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Susceptibility of Lipopolysaccharide-Responsive and -Hyporesponsive Ity^s Mice to Infection with Rough Mutants of *Salmonella typhimurium*

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The R5 (chemotype Rb) but not the R10 (chemotype Rd) mutant of murine pathogen *Salmonella typhimurium* 395MS was extremely virulent in intraperitoneal infections of C57BL/10ScCr mice carrying the *ity^s* and *lps^d* alleles. C57BL/6J (*ity^s lps^n*) and C3H/HeJ (*ity^r lps^d*) mice showed a much higher resistance to the R5 mutant. Further studies were performed with peritoneal macrophages in vitro in order to elucidate susceptibility in lipopolysaccharide (LPS)-hyporesponsive mice carrying Ity^s. The intracellular killing capacity of the Ity^s Lps^d macrophages was lower than that of the Ity^s Lps^n macrophages for the R5 mutant and may partly explain the increased susceptibility of the Ity^s Lps^d mice. The deep rough mutant, R10, was rapidly killed intracellularly by the Ity^s Lps^d macrophages. Processing of the bacteria in macrophages that had phagocytosed R5 or R10 bacteria was followed for up to 18 days by endotoxin measurements (*Limulus* assay) and immunostaining, with monoclonal antibodies to various parts of the LPS molecule being used. Only 0.1% or less of the macrophage-associated bacteria remained alive after 48 h of incubation, and none were alive on day 7. Although immunostaining showed that LPS was present in both the Lps^d and Lps^n macrophages during the whole incubation period of 18 days, endotoxin activity in the Lps^d macrophages on day 7 was lower than that in the Lps^n macrophages, indicating that qualitative modifications of the chemical composition or physical state of the LPS molecule occurred. The interleukin-6 response in the Ity^s Lps^d macrophages was delayed and of shorter duration compared with that in the Ity^s Lps^n macrophages. The results suggest that the difference between the LPS-hyporesponsive and -responsive Ity^s mice in susceptibility to infection with the R5 mutant was due to the lower activation state of the Lps^d macrophages during infection, leading to a lower intracellular killing rate and a lower interleukin-6 response. Deep rough mutant R10 was highly sensitive to the intracellular bactericidal systems of the macrophages. A rapid killing of the bacterium should restrict the infection and may partly compensate for a diminished inflammatory response. The persistence of LPS within the cells is discussed.

Natural resistance of inbred mice to *Salmonella typhimurium* is partly genetically determined (18, 37, 40). At least five different gene loci have been identified. Three of these genes (*ity*, *lps*, and the susceptibility gene of C3Heb/FeJ) are expressed during the early phase of infection (10, 11, 23, 30, 31, 51), and the other two (*xid* and the late-phase susceptibility gene) are expressed in the later phase of the infection (18, 32, 43). In comparison with the *ity^r* genotype, the *ity^s* genotype is characterized by a lower intracellular killing rate for both macrophages and granulocytes (23, 49, 50). The *ity^s* genotype also allows a more rapid growth of *S. typhimurium* in vivo (4, 5). The *lps^d* gene results in the defective response of macrophages, monocytes, and lymphocytes to lipopolysaccharide (LPS), leading to a diminished induction of inflammatory mediators and cytokines (2, 6, 45, 52). Thus, mice expressing this gene tolerate high doses of endotoxin.

The bacterial LPS constitutes one of several virulence factors of *S. typhimurium*. Rough *Salmonella* mutants which are less virulent because of the synthesis of incomplete LPS molecules may still retain their ability to survive intracellularly (9, 14, 24, 33). Studies on the degradation of bacteria and their LPSs in macrophages have suggested that the LPS is selectively retained (8). LPS, which is a strong inducer of inflammatory mediators, may have a long-term modulating effect on macrophages and their effector functions because of its persistence in the cells and tissues (7, 13). No differences between LPS-responsive and -hyporesponsive macrophages with regard to their chemical degradation of purified LPS in vitro have been observed so far (28, 35).

