# Induction of γ/δ T Cells in Murine Salmonellosis by an Avirulent but Not by a Virulent Strain of *Salmonella choleraesuis*

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## Summary

To elucidate the relationship between the virulence of intracellular bacterium and its ability to induce γ/δ T cells in the host during infection, we examined the differences in appearance of γ/δ T cells in mice infected with *Salmonella choleraesuis* virulent strain RF-1 carrying a virulence plasmid of 50 kb, and with avirulent strain 31N-1 cured of the 50-kb plasmid. The number of γ/δ T cells in the peritoneal cavity was increased to a significant level on day 3 after an intraperitoneal infection with a sublethal dose ($5 \times 10^4$ colony-forming units) of avirulent strain 31N-1. On the other hand, no increase in the number of γ/δ T cells was evident in the peritoneal cavity at any stage after infections with various doses of virulent strain RF-1, although the numbers of the bacteria were drastically increased. Similar to that seen in the peritoneal cavity, the number of γ/δ T cells in the liver was significantly increased after an intraperitoneal infection with avirulent strain 31N-1 but not with virulent strain RF-1. The early appearing γ/δ T cells during salmonellosis with avirulent stain 31N-1, which preferentially used Vγ1/Vδ6, showed blastogenesis in response to purified protein derivative (PPD) derived from *Mycobacterium tuberculosis*. The γ/δ T cells also responded to the peritoneal adherent cells in mice infected with avirulent strain 31N-1 6 d previously, which expressed a high level of endogenous heat-shock protein (hsp) homologous to the mycobacterial 65-kD hsp. The expression of the hsp, however, was not prominent in the adherent cells in mice infected with virulent strain RF-1. These results suggest that the γ/δ T cells specific for PPD may play important roles in host defense against murine salmonellosis, and that the virulence of *Salmonella* may be inversely correlated with its ability to induce endogenous hsp in the infected macrophages, which in turn stimulate the γ/δ T cells in the host during salmonellosis.

The γ/δ T cells, which represent the first T cells in ontogeny and display more limited diversity than α/β T cells (1–3), are only present in much smaller numbers in the peripheral lymphoid tissues but are relatively abundant in the epithelia of epidermis, intestine, uterus, and tongue (4). There have been several lines of evidence indicating that CD1c and thymus leukemia antigens, both relatively nonpolymorphic class I–like molecules, are important components of the ligands for γ/δ T cells (5, 6). On the other hand, several studies have demonstrated that the γ/δ T cells are increased during infection with various pathogens, including *Mycobacterium leprae* (7), *M. tuberculosis* (8), *M. bovis* (9), *Leishmania donovani* (7), *Listeria monocytogenes* (10), *Plasmodium falciparum* malaria (11), HIV (12), and influenza A virus (13). A significant fraction of these γ/δ T cells are found to specialize in recognizing mycobacterial antigens, including the 65-kD heat-shock proteins (hsp)[1], which have been previously implicated as immunodominant antigens (6–8, 10, 14–18). The 65-kD hsp are widely distributed in nature and are highly conserved proteins between eukaryotes and prokaryotes (19). Under a variety of stress conditions such as heat shock, nutrient deprivation, and oxygen radicals, eukaryotic cells produce hsp, including the molecules homologous to the mycobacterial 65-kD hsp to preserve cellular functions (14). The γ/δ T cells recognizing the conserved epitopes on endogenous and bacterial hsp may represent a primitive arm of immunity devoted to the elimination of transformed, infected, or otherwise stressed autologous cells (17, 20, 21).

[1] *Abbreviations used in this paper:* BCG, Bacillus Calmette Guérin; FCM, flow cytometry; hsp, heat-shock protein; MNC, mononuclear cells; NCP, nitrocellulose paper; PEC, peritoneal exudate cells; PPD, purified protein derivatives; *sp*, species.

*Salmonella* species (*sp*) are facultative pathogens that are capable of surviving and persisting within mammalian host macrophages. The virulence of *Salmonella sp* is associated with its ability to survive within macrophages. Some virulence factors of *Salmonella sp* have been reported. The O antigen, a polymer of short repeat units of LPS on the surface of the bacteria, is a well-characterized virulence factor, which affects virulence mainly by modulating complement activation and consequently modulating bacterial phagocytosis by macrophages (22). The hsp of *Salmonella sp* are also thought to be a virulence factor because the hsp are linked with defense molecules against toxic molecules such as reactive oxygen metabolites in macrophages (23–25). Furthermore, high molecular weight plasmids have been found to be associated with virulence of several strains of *Salmonella* although the plasmid functions contributing to virulence have yet to be elucidated (26–31).

