial growth in macrophages regardless of the *Ity* phenotypes of the cells, and that the bactericidal capacity of both *Ity*^{r} and *Ity*^{s} macrophages does not differ from each other against *S. typhimurium* opsonized with *Ity*^{r} serum.

C3 consumption in the alternative pathway

Consumption of C3 resulting from the activation of the alternative pathway was compared between *Ity*^{r} and *Ity*^{s} mouse sera after 25% Mg^{2+} EGTA-chelated serum was incubated with 5×10^7 heat-killed *S. typhimurium* LT2 (Table 3). Strain LT2 consumed at least 52·5% of total C3 in *Ity*^{r} mouse serum, whereas it led to only lower levels of C3 consumption which averaged 20·9% of total C3 in *Ity*^{s} mouse serum. Mg^{2+} EGTA chelation of each serum by itself (i.e. in the absence of bacteria) did not lead to C3 consumption (data not shown). These results indicate that C3 of *Ity*^{r} mouse serum is consumed via the alternative pathway by *S. typhimurium* to a greater extent than that of *Ity*^{s} mouse serum, which is in agreement with the difference in the assay of C3 deposition (Table 1) as well as IAHA titres (Table 2).

Effect of opsonization of *S. typhimurium* with A/J serum on resistance of *Ity*^{s} mice

Opsonization with A/J serum resulted in about a two-fold increase ($P<0·01$) in the median survival time (MST) of three