As a part of our studies of chronic *Salmonella* infection in mice, we analyzed the virulence of three rough mutants of *S. typhimurium* 395MS (two of the Rb and one of the Rd chemotype) in mouse strains differing in their *Salmonella* susceptibility genes *ity* and *lps*. A dramatic difference in lethality between Ity^s Lps^n and Ity^s Lps^d mice was found. The Rb mutant was extremely virulent in intraperitoneal infections of Ity^s Lps^d mice. In this study, we report that the Lps^d macrophages of Ity^s mice were less efficient in their intracellular killing of the Rb mutant as studied in vitro. The delayed, lower, and more transient interleukin-6 (IL-6) response to infection with the *Salmonella* mutant by the Lps^d macrophages compared with that by the Lps^n macrophages also demonstrated the importance of LPS as a triggering factor for host defense in gram-negative infections. With regard to the processing of bacterial LPS in macrophages infected in vitro, we found that the antigenic LPSs of the *Salmonella* mutants persisted during the whole observation period of 18 days. However, somewhat different patterns with regard to the *Limulus* activity of the endotoxin in the cells were seen for the Lps^d and Lps^n macrophages after 1 week of incubation.

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MATERIALS AND METHODS

Bacterial strains. The R mutants derived from smooth parent *S. typhimurium* 395MS (MS), viz., 395MR5 (R5; chemotype Rb), 395MR6 (R6; chemotype Rb), and 395MR10 (R10; chemotype Rd), have been described earlier (16, 17, 21). All three mutants contain haptenic O antigens. In addition, R5 is a leaky mutant synthesizing O-specific LPS (O:1, 4, 5, and 12) (16). The R5 mutant was positive for immunostaining and immunofluorescence, with a murine monoclonal antibody being used against the O5 antigen, whereas R6 and R10 were negative. *Yersinia enterocolitica* CCUG 8233 and *Campylobacter jejuni* CCUG 8656 were isolates from patients. All bacterial strains except *C. jejuni* were cultivated in nutrient broth at 37°C overnight and then were washed twice in phosphate-buffered saline (PBS). *C. jejuni* was cultured on blood agar plates in a microaerophilic milieu at 42°C. The concentrations of the bacterial suspensions were measured by optical density at 650 nm. Bacterial concentrations were also checked by culturing the suspensions on nutrient agar plates overnight.

Mouse strains. Genotype *ity*^{s} *lps*^{n} (C57BL/6J) and genotype *ity*^{s} *lps*^{d} (C57BL/10ScN or C57BL/10ScCr) mice were obtained from Bomholtgård Breeding and Research Centre Ltd. or Harlan Sprague Dawley Inc. (Indianapolis, Ind.). Only one designation (C57BL/10ScCr) was used for the two Ity^s Lps^d strains. C3H/HeJ mice (original breeding stock was from Jackson Laboratories, Bar Harbor, Maine) and C3H/HeN mice (original breeding stock was from Charles River Laboratories, Margate, Kent, United Kingdom) were bred at the animal facilities at the Institute of Clinical Bacteriology, Immunology, and Virology, University of Göteborg. Male mice were used at 6 to 8 weeks of age.

Mouse lethality. Mice were injected intraperitoneally with 0.2 ml of bacterial suspension. The deaths of mice were recorded up to 1 month after the injection of bacteria. In several mice, the livers and spleens were analyzed for bacterial growth of *S. typhimurium.*

Intracellular killing by macrophages in vitro. The in vitro assay was performed according to the method of Lissner et al. (23). Resident peritoneal macrophages were used. In short, peritoneal macrophages were harvested and seeded into 24-well plates (Nunc, Roskilde, Denmark) or into 8-well chamber slides (Nunc). Approximately 4×10^5 cells were added to each well in 200 μl of Iscove's medium with 10% heat-inactivated fetal bovine serum (FBS) and 100 μg of gentamicin sulfate per ml. An additional 500 a μl of medium was added after a 90-min incubation at room temperature. After 6 h of incubation at 37°C, the medium was removed and 500 μl of Iscove's medium with 10% FBS was added. After an overnight incubation, adherent cells were washed with Dulbecco's PBS (pH 7.4) and 500 μl of the bacterial suspension was added to each well (for a ratio of 20 bacteria per one adherent cell). The bacteria had been opsonized by incubation for 30 min at 37°C with 5 or 10% fresh heat-inactivated homologous mouse serum. After 50 min of incubation at 37°C, the suspension containing bacteria was removed and the wells were washed three times. The cells were lysed with 0.5% sodium deoxycholate. The number of viable bacteria present in the cells was analyzed by plating the properly diluted lysate on nutrient agar plates. For an estimation of the number of adherent cells, a lidocaine-antibiotic solution (10 μg of lidocaine and 50 μg of gentamicin per ml) was added to the well. After 4 h of incubation, the released cells were counted in a hemacytometer. Infected cells incubated for longer periods of time were overlaid with Iscove's medium containing 5% FBS and 5 μg of gentamicin per ml. The viability of the cells was approximately 98 to 99% during the whole incubation period, except for the 18- to 23-day cell cultures, for which it decreased somewhat (90 to 98%). At 2 days of incubation and after, the cell count of the wells containing the C57BL/6 cells was ca. 40 to 50% lower than that of the wells containing C57BL/10ScCr cells.