We have previously shown that the early appearing $\gamma/\delta$ T cells specific for the mycobacterial 65-kD hsp play an important role in protection against infection with *L. monocytogenes* (10, 32). In the present report, to elucidate the role of the 65-kD hsp–reactive $\gamma/\delta$ T cells in murine salmonellosis and to search for the relationship between the virulence of the bacteria and the appearance of $\gamma/\delta$ T cells in the host, we have examined the differences in induction of $\gamma/\delta$ T cells in mice infected with a virulent strain of *S. choleraesuis* carrying a virulence plasmid of 50 kb and in mice infected with an avirulent strain cured of the 50-kb plasmid (26). Our results reveal that the early appearing $\gamma/\delta$ T cells in murine salmonellosis are specialized to recognize PPD derived from *M. tuberculosis* and that the virulence of the organism is inversely correlated with its ability to induce the $\gamma/\delta$ T cells in the host during salmonellosis. The implications of these findings for the relation between the virulence of *Salmonella* and the responses of $\gamma/\delta$ T cells in murine salmonellosis are discussed.

## Materials and Methods

*Animals.* Female BALB/c mice, 7 wk of age, were purchased from the Japan SLC (Hamamatsu, Japan). Mice were housed under standard conditions and offered feed and water ad libitum.

*Microorganisms.* *Salmonella choleraesuis* strain RF-1, which has a virulence plasmid of 50 kb, was used as a virulent strain, and strain 31N-1, which is a derivative cured of the 50-kb plasmid, was used as an avirulent strain (26). The approximate intraperitoneal $LD_{50}$ of strain RF-1 for BALB/c mice was 100 CFU, and that of strain 31N-1 was $10^7$ CFU, maintained by several passages through mice. Organisms suspended in tryptic soy broth (Difco Laboratories, Detroit, MI) containing 15% glycerol (Nakarai Tesque Inc., Kyoto, Japan) were kept at −70°C. The bacteria were grown in tryptic soy broth at 37°C for 18 h, harvested, washed three times, and suspended in PBS. The concentration of bacteria was quantitated by plate counts.

*Bacterial Growth in the Peritoneal Cavity.* Mice were injected intraperitoneally with 100 CFU of strain RF-1 or $5 \times 10^4$ CFU of strain 31N-1 in 0.2 ml of PBS. At indicated times after inoculation, peritoneal exudates were obtained from the peritoneal cavity by lavage with 4 ml of HBSS and were serially diluted with HBSS. Serial dilutions of the exudate samples were plated in tryptic soy agar to determine the viable number.

*Bacterial Growth in the Liver.* Mice were injected intraperitoneally with the same dose as described above. For enumeration of viable counts in the liver, the liver was perfused with 20 ml of sterile HBSS to wash out bacteria in the blood vessels immediately after mice were bled. Bacterial counts from the liver were performed at various intervals according to the method described by Tripathy and Mackaness (33). The medium used for culture was tryptic soy agar, and the CFU per organ was determined after a 48-h culture.

*Cell Preparation.* Mice were killed 3, 6, or 10 d after the bacterial injection. Uninfected control mice were used in parallel. Peritoneal exudate cells (PEC) were prepared by centrifuging peritoneal exudates at 110 *g* for 10 min and suspended at a concentration of $10^6$/ml in RPMI 1640 containing 10% FCS, 100 U/ml penicillin, 100 μg/ml of streptomycin, and 10 mM Hepes. Cells were plated in wells of 24-well flat-bottomed tissue culture plates (Corning Glass Works, Corning, NY) and allowed to adhere for 2 h at 37°C in a humidified atmosphere of 95% air and 5% $CO_2$. Nonadherent cells were plated again, cultured, and nonadherent cells were used as mononuclear cells (MNC). Adherent cells in wells were washed several times with warm HBSS. Adherent cells were collected by scraping with a rubber policeman, washed, and counted. This procedure retained >95% of the adherent cells that were macrophages, as determined by esterase staining and sheep erythrocyte phagocytosis. Liver MNC were prepared as previously described (34) with a slight modification. Briefly, the liver was perfused with 20 ml of sterile HBSS to eliminate blood in the liver. The liver was pressed through 100-gauge stainless steel mesh, and suspended in RPMI 1640. After washing with the medium, the cell pellet was resuspended in 15 ml of the medium, and cell suspensions were centrifuged at 50 *g* for 1 min. The supernatant was harvested, spun down, resuspended in 8 ml of 44% Percoll (Sigma Chemical Co., St. Louis, MO), and layered on 5 ml of 67.5% Percoll in a 15-ml tube, as described by Cerf-Bensussan et al. (35). The gradient was spun down at 600 *g* at 20°C for 20 min. Lymphocytes at the interphase were harvested, washed twice, and suspended in HBSS. Thymus, spleen, and mesenteric lymph nodes (MLNs) were obtained from mice by the conventional method.