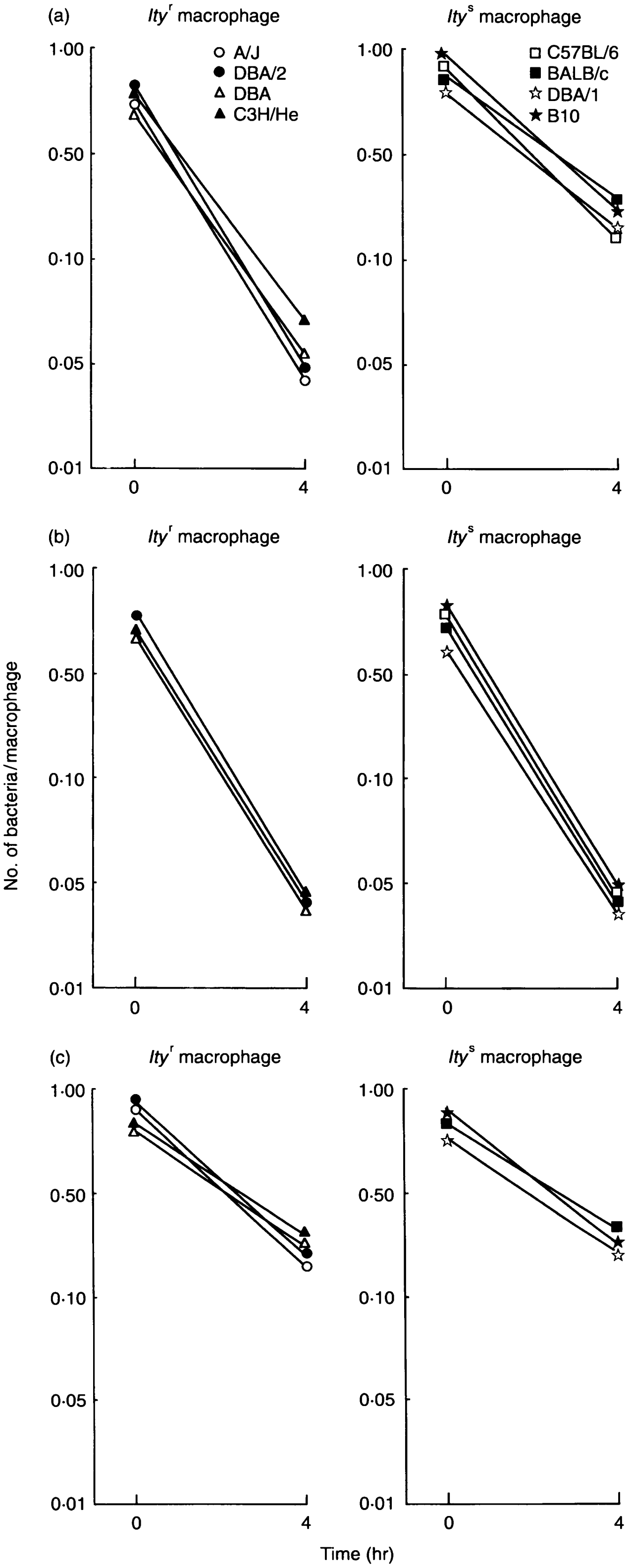

Figure 1. Anti-*Salmonella* activity in resident peritoneal macrophages from *Ity*^{r} and *Ity*^{s} mice. Macrophages were harvested from *Ity*^{r} (see key) and *Ity*^{s} (see key) mice. Cells were infected *in vitro* with *S. typhimurium* LT2 opsonized with fresh homologous serum (a), A/J serum (b), and C57BL/6 serum (c). Macrophage lysates were prepared at 0 (at the end of a 50-min phagocytosis period), and 4 hr (4 hr after the completion of phagocytosis). The number of viable salmonellae was determined at each time-point. The results were obtained from three experiments and are expressed as the number of viable cell-associated bacteria/the number of viable macrophages counted. Each point represents the average of nine culture dish wells. Standard deviation (SD) was within 1% at each point.

Table 3. Consumption of C3 after incubation of 25% Mg^{2+} EGTA-chelated mouse serum with *S. typhimurium* LT2

Source of mouse serum	C3 consumption (% of total C3)
Ity^r	
A/J	52·5±7·3
DBA/2	58·7±6·4
CBA	56·3±5·9
C3H/He	57·4±6·8
Ity^s	
C57BL/6	22·3±5·7
BALB/c	20·3±4·8
DBA/1	21·7±5·2
B10	22·3±3·9

C3 concentrations were quantified by SRID after 25% Mg^{2+} EGTA-chelated serum was incubated at 37° for 30 min with 5×10^7 heat-killed *S. typhimurium*. Data were obtained from two experiments using three mice of each strain per experiment. Results are expressed as the mean±SD for six assays. The difference in the mean of C3 consumption between Ity^r and Ity^s mice was significant ($P<0·05$).

Ity^s mouse strains, compared with infection with the same dose of *S. typhimurium* opsonized with heat-inactivated A/J serum (Table 4). In contrast, opsonization of *S. typhimurium* with Ity^s serum neither affected resistance of Ity^r mice nor increased Ity^s mouse resistance (data not shown). These findings indicate that opsonization with Ity^r serum seems likely to reduce the virulence of *S. typhimurium* for Ity^s mice, thereby modifying the consequence of infection.

Effect of opsonization with Ity^r serum on the cytokine profile in Ity^s mice

Competitive PCR analyses demonstrated that the expression level of IFN-γ mRNA in C57BL/6 mice was 1·85 times on day 3 and 1·43 times on day 5, respectively, as high as that in A/J mice (Fig. 2a and b). A/J mice exhibited a slightly higher level of IL-12 p40 mRNA expression on day 1 compared with C57BL/6 mice, while on days 3 and 5 mRNA for this cytokine was expressed in C57BL/6 mice at least 1·76 times as high as that in A/J mice. In contrast, IL-10 mRNA was expressed in C57BL/6 mice more than 21 times as high as that in A/J mice on days 3 and 5. In C57BL/6 mice, however, infection with LT2 opsonized with fresh A/J serum reduced the expression of IL-10 mRNA to the same levels as seen with infected A/J mice (Fig. 2c). The expression of both IFN-γ and IL-12 p40 mRNAs was also reduced in C57BL/6 mice after infection with fresh A/J serum-opsonized LT2. As a result of opsonization of *S. typhimurium* with A/J serum, the expression profiles of these three cytokines in C57BL/6 mice were similar to those observed in infected A/J mice during the first 5 days of infection.