The MIC and MBC of gentamicin were determined for the *S. typhimurium* 395 R mutants. The MIC and MBC were 1.25 μg/ml for all mutants.

Monoclonal antibodies and biotinylation. The murine monoclonal antibodies that were used have been described elsewhere (25, 26). One monoclonal antibody was specific for the O5 antigen (anti-O5) of the O-specific polysaccharide repeating unit of the *S. typhimurium* LPS molecule. Another antibody reacted with the LPSs of several different *Salmonella* species (anti-SalmLPS). No reaction, however, between this antibody and R10 LPS or an unrelated LPS, such as *Escherichia coli* O4 LPS, was observed. The exact immunodeterminant to which the antibody binds has not yet been established. The third antibody was specific for lipid A. This antibody did not react with LPSs from various smooth bacteria. In order to biotinylate the antibodies, supernatants from cell cultures were purified by ammonium sulfate precipitation. After dialysis against PBS, the solutions were purified with the Affi-Gel protein A MAPS II kit (Bio-Rad). Biotin *N*-hydroxysuccinimide ester (BHSE) was obtained from Bio-Yeda (Rehovot, Israel). The procedure described by Bayer and Wilchek was followed (3). Briefly, 20 μl of BHSE dissolved in dimethyl sulfoxide (0.55 mg/ml) was added to 1 mg of protein in 1 ml of 0.1 M $NaHCO_3$ (pH 7.4). The mixture was allowed to stand for 4 h at room temperature and thereafter was dialyzed extensively against PBS. Working dilutions of the antibodies were determined by immunostaining of R5 and R10 bacteria.

Immunostaining of *Salmonella* antigens in macrophages. The in vitro assay for intracellular killing was performed as described above, except that 8-well chamber slides were used for the incubation of the infected cells. Every third day, half of the old medium in the wells was replaced by fresh medium. After incubation for 1 h, 18 h, 48 h, 7 days, 14 days, and 18 days, the cells were washed in PBS and fixed for 1 h at room temperature with 2% paraformaldehyde in PBS according to the method of Lang et al. (20). The chamber frames were removed. The slides were washed and stored at 4°C in sterile PBS overnight or for not longer than 3 days. PBS containing 10% FBS was added to the slides, which were incubated for 20 min. The slides were treated with 0.2% Triton X-100 and washed in PBS. Incubation with 10% FBS in PBS was repeated (20). After being washed, the slides were incubated for 2 h at room temperature with the biotinylated monoclonal antibody solutions diluted in 1% bovine serum albumin–PBS. The slides were washed three times and incubated with the ABC-AP kit (Vector Laboratories). Vector Red was used for staining. Mounting was performed in Kaiser's glycerin-gelatin (Merck, Darmstadt, Germany).

TABLE 1. Lethality for different mouse strains infected intraperitoneally with *S. typhimurium* R mutants R5 (chemotype Rb), R6 (chemotype Rb), and R10 (chemotype Rd)[a]

Mouse strain	*Salmonella* susceptibility genes	Organism	No. surviving/total no. infected (% surviving)
C3H/HeN	ity^r lps^n	R5	5/5 (100%)
C3H/HeJ	ity^r lps^d	R5	7/7 (100%)
C57BL/6J	ity^s lps^n	R5	56/62 (90%)
C57BL/6J		R6	9/9 (100%)
C57BL/10ScCr	ity^s lps^d	R5	0/23 (0%)
C57BL/10ScCr		R6	0/5 (0%)
C57BL/10ScCr		R10	29/29 (100%)
C57BL/10ScCr		*C. jejuni*	5/5 (100%)
C57BL/10ScCr		*Y. enterocolitica*	5/5 (100%)

[a] Each mouse received 10^7 bacteria per 0.2 ml of bacterial suspension. Deaths were recorded over 4 weeks.

Chromogenic *Limulus* amoebocyte lysate assay. A chromogenic *Limulus* lysate assay kit (Coatest; Chromogenix, Mölndal, Sweden) was used for the quantification of bacterial endotoxin. *E. coli* O111 LPS, included in the kit, was used as the endotoxin standard (100 pg of O111 LPS corresponded to 1.2 endotoxin units). In short, 50 μl of sample or standard was mixed with 50 μl of lysate solution in a microplate well and incubated for 10 min in a dry heat incubator at 37°C. A total of 100 μl of the substrate solution (pentapeptid-paranitroanilin) was added to the well and incubated for another 3 min. The enzyme reaction was stopped by adding 100 μl of acetic acid (20%). The microplate was read in a spectrophotometer at 405 nm.