*Antibodies.* Anti-TCR $\alpha/\beta$ mAb (H57-597) were a kind gift of Dr. R. Kubo (National Jewish Center for Immunology and Respiratory Medicine, Denver, CO). Anti-CD3 mAbs (145-2C11) were the gift of Dr. J. A. Bluestone (University of Chicago, Chicago, IL). Biotin-conjugated anti-TCR $\gamma/\delta$ mAbs were purchased from Phar Mingen (San Diego, CA). PE-conjugated anti-Thy-1.2 mAb and FITC-conjugated goat anti–hamster IgG were purchased from Caltag Laboratories Inc. (South San Francisco, CA). PE-conjugated anti-L3T4 mAb and biotin-conjugated anti-Lyt-2 mAb were from Becton Dickinson & Co. (Mountain View, CA). FITC-conjugated streptavidin was from Dakopatts (Glostrup, Denmark). Red613-conjugated streptavidin was from Gibco BRL (Gaithersburg, MD). mAb IA10, which was raised against *Mycobacterium bovis* and specific for an epitope located between amino acids 172 and 224 of the bacterial 65-kD hsp, was provided by Dr. J. De Bruyn (Institut Pasteur du Brabant, Bruxelles, Belgium). Antibodies against TCR $\alpha/\beta$ and CD3 were obtained by growing hybridoma cells in serum-free medium (101; Nissui, Tokyo, Japan) and collecting supernatant.

*Flow Cytometric (FCM) Analysis.* For three-color analysis of T cell subsets, single-cell suspensions were stained with FITC anti-CD3 mAb, PE anti-CD4 mAb, and Red613 anti-CD8 mAb, and analyzed with a FACScan® (Becton Dickinson & Co.). Live cells were gated by forward and side scattering. Analysis gate was set on $CD3^+$ cells, and expression of CD4/CD8 molecules was displayed as a dot plot. For two-color analysis, cells were stained with PE anti-Thy-1.2 mAb and FITC anti-TCR $\alpha/\beta$ mAb or FITC anti-

TCR $\gamma/\delta$ mAb. For in vivo experiments, data were presented as two-dimensional contour maps. To obtain the subpopulation percentage, total counts were integrated in a selected area of control plots. For in vitro experiments, the analysis gate was set on Thy-1.2$^+$ cells and expression of TCR $\alpha/\beta$ and $\gamma/\delta$ were displayed as a single histogram.

*V Gene Segment Usage Analysis.* Total RNA was extracted with acid guanidinium thiocyanate-phenol-chloroform extraction method (36) from nonadherent cells of the peritoneal exudate cells (PEC), which were collected on day 6 after an intraperitoneal injection with *S. choleraesuis* strain 31N-1. cDNA synthesis and PCR were performed as described by Saiki et al. (37) using a cDNA cycle kit (Invitrogen Corp., San Diego, CA). RNA (5 $\mu$g) was primed either with 20 pmol of $\gamma$ chain C region (C$\gamma$) primers (5'-CTTATGGAGATTTGTTTCAGC-3') or 6.7 pmol of $\delta$ chain J region (J$\delta$) primers (5'-TTGGTTCCACAGTCACTTGG-3') in 21-$\mu$l reaction mixtures for reverse transcription. Synthesized cDNA was used at 2 $\mu$l. 5' and 3' primers were presented at 800 nM. The PCR was performed on a thermal reactor (Hybaid Ltd., Middlesex, UK). PCR cycles were run for 1 min at 94°C, followed by 1 min at 54°C, and 30 s at 72°C. Before the first cycle, a denaturation step for 7 min at 94°C was included, and after 28 cycles the extension was prolonged for 4 min at 72°C. The 3' primer for the $\gamma$ or $\delta$ PCR is above the C primer and J$\delta$ primer, respectively. The 5' V primers are as follows: V$\gamma$1/2 (ACACAGCTATACATTGG-TAC); V$\gamma$2 (CGGCAAAAAACAAATCAACAG); V$\gamma$4 (TGT-CCTTGCAACCCCTACCC); V$\gamma$5 (TGTGCACTGGTACCAA-CTGA); V$\gamma$6 (GGAATTCAAAAGAAAACATTGTCT); V$\gamma$7 (AAGCTAGAGGGGTCCTCTGC); V$\delta$1 (ATTCAGAAGGCAA-CAATGAAAG); V$\delta$2 (AGTTCCCTGCAGATCCAAGC); V$\delta$3 (TTCCTGGCTATTGCCTCTGAC); V$\delta$4 (CCGCTTCTCTGTG-AACTTCC); V$\delta$5 (CAGATCCTTCCAGTTCATCC); V$\delta$6 (TCA-AGTCCATCAGCCTTGTC); V$\delta$7 (CGCAGAGCTGCAGTG-TAACT); V$\delta$8 (AAGGAAGATGGACGATTCAC).