Table 4. The median survival time (MST) of Ity^s mice after infection with *S. typhimurium* LT2 opsonized with fresh A/J serum

Ity^s mouse strain	MST (days) opsonization with A/J serum		Fold increase in MST (A/B)
	Fresh (A)	Heat-inactivated (B)	
C57BL/6	12·8	6·7	1·9
BALB/c	10·9	6·2	1·8
DBA/1	13·8	7·1	1·9
B10	10·2	5·4	1·9

Each group consisted of 10 mice intravenously infected with 5000 CFU of *S. typhimurium* LT2 opsonized with fresh A/J serum (A) or heat-inactivated A/J serum (B). Results are expressed as the mean of MST obtained from three separate experiments. Difference in the mean of MST between A and B was significant ($P<0·05$) for each strain.

Effect of anti-mouse C3 antibody on resistance of A/J mice to *S. typhimurium*

To examine whether C3 activation is involved in the early resistance of Ity^r mice to *S. typhimurium* infection, A/J mice were treated with goat IgG to mouse C3 intraperitoneally, and then infected intravenously with 5000 CFU of strain LT2. Treatment of A/J mice with anti-mouse C3 antibody significantly enhanced bacterial growth in the spleen on day 3, the level of which was comparable to that in C57BL/6 mice receiving control IgG (Table 5). To ensure that anti-C3 antibody treatment was effective, serum C3b levels were determined by IAHA on days 1 and 3. In treated A/J mice, serum C3b levels did not increase after infection and they remained at the same levels as seen with infected C57BL/6 mice. In addition, treatment of anti-C3 IgG antibody induced the high level of IL-10 mRNA expression in the spleen of A/J mice 3 days after infection (IL-10/β-actin=0·48 in antibody treated A/J mice, <0·01 in untreated A/J mice). In contrast, administration of control IgG neither affected C3b generation nor induced the expression of IL-10 mRNA in infected A/J mice (IL-10/β-actin<0·01).

DISCUSSION

Results presented here confirm our previous data[16] showing that a high level of C3 activation occurred in Ity^r mice during

Table 5. Effect of the purified anti-mouse C3 IgG on resistance of A/J mice to *S. typhimurium*

Mice	Treatment*	Bacterial count† (log$_{10}$ CFU/spleen) at day 3	Serum C3b levels‡ (log$_2$ titres) at day 3
A/J	Untreated	2·94±0·61	5
	Anti-C3 IgG	5·42±0·94	2
		$P<0·01$	
	Control IgG	3·04±0·72	5
C57BL/6	Control IgG	5·96±0·88	1

*Mice received intraperitoneally 1·2 mg of the indicated IgG 1 hr before and 24 hr after intravenous infection with 5000 CFU of strain LT2.

Data were obtained from two separate experiments.

†Results are the geometric mean number of bacteria recovered from spleens of six mice per group±SD.

‡Serum C3b levels were determined by IAHA. Results are the mean of six mice per group.