Pooled lysates ($n = 4$) of infected macrophages collected immediately after infection and at 1, 2, and 7 days after infection were analyzed in duplicate for their endotoxin contents.

IL-6 assay. A subclone (B9) of cell line B13.29 which is dependent on IL-6 for growth was used (1, 15, 19). The cells were harvested from the tissue culture flasks and seeded into microtiter plates (Nunc) at a concentration of 5,000 cells per well. Samples (diluted 1:2 and 1:5) or IL-6 standard was added to the cells and incubated for 68 h in Iscove's modified Dulbecco's medium supplemented with 5×10^{-5} M β-mercaptoethanol, 5% fetal calf serum (Sera-lab, Sussex, United Kingdom), and gentamicin (100 μg/ml). [^{3}H]thymidine was added 4 h prior to the harvesting of the cells.

RESULTS

Virulence of *S. typhimurium* R mutants. Mouse strains differing in their *Salmonella* susceptibility genes *ity* and *lps* were infected intraperitoneally with the *S. typhimurium* R mutants shown in Table 1. Ninety percent of *S. typhimurium*-susceptible Ity^s Lps^n mouse strain C57BL/6J (6J) survived infection with the R5 mutant. However, its endotoxin-hyporesponsive counterpart (Ity^s Lps^d), C57BL/10ScCr (10ScCr), was much more susceptible to both R5 and R6, and all the animals died. A dose-response experiment with R5 showed that even the lowest dose tested, 100 R5 bacteria killed the 10ScCr mice (data not shown). Infections with deep rough mutant R10 did not result in any deaths, although a persistent infection was established in several animals of the 10ScCr strain. Infections with two other human pathogens, *C. jejuni* and *Y. enterocolitica*, also resulted in 100% survival of the 10ScCr mice. No chronic infections were found after the sacrifice of these animals.

In vitro intracellular bactericidal activity of Ity^s macrophages differing in LPS responsiveness. The intracellular bac-

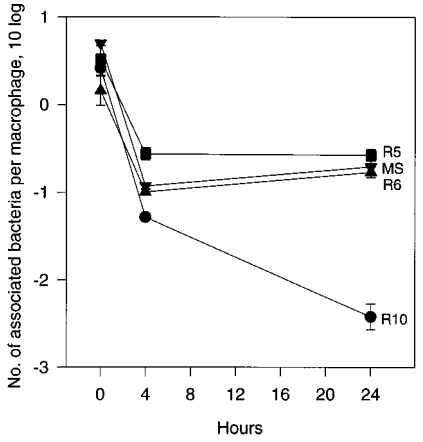

FIG. 1. Intracellular killing of R5, R6, R10, and MS by macrophages from C57BL/6J (Ity^s Lps^n) mice. Resident peritoneal macrophages were infected in vitro with the *S. typhimurium* mutants. Each point represents the mean number (log) ± standard error of the mean of cell-associated bacteria per macrophage. The bacterial counts were averaged from four culture dish wells per time point. The macrophage numbers were averaged from two infected culture dish wells per time point.

tericidal activity of macrophages has been considered important in the defense against *S. typhimurium*. We therefore analyzed the effects of the *lps* genotype on the intracellular killing in the Ity^s macrophages. First, the killing rates for the three R mutants and the parental strain in the Lps^n macrophage were compared. After 4 h of incubation, the intracellular killing rate for R10 was greater than those for R5, R6, and MS (Fig. 1). After another 20 h, almost all R10 mutants were dead, whereas R5, R6, and MS remained unchanged. The slightly higher intracellular survival of R5 compared with R6 was confirmed in repeat experiments (data not shown).

The intracellular killing rates for the most resistant mutant, R5, and the most sensitive one (R10) by macrophages of the Ity^s Lps^n and Ity^s Lps^d strains were compared. The intracellular bactericidal activities of macrophages from both mouse strains were almost identical for the R10 mutant (Fig. 2). A

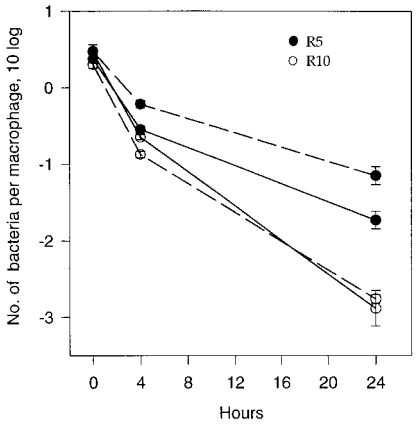

FIG. 2. Intracellular killing of R5 and R10 by macrophages from C57BL/6J (Ity^s Lps^n) (solid lines) and C57BL/10ScCr (Ity^s Lps^d) (broken lines) mice. Resident peritoneal macrophages were infected in vitro with the *S. typhimurium* mutants. Each point represents the mean number (log) ± standard error of the mean of cell-associated bacteria per macrophage. The bacterial counts were averaged from four culture dish wells per time point. The macrophage numbers were averaged from two infected culture dish wells per time point.