20 $\mu$l of PCR products was subjected to electrophoresis on a 1.8% agarose gel (Nakarai Tesque, Kyoto, Japan) and transferred to Gene Screen Plus (New England Nuclear, Boston, MA). The Southern blots of $\gamma$ and $\delta$ PCR products were hybridized with MNG6 cDNA containing C$\gamma$2 gene, J$\delta$1 probe (oligonucleotide; 5'-TTGGTTCCACAGTCACTTGG-3'). After hybridization, the filters were incubated in 1 M NaCl, 1% SDS, 10% dextran sulfate, and 100 $\mu$g/ml heat-denatured salmon sperm DNA for 18 h at 60°C (C$\gamma$ probe) (38) or 50°C (J$\delta$1 probe), and then the filters were washed in 2× SSC, 1% SDS for 10 min at 60°C, and exposed to x-ray films at −70°C.

*In Vitro Assay.* Nonadherent PEC (2 × $10^6$) on day 6 after infection were incubated with syngeneic normal adherent PEC (2 × $10^4$) in the presence or absence of purified protein derivatives (PPD) derived from *M. tuberculosis* (30 $\mu$g/ml) in 24-well plates for 48 h in a $CO_2$ incubator. For elucidation of the role of macrophages in induction of $\gamma/\delta$ T cells, normal nonadherent PEC (2 × $10^6$) were incubated with syngeneic adherent PEC (2 × $10^6$) on day 6 after infection for 48 h. After incubation, cells were washed with HBSS, and FACS® analysis was performed.

*SDS-PAGE.* SDS-PAGE was performed according to a modified procedure of Laemmli (39). All samples were heated at 100°C for 5 min and reduced by 2-ME. Gels were washed in 45% methanol-10% acetic acid for 10 min, stained with Fast stain (Zoion Research Inc., Allston, MA) for 20 min, and then destained in a solution of 40% methanol-4% acetic acid. PPD (25 $\mu$g) and recombinant 65-kD hsp (10 $\mu$g) derived from *M. bovis* (kindly provided by Dr. R. van der Zee, Laboratory of Bacteriology, National Institute of Public Health and Environment Protection, the Netherlands) were used for positive controls.

*Western Blot Assay.* Western blot technique of Burnette was used (40) with a slight modification. After electrophoresis, gels were immediately transferred to nitrocellulose paper (NCP) at a constant voltage (80 V) for 3 h in prechilled electrode buffer (192 mM glycine, 25 mM Tris base, 20% methanol, 0.03% SDS). After transfer, the NCP was rinsed in washing buffer (20 mM Tris, 150 mM NaCl, 2 mM EDTA-2Na), and then incubated for 1 h at 25°C with blocking buffer (washing buffer containing 0.05% Triton X-100, 0.02% SDS, 2% BSA). The NCP was incubated at 25°C for 2 h in a 1:100 dilution of IA10 in a 0.05% BSA-blocking buffer. After being washed in washing buffer, the NCP was incubated at 25°C for 1 h with optimal horseradish peroxidase–conjugated goat anti-mouse Ig (Serotec Ltd., Bicester Oxon, UK) dilutions as determined experimentally. After washing in washing buffer, the NCP was stained with an immunostain kit (Konica, Tokyo, Japan).

## Results

*Kinetics of Bacterial Growth and T Cells Bearing TCR $\alpha/\beta$ or TCR $\gamma/\delta$ in the Peritoneal Cavity and Liver during S. choleraesuis Infection.* The growth kinetics of *S. choleraesuis* in the peritoneal cavity and liver were monitored for 10 d after an intraperitoneal challenge with 50 CFU of strain RF-1 or 5 × $10^4$ CFU of strain 31N-1. The numbers of bacteria in both peritoneal cavity and liver increased steadily with time in mice infected with strain RF-1. Whereas, in mice infected with strain 31N-1, the numbers of viable *Salmonella* decreased lineally, although the challenge dose of strain 31N-1 was much higher than that of strain RF-1 (Fig. 1).