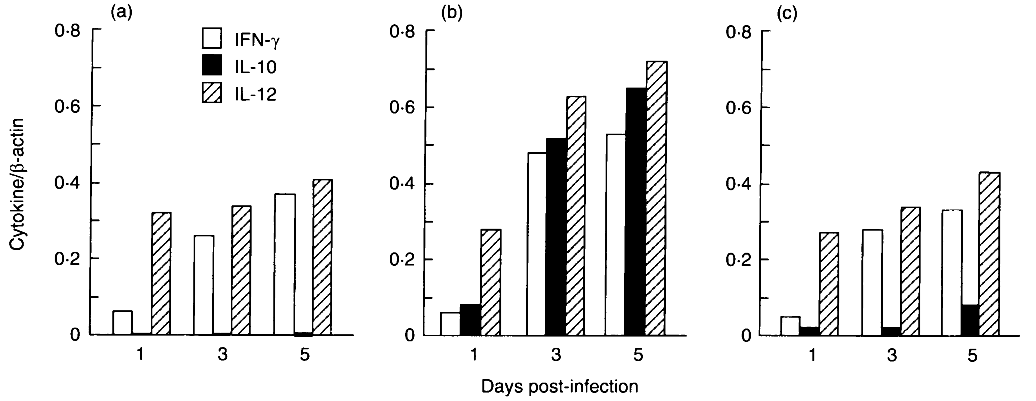

Figure 2. Effect of opsonization of *S. typhimurium* LT2 with fresh A/J serum on the expression of mRNAs for IFN-γ IL-10 and IL-12 in the spleen of infected C57BL/6 mice. (a) A/J mice after infection with strain LT2, (b) C57BL/6 mice after infection with strain LT2, (c) C57BL/6 mice after infection with strain LT2 opsonized with fresh A/J serum.

the initial phase of *S. typhimurium* infection, and demonstrate that the difference in levels of C3 activation by *S. typhimurium* between *Ity*^{r} and *Ity*^{s} mice was closely related to that in the cytokine profile between them at the early phase of infection.

It is known that the expression of *Ity*^{r} gene regulates the net growth of *S. typhimurium* in macrophages during the early phase of infection. Lissner *et al.*[15] have demonstrated, using *Ity* congenic mice, that the basis for differential net growth of *S. typhimurium* in *Ity*^{r} and *Ity*^{s} macrophages is a variation in the degree of *Salmonella* killing, although the *in vivo* uptake of *S. typhimurium* by resident macrophages of the reticuloendothelial system is not influenced by *Ity* genotype. Various mechanisms have been proposed by which *Ity*^{r} macrophages kill *S. typhimurium* differentially faster than do *Ity*^{s} macrophages. It is postulated that *S. typhimurium* may fail to trigger an adequate respiratory burst in *Ity*^{s} macrophages, thereby diminishing the intracellular oxygen-dependent bactericidal events. However, there are several reports[16,23] demonstrating no difference in chemiluminescence response of *Ity*^{r} and *Ity*^{s} macrophages. Thus, it must be determined as to which factors may contribute to the full expression of *Ity in vivo.*

Our previous study[16] has shown that *Salmonella* lipopolysaccharide activates C3 in A/J serum to a greater extent than in C57BL/6 serum. Quantitative analysis in this study showed that higher levels of C3 deposition onto the surface of *S. typhimurium* as well as C3b generation occurred in *Ity*^{r} serum than in *Ity*^{s} serum. More interestingly, opsonization of *S. typhimurium* with A/J serum significantly suppressed intracellular bacterial growth in *Ity*^{s} macrophages. In contrast, *Ity*^{r} macrophages killed *S. typhimurium* opsonized with C57BL/6 serum only at the same levels as observed for *Ity*^{s} macrophages against *S. typhimurium* opsonized with either C57BL/6 serum or each *Ity*^{s} homologous serum. These findings strongly suggest that high levels of C3 activation at the initial interaction between *S. typhimurium* and mouse serum can modify the consequent intracellular growth of bacteria in macrophages of both *Ity*^{r} and *Ity*^{s} mice, and that even lower levels of C3b generation, which was observed for *Ity*^{s} serum after incubation with *S. typhimurium*, may be sufficient for both macrophages to ingest the bacteria.