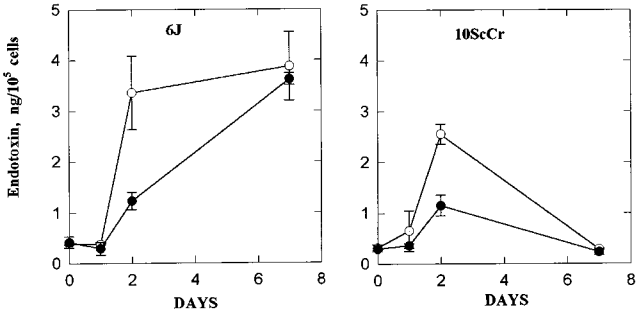

FIG. 3. Amount of endotoxin in lysate from resident peritoneal macrophages infected with *S. typhimurium* R5 (●) or R10 (○). The samples constituted a pool of cell lysate from cultures in quadruplicate. Quantification was made by the *Limulus* assay. The samples were run in duplicate on two different occasions. Standard errors of the means are indicated.

significant difference, however, was observed for R5. The intracellular killing rate was significantly lower in the Lps^d macrophages ($P < 0.005$ at 4 h and $P < 0.02$ at 24 h by Student's *t* test).

Intracellular killing, *Limulus* activity, and LPS immunoreactivity of *S. typhimurium* mutants phagocytosed by Ity^s macrophages in vitro. Macrophages from 6J or 10ScCr were infected with R5 or R10 and incubated over a longer period of time. The intracellular killing of the R5 and R10 mutants was assessed at 24 h, 48 h, 7 days, and 20 days. At 48 h, approximately 0.1% of the bacteria were still alive intracellularly compared with the number of bacteria present directly after the infection (when there were approximately 10^6 bacteria per 1.5 $\times$ 10^5 macrophages). At 7 days of incubation, no live bacteria were found, though sporadically one or another of the quadruplicate cell cultures contained a few bacteria.

The endotoxin contents of the infected macrophages as analyzed by the *Limulus* assay were almost identical at time zero for both the *Salmonella* mutants and the two mouse strains (Fig. 3). While similar endotoxin amounts were found at time zero and on day 1, a change occurred on day 2. The intracellular endotoxin levels in the macrophages of both mouse strains increased, although the highest increase was observed in macrophages infected with the R10 mutant ($P < 0.01$ [including both mouse strains] by Student's *t* test). On day 7, the endotoxin activity had increased further in the 6J macrophages. A different pattern was observed for the 10ScCr macrophages on day 7, with the return of the endotoxin levels to the same values as for days 1 and 2 (6J versus 10ScCr, $P < 0.002$).

In similar sets of cell cultures, the intracellular processing of the bacteria was monitored for 18 days by immunostaining with monoclonal anti-LPS antibodies (Table 2) (Fig. 4). A strong reaction with the anti-O5 antibody was observed in both strains of macrophages which had phagocytosed R5 until the seventh day of incubation. Thereafter, at days 14 and 18, a much weaker reaction involving fewer positive cells was seen. With the antibody directed against an epitope common to several *Salmonella* LPSs being used, the immunostaining of the R5-infected macrophages was negative at 1 h but became weakly positive at 18 h and strongly positive at 48 h, with a higher frequency of positive cells lasting to the end of the experiment (18 days). The anti-lipid A antibody which reacts with the R10 bacteria gave positive reactions at all time intervals in both strains of macrophages that had phagocytosed these bacteria. After 1 h of incubation, however, only a small fraction of the cells was positive. An intermediate reaction with this antibody

TABLE 2. Immunostaining of macrophages from C57BL/6J (Ity^s Lps^n) or C57BL/10ScCr (Ity^s Lps^d) mice infected with *S. typhimurium* R mutants R5 or R10 in vitro