Next, we examined the kinetics of $\gamma/\delta$ T cells in the peritoneal cavity and liver during salmonellosis, FCM analysis for expression of Thy-1.2 and TCR $\gamma/\delta$ was carried out on the nonadherent PEC and MNC in the liver on days 3, 6, or 10 after infection. A typical result was shown in Fig. 2 and the mean of nine mice was summarized in Table 1. The proportion of $\gamma/\delta$ T cells in the peritoneal cavity increased significantly from <2% in normal mice to 6% on day 6 after an intraperitoneal injection with strain 31N-1 and then decreased on day 10 after infection. On the other hand, no increase in the frequency of $\gamma/\delta$ T cells in the nonadherent PEC was evident at any stage after infection with strain RF-1. The absolute number of the nonadherent PEC was significantly increased from 2.0 × $10^6$/mouse in normal mice to 4.5 × $10^6$/mouse or 3.5 × $10^6$/mouse on day 6 after infection with strain 31N-1 or RF-1, respectively. The number of MNC in the liver in normal mice was ~2.5 × $10^6$/mouse and remained at a constant level after infection with *S. choleraesuis*, irrespective of strain 31N-1 or RF-1. The proportion of $\gamma/\delta$ T cells in the liver increased significantly to the maximum on day 6 after infection with strain 31N-1 and remained at the higher level on day 10 after infection. On the other hand, no significant increase in the number of $\gamma/\delta$ T cells was detected on days 3 and 6 after infection with strain RF-1, although an appreciable level of $\gamma/\delta$ T cells appeared on day 10 after infection. To further confirm the lack of appearance of $\gamma/\delta$ T cells in mice infected with strain RF-1, we examined the expression of TCR $\gamma/\delta$ on the nonadherent PEC and liver lymphocytes in mice infected with a high dose (5 × $10^4$ CFU) of strain RF-1. Since all mice died within 5 d after infection with the dose of strain RF-1, the expression

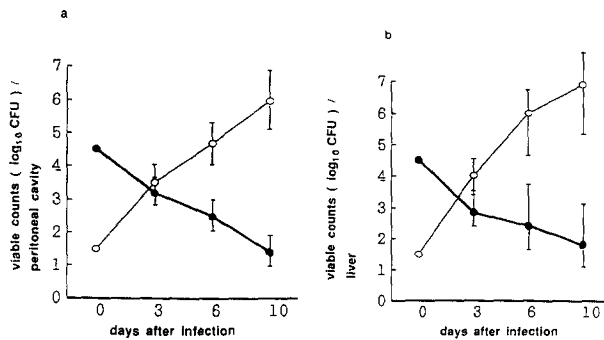


**Figure 1.** (*a*) Bacterial growth of *S. choleraesuis* in the peritoneal cavity after an intraperitoneal challenge with *S. choleraesuis*. Mice were injected intraperitoneally with 50 CFU of strain RF-1 (○) or 5 × $10^4$ CFU of strain 31N-1 (●). Data were obtained from three separate experiments and were expressed as the mean ± SD for nine mice at each time point. (*b*) Bacterial growth of *S. choleraesuis* in the liver after an intraperitoneal challenge with *S. choleraesuis*. Mice were injected intraperitoneally with 50 CFU of strain RF-1 (○) or 5 × $10^4$ CFU of strain 31N-1 (●). Data were obtained from three separate experiments and were expressed as the mean ± SD for nine mice at each time point.

of TCR $\gamma/\delta$ was examined only on the T cells on day 3 after infection. The $\gamma/\delta$ T cells were not significantly increased in these mice even after infection with a high dose of strain RF-1 (data not shown). These results suggest that the virulent strain RF-1 of *S. choleraesuis* may be incapable of inducing of $\gamma/\delta$ T cells in the infected host.

*V Gene Segment Usages by the Early Appearing $\gamma/\delta$ T Cells during Salmonellosis by Avirulent Strain 31N-1.* To determine the V$\gamma$/V$\delta$ gene usages by the early appearing $\gamma/\delta$ T cells during salmonellosis, nonadherent PEC were pooled from mice on day 6 after an intraperitoneal injection of strain 31N-1. RNA from nonadherent PEC was reversely transcribed into cDNA and amplified by PCR using primers for C$\gamma$ or J$\delta$ and various V$\gamma$ or V$\delta$ segments, respectively. The amplified DNA was detected by Southern blot analysis using C$\gamma$- and J$\delta$1-specific probes. As shown in Fig. 3, the $\gamma/\delta$ T cells in PEC from normal mice preferentially used V$\gamma$2/V$\delta$6, although the V repertoire was somewhat diversified, and V$\gamma$1/2/V$\delta$6 predominated notably in the T cells in PEC on day 6 after an intraperitoneal infection with *S. choleraesuis* strain 31N-1. These results suggest that V$\gamma$1/2/V$\delta$6 T cells may selectively expand after infection with *Salmonella*.

*Induction of $\gamma/\delta$ T Cells by PPD Derived from M. tuberculosis.* Happ et al. (41) have reported that PPD-reactive $\gamma/\delta$ T cells preferentially express V$\gamma$1/V$\delta$6 gene products. To determine the possible ligands for the early appearing $\gamma/\delta$ T cells in salmonellosis, nonadherent PEC on day 6 after infection with *S. choleraesuis* were cultured with PPD from *M. tuberculosis* in the presence of syngeneic adherent PEC (Fig. 4). After 48 h of culture, the proliferating cells were analyzed by two-color staining with anti-Thy-1.2 mAb and anti-TCR $\gamma/\delta$ mAb. When the nonadherent PEC on day 6 after intraperitoneal infection with strain 31N-1 were cultured with PPD, $\gamma/\delta$ T cells were significantly increased. TCR $\gamma/\delta$ was expressed on ∼ 34% of nonadherent PEC from mice infected with strain 31N-1 in the presence of PPD, whereas only 15% expressed TCR $\gamma/\delta$ on their surface in the absence of PPD. On the other hand, such an increase of $\gamma/\delta$ T cells was not evident when the nonadherent PEC from mice infected with strain RF-1 were cultured with PPD and syngeneic adherent PEC.