It is known that the net growth of bacteria in the spleen of *Ity*^{r} mice is similar to that of *Ity*^{s} mice during the first 48 hr of infection.[1,2] The present findings obtained from the *in vitro* study suggest that the full expression of *Ity* may not be achieved *in vivo* unless serum C3 is activated by *Salmonella* lipopolysaccharide to a great extent via the alternative pathway. This postulate is supported by the following experimental facts: first, infection with *S. typhimurium* opsonized with *Ity*^{r} serum resulted in approximately a twofold increase in MST of infected *Ity*^{s} mice, and second, administration of anti-C3 antibody decreased the early resistance of A/J mice in association with decreased levels of serum C3 concentrations.

In this study, infection with a virulent *S. typhimurium* induced the same level of IFN-γ mRNA expression in the spleen of *Ity*^{s} mice as observed for the *Ity*^{r} mice. On the other hand, mRNA for IL-10 was expressed at much higher levels in *Ity*^{s} mice than in *Ity*^{r} mice. These findings are in agreement with the previous data reported by Pie *et al.*[14]; both IL-10 and IFN-γ mRNAs were expressed in the spleen of C57BL/6 mice to a greater extent than in that of CBA mice (*Ity*^{r}). Our previous study[16] demonstrated that recombinant murine IFN-γ enhanced the intracellular killing activity of macrophages from C57BL/6 mice to a greater extent against *S. typhimurium* opsonized with A/J serum than against that opsonized with C57BL/6 serum. Thus, an insufficiency of C3b generation in C57BL/6 serum, as a result from C3 activation by *Salmonella* lipopolysaccharide, may account for the failure of C57BL/6 macrophages to suppress the intracellular growth of bacteria to the same extent as did A/J cells, despite the high level of IFN-γ mRNA expression in C57BL/6 mice.

Neutralization of IL-10 with a monoclonal antibody does not increase resistance of *Ity*^{s} mice to *S. typhimurium*; so that, the high level of IL-10 mRNA expression in the spleen of C57BL/6 mice seems to be a consequence rather than the cause of their susceptibility to *S. typhimurium*.[14] However, IL-10 is capable of suppressing IFN-γ production by T cells and natural killer cells,[24,25] and also inhibiting production of reactive nitrogen oxides in IFN-γ-activated macrophages.[26] Suppression of IL-10 production therefore appears to enhance the anti-*Salmonella* activity of macrophages when they are stimulated with IFN-γ. In addition, infection with an avirulent strain of *S. typhimurium* is found to induce a clear increase in the number of IFN-γ-producing cells in *Ity*^{s} mice,[12] and also to decrease the expression of IL-10 mRNA in *Ity*^{s} mice.[14] The present findings indicate that opsonization of a virulent *S. typhimurium* with A/J serum reduced the expression of IL-10 mRNA in the spleen of C57BL/6 mice to a great extent. Thus, opsonization of *S. typhimurium* with *Ity*^{r} serum seems likely to reduce its virulence.[27]

Finally, previous studies from our laboratory[16] showed that either zymosan or *Listeria monocytogenes* activated *Ity*^{s} mouse complement to generate C3b to a greater extent than *Ity*^{r} mouse complement. This fact suggests that *Ity*^{r} mouse complement is strongly activated upon stimulation with *Salmonella* lipopolysaccharide but not upon stimulation with surface components of Gram-positive organisms. This difference is related to the findings that C57BL/6 mice are inherently resistant to *L. monocytogenes* but highly susceptible to *S. typhimurium*, and the reverse is true for A/J mice.[28] With these findings, high levels of C3b generation in mouse serum appear to be involved in the early resistance of mice to the facultatively intracellular pathogens.

In conclusion, the high level of C3 activation via the alternative pathway is crucial to the full expression of *Ity* in *Ity*^{r} mice during the early phase of infection with a virulent strain of *S. typhimurium*. Moreover, the present findings predict that the most likely explanation for the role of complement in *Ity*^{r} serum may be to reduce the virulence of *S. typhimurium* but not to enhance intracellular killing mechanisms in macrophages. Our study is in progress to confirm the present results using *Ity* congenic mice, and also to determine whether the *Ity* gene is closely linked to the C3 gene.

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