Salmonella mutant	Mouse strain	Monoclonal antibody	Frequency of stained cells at[a]:					
			1 h	18 h	48 h	7 days	14 days	18 days
R5	6J	Anti-O5	++	+++	+++	+++	+	(+)
	10Sc	Anti-O5	+++	+++	+++	++	(+)	−
	6J	Anti-SalmLPS	−	(+)	+++	+++	++	+++
	10Sc	Anti-SalmLPS	−	(+)	++	+++	+++	+++
	6J	Anti-lipid A	−	−	−	−	−	ND
	10Sc	Anti-lipid A	−	−	−	(+)	−	++
R10	6J	Anti-O5	−	−	(+)	−	−	−
	10Sc	Anti-O5	−	−	−	−	−	−
	6J	Anti-SalmLPS	−	−	−	−	−	−
	10Sc	Anti-SalmLPS	−	−	−	−	−	−
	6J	Anti-lipid A	(+)	+++	+++	+++	++	+++
	10Sc	Anti-lipid A	+	+	++	++	+++	+++

[a] −, 0%; (+), less than 1%; +, 1 to 25%; ++, 26 to 75%; +++, more than 75%; ND, not determined.

was found in macrophages from the 10ScCr strain with intracellularly killed R5 after 18 days of incubation. No positive cells were observed when the anti-O5 or anti-*Salmonella* LPS antibody was used in the immunostaining of the R10-infected macrophages.

IL-6 response of infected macrophages in vitro. The IL-6 response during the intracellular killing of R5 and R10 bacteria by macrophages from 6J or 10ScCr was followed (Fig. 5). The IL-6 response in 6J macrophages was faster and higher and lasted longer than that in 10ScCr. The IL-6 responses in 6J macrophages for the R10 and R5 mutants were similar only within the first 24 h. The R5-induced IL-6 response peaked at day 7, while the increase was slower for R10, reaching a peak at day 18. In 10ScCr macrophages, the R10 mutant seemed to have induced a somewhat higher IL-6 secretion compared with that induced by R5.

DISCUSSION

The *ity* gene (also called *bcg*, *lsh*, or *Nramp*) has been suggested to be a major regulator of innate resistance to intracellular bacteria, including *S. typhimurium* (36, 38, 44, 51). The macrophage constitutes an important effector cell in resistance to *S. typhimurium*. Even though the *ity* gene product is not known, it was recently suggested to regulate gamma interferon production by NK cells via the macrophages (39). In this investigation, we studied the contribution of the *lps^d* gene to the susceptibility of Ity^s mice to some rough mutants of *S. typhimurium*, which are less virulent than their parental strain (9). Mice carrying only the *ity^s* or *lps^d* genotype were not susceptible to the Rb mutant (R5). Carrying the *lps^d* genotype in addition to *ity^s*, however, dramatically changed resistance to the R5 mutant (Table 1). All Ity^s Lps^d mice died, even at such a low dose as 100 bacteria, while only 10% in the Ity^s Lps^n group died, even at a 100,000-fold higher dose. This difference in susceptibility was probably partly due to less effective intracellular killing of the R5 mutant by the Ity^s Lps^d phagocytes, as was seen in the macrophage assay (Fig. 2). Thus, the *lps* genotype in an Ity^s background seems to affect the ability of the whole animal or macrophages from that animal to kill rough *Salmonella* bacteria. Although the two Ity^s mouse strains C57BL/6 and C57BL/10 used in our study have a close genetic relationship, it cannot be excluded that the observed differences may partly have been due to hidden variables which could not be assessed.

A low activation state of the LPS-hyporesponsive macrophages may explain the reduced intracellular killing capacity. It has been shown that *Mycobacterium bovis* BCG infection of Lps^d mice did not result in activated macrophages, as it did for Lps^n mice (34, 41). In another study, infection with an avirulent *S. typhimurium* strain resulted in activated macrophages for both Lps^n and Lps^d mice. In this case, however, a difference between the Lps^d and Lps^n macrophages in terms of the activation state was found by the different bacterial doses needed for the activation (42).

For the Rd mutant (R10), the intracellular killing in vitro by macrophages of both Ity^s Lps^d and Ity^s Lps^n mice was very rapid (Fig. 2). Thus, the phenotypic expression of the *ity^s* gene in the macrophage, i.e., low intracellular killing rate, was not seen with the R10 mutant. The mechanisms by which the deep rough mutant is rapidly killed intracellularly are less well known. The deep rough Rd mutant synthesizes only the inner part of the core region of the LPS. In addition, the cell wall composition of protein and phospholipids has changed in the Rd mutant compared with that of other chemotypes (Ra to Rc) (46). These changes of the cell wall in R10 may make it more sensitive to the bactericidal systems in the macrophage.