Born et al. (42) have shown that PPD-reactive $\gamma/\delta$ T cells expressing V$\gamma$1/V$\delta$6 recognize an epitope in the murine 65-kD hsp corresponding to the amino acids at positions 180–196 in the sequence of the mycobacterial 65-kD hsp. Therefore, it is possible that the early appearing $\gamma/\delta$ T cells expressing V$\gamma$1/V$\delta$6 during salmonellosis are specialized to recognize the endogenous 65-kD hsp derived from infected macrophages.

**Table 1.** *Kinetics of $\gamma/\delta$ T Cells in the Peritoneal Cavity and Liver after an Intraperitoneal Challenge with S. choleraesuis*

| Strain | Challenge dose | Peritoneal cavity | | | | Liver | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | Before | Day 3 | Day 6 | Day 10 | Before | Day 3 | Day 6 | Day 10 |
| | *CFU* | *%/lymphocytes* | | | | | | | |
| RF-1 (virulent) | 50 | 1.5 ± 0.3* | 1.6 ± 0.3 | 1.2 ± 0.4 | 2.8 ± 0.3 | 4.4 ± 0.4 | 2.9 ± 0.3 | 4.0 ± 0.5 | 7.8 ± 2.0 |
| | 5 × $10^4$ | 1.5 ± 0.3 | 2.5 ± 0.5 | ND‡ | ND‡ | 4.4 ± 0.4 | 7.0 ± 1.3 | ND‡ | ND‡ |
| 31N-1 (avirulent) | 50 | 1.5 ± 0.3 | 1.4 ± 0.3 | 1.5 ± 0.5 | 1.3 ± 0.5 | 4.4 ± 0.4 | 5.0 ± 0.7 | 4.5 ± 0.7 | 4.7 ± 0.3 |
| | 5 × $10^4$ | 1.5 ± 0.3 | 6.2 ± 0.5§ | 4.7 ± 0.9§ | 4.0 ± 0.9§ | 4.4 ± 0.4 | 7.4 ± 0.9 | 16.6 ± 2.4§ | 15.1 ± 3.1§ |

Mice were infected intraperitoneally with 50 or 5 × $10^4$ CFU of strain RF-1 or 31N-1. FCM analysis for expression of Thy-1.2 and TCR $\gamma/\delta$ was carried out on the nonadherent PEC and MNC in the liver on day 3, 6, or 10.

* Data were obtained from three different experiments and were expressed as mean ± SD of nine mice.

‡ All mice were dead by 5 d after intraperitoneal infection with strain RF-1 (5 × $10^4$ CFU).

§ $p < 0.01$. Statistical significance was determined by the student's *t* test.