The IL-6 response in Lps^d macrophages in vitro was delayed and was lower and of shorter duration (Fig. 5). This finding was expected, since LPS has been shown to be a major inducer of IL-6. The ability of the host cells to rapidly mount an inflammatory mediator response (IL-1, IL-6, IL-8, and tumor necrosis factor) during invasion by bacteria is important for recruiting phagocytic cells as well as for increasing the bactericidal activity of phagocytes. Accumulation of phagocytes at the site of infection would prevent spreading of the bacteria. In LPS-hyporesponsive mice, defective cell recruitment obviously contributes to the increased susceptibility to infections by gram-negative bacteria (22, 47, 48, 53). Thus, it appears that LPS activation of macrophages leads to the secretion of cytokines and to a higher intracellular killing rate in Ity^s mice. These are important complementary events in restricting *Salmonella* infections.

The immunoreactive LPS persisted equally well in both the Lps^d and Lps^n macrophages infected in vitro (Table 2). The anti-SalmLPS and anti-lipid A antibodies showed strong reactivities for the R5 and R10 LPSs, respectively, throughout the study. It is well known that LPS can persist for a long time in host cells or tissues still expressing biological activity (12, 54). The polysaccharide part of the LPS molecule is very resistant to degradation. Studies on the degradation of *Salmonella abortusequi* LPS in the liver of rats showed, however,

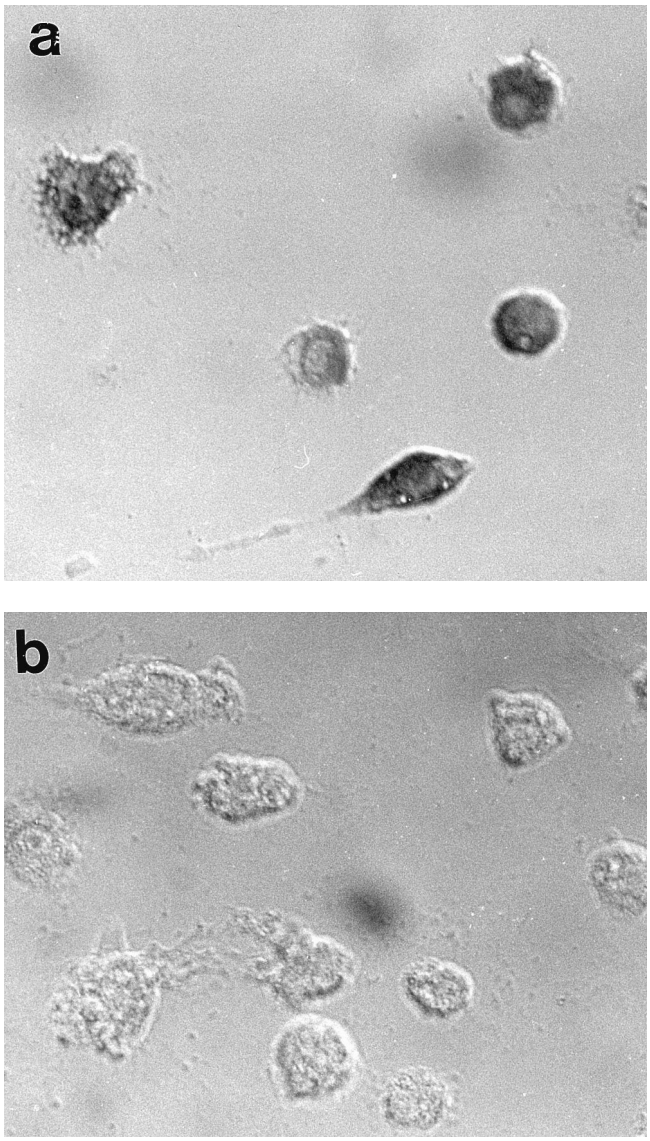

FIG. 4. Resident peritoneal macrophages from C57BL/10ScCr (Ity^s Lps^d) mice immunostained for the presence of *S. typhimurium* LPS or lipid A. The cells were infected with the leaky *S. typhimurium* R5 mutant 14 days prior to the immunostaining. Magnification, ×500. Immunostaining was performed with the monoclonal anti-SalmLPS antibody (a) and the monoclonal anti-lipid A antibody (b). Positively stained cells (appearing as dark grey to black cells) were observed only in panel a. The frequency of stained cells in panel a was more than 75% (Table 2).

that abequose (the immunodeterminant part of the O5 antigen) could be removed from the LPS molecules (12, 13). Our results also suggested that abequose was removed from the LPS of the leaky R5 mutant, since the reaction with the specific anti-O5 antibody after 2 weeks of incubation was very weak compared with that in the first week.