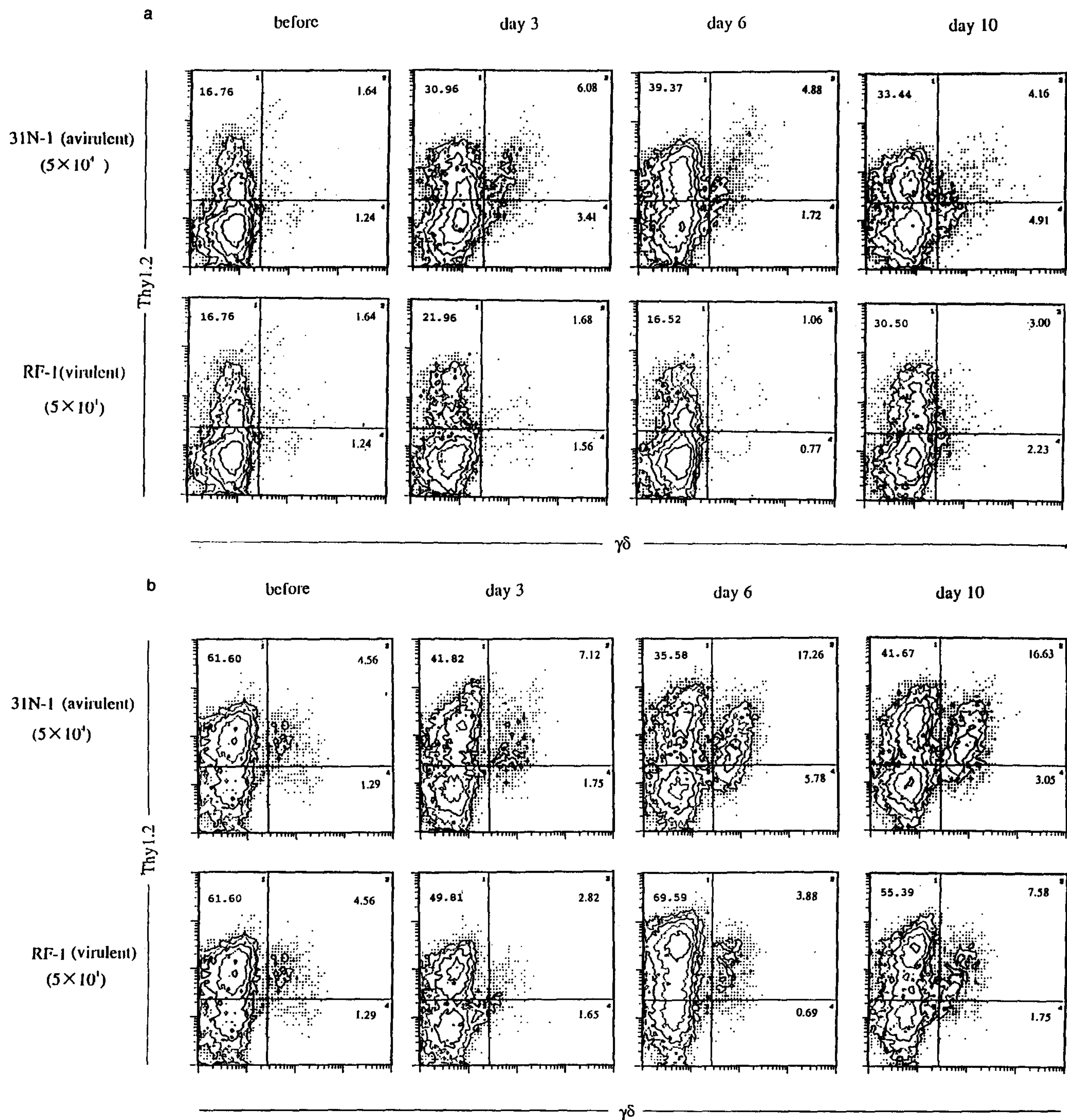


**Figure 2.** (*a*) Kinetics of $\gamma/\delta$ T cells in the peritoneal cavity after an intraperitoneal challenge with *S. choleraesuis*. Mice were injected intraperitoneally with 50 CFU of strain RF-1 or $5 \times 10^4$ CFU of strain 31N-1. Nonadherent peritoneal cells were stained with PE-conjugated anti-Thy-1.2 mAb and biotin-anti-TCR $\gamma/\delta$ mAb followed by FITC-streptavidin. (*b*) Kinetics of $\gamma/\delta$ T cells in the liver after an intraperitoneal challenge with *S. choleraesuis*. Mice were injected intraperitoneally with 50 CFU of strain RF-1 or $5 \times 10^4$ CFU of strain 31N-1. Cells were stained with PE-conjugated anti-Thy-1.2 mAb and biotin-anti-TCR $\gamma/\delta$ mAb followed by FITC-streptavidin.

To elucidate the roles of the infected macrophages in induction of $\gamma/\delta$ T cells, normal nonadherent PEC, which contain an appreciable number of $V\gamma1/V\delta6$ T cells, were cultured with syngeneic adherent PEC infected with strain 31N-1 in the absence of PPD. As shown in Fig. 5, the proportion of $\gamma/\delta$ T cells increased significantly in response to adherent PEC infected with strain 31N-1 even in the absence of PPD. These results suggest that macrophages infected with strain