The lack of or weak immunostaining of R5 bacteria during the first 18 h of incubation with the anti-SalmLPS antibodies was due to the pretreatment of the bacterial cells with homologous mouse serum for opsonization (unpublished results). The binding of serum components to the bacterial cells resulted in the shielding of antigenic sites on the LPS molecule that react specifically with the anti-SalmLPS antibodies. The reactivity of the anti-lipid A antibodies was also affected by the serum treatment of the R10 bacteria, but to a lesser extent.

The LPS content of the macrophages during the first 24 h of incubation was constant as measured by the *Limulus* assay (Fig. 3). The increase in *Limulus* activity after 2 days of incubation could not be due to an increase in the number of bacteria, since only 0.1% of the bacteria were alive at this time. Since cell wall fragments containing LPS express a significantly higher *Limulus* activity than LPS present in the cell walls of intact bacteria, release of LPS because of the disintegration of bacterial cells results in an increase in the *Limulus* activity, as was seen with the infected macrophages (26). The higher *Limulus* activity found at 7 days of incubation in the Ity^s Lps^n macrophages compared with that in the Ity^s Lps^d macrophages suggests that the *lps* gene may influence the processing of endotoxin at a later stage. Modification of the biological activity of the endotoxin by further changes in its physical state or changes in the lipid A part of the LPS molecule could have taken place (28, 29, 35). It also cannot be excluded that more endotoxin was retained within the Ity^s Lps^n macrophages. This finding, which shows for the first time a difference between Lps^n and Lps^d macrophages in the processing of LPS, needs to be investigated further.

In vitro studies by Duncan et al. (8) on the degradation of heat-killed *E. coli* bacteria by macrophages from C3H/HeJ (Ity^s Lps^d) and C3HeB/FeJ (Ity^s Lps^n) mice suggested that the macrophage-processed LPS was selectively retained. The LPS expressed an enhanced immunostimulatory activity (Il-1 production and mitogenicity) compared with that expressed by the phenol-extracted LPS (7, 8). These enhanced biological activities of the processed LPS may benefit the host by enhancing the antiinfective response but may be detrimental by sustaining an inflammatory reaction after the infection has been effectively combated. In vitro studies of the degradation of purified LPS by human polymorphonuclear leukocytes or murine macrophages have shown that fatty acids, hydroxy fatty acids, and phosphates on the lipid A molecule are partly removed, thereby modifying the biological activity (28, 29, 35).

Although the survival rate of the R10-infected, LPS-hyporesponsive mice was 100%, a persistent infection of the spleen, mesenterial lymph nodes, or liver occurred in several mice. These experimental infections with R mutants are currently being explored as models for studying chronic infections localized to the mononuclear phagocytic system. In addition, we have also observed joint swelling and an increased frequency of alopecia, which may have similarities to reactive arthritis seen in some patients after *Salmonella* infections. The persistence of

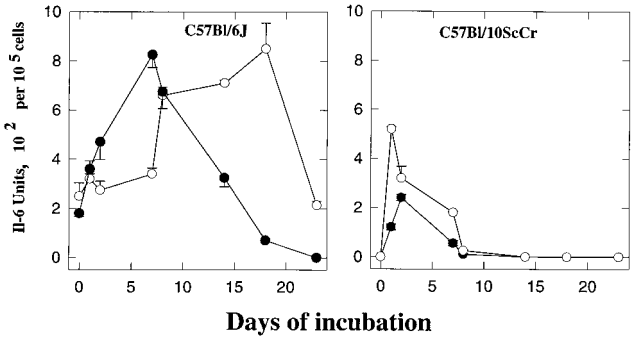

FIG. 5. The IL-6 response of macrophages from C57BL/6J (Ity^s Lps^n) or C57BL/10ScCr (Ity^s Lps^d) mice infected with the R5 (●) or R10 (○) mutant. Cell culture medium from four wells were pooled and analyzed. The samples were analyzed in triplicate (standard error bars are indicated).

antigenic material and a possible change in the processing of LPS by macrophages may have some bearing on this development (27).

In summary, we have shown the importance of the *lps* gene in conferring protection in the early defense against R mutants of *S. typhimurium* in the susceptible host by increasing the intracellular killing rate of macrophages and inducing a more rapid and stronger cytokine response. Also, the processing of bacteria is to some extent dependent on LPS responsiveness.

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