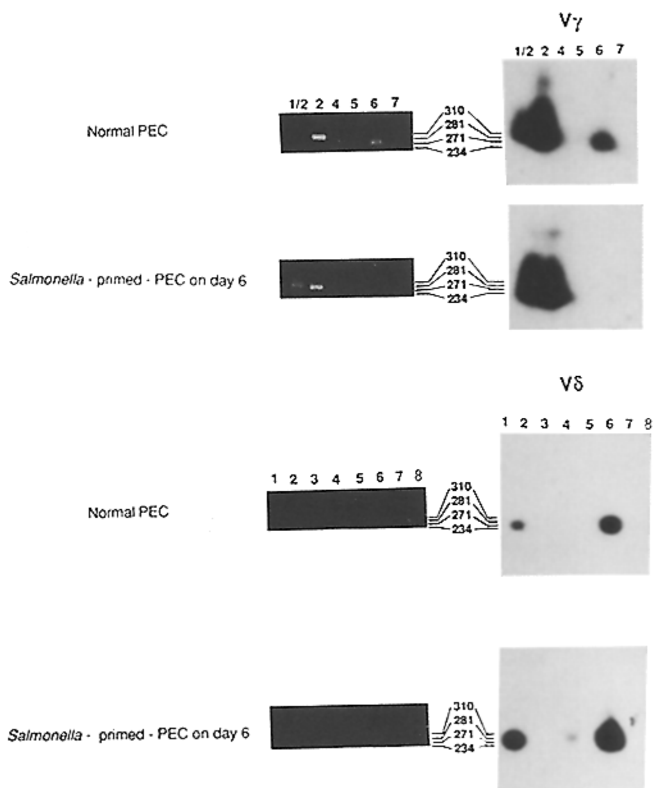


**Figure 3.** Vγ and Vδ gene usage in nonadherent PEC derived from unprimed mice or mice infected with *S. choleraesuis* strain 31N-1 6 d previously. Total RNA extracted from nonadherent PEC were reverse transcribed into cDNA and amplified by PCR using primers for Cγ or Jδ1 and various Vγ or Vδ segments, respectively. The Southern blot of γ-PCR products was hybridized with MNG6. The Southern blot of δ-PCR products was hybridized with oligonucleotide probes for Jδ1.

31N-1 stimulate a significant fraction of the γ/δ T cells in normal mice.

*Identification of Molecules Homologous to the Mycobacterial 65-kD hsp in PEC Infected with S. choleraesuis Strain 31N-1.* To determine the difference in expression of endogenous hsp homologous to the mycobacterial 65-kD hsp, lysates of macrophages infected with each strain of *S. choleraesuis* were run under reducing conditions in an SDS-PAGE system, electroblotted, and stained with mAb IA10, which is specific for an epitope located between amino acids 172 and 224 of the mycobacterial 65-kD hsp, and crossreact a 68–70-kD band in macrophages subject to stimulation with IFN-γ (43). An ∿70-kD band was detected in the lysates of macrophages infected with strain 31N-1, whereas only a very weak 70-kD band was detected in the lysate of macrophages infected with strain RF-1 (Fig. 6). The 70-kD band is thought to correspond to the endogenous hsp derived from autologous stressed cells, because no band was detected in both lysates of *S. choleraesuis* strain RF-1 and 31N-1 by IA10 mAb (data not shown). Therefore, it is suggested that macrophages infected with an avirulent strain of *S. choleraesuis* express a large amount of endogenous hsp, including a homologue to the mycobacterial 65-kD hsp, but those infected with a virulent strain may express only a few, if any, endogenous hsp. It is also suggested that the appearance of γ/δ T cells during salmonellosis may be closely linked with expression of endogenous hsp in the infected macrophages.

## Discussion

We have obtained the first evidence for an inverted correlation between virulence of *Salmonella* and its ability to induce

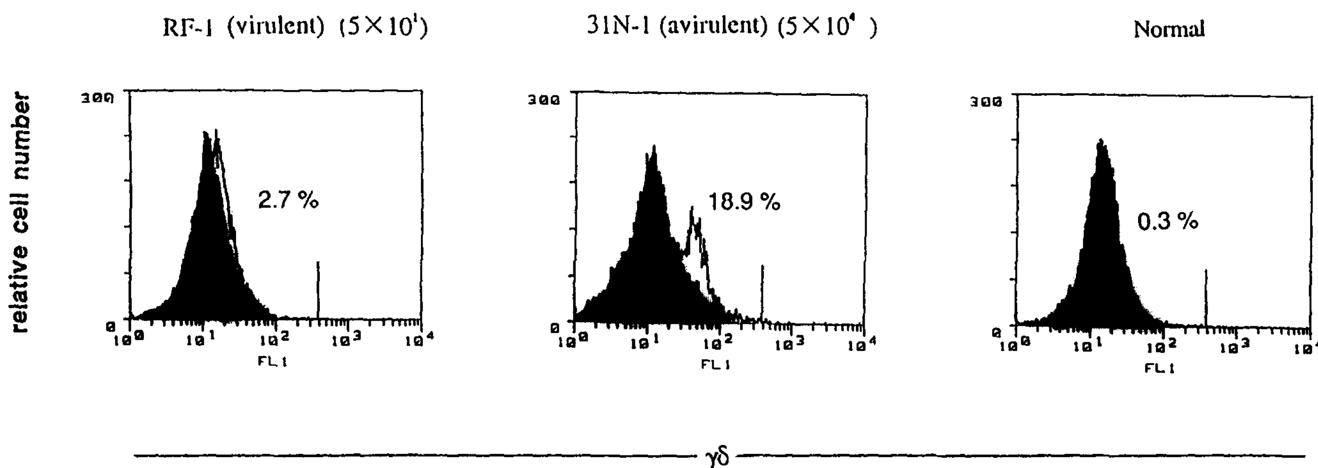


**Figure 4.** Expression of TCR γ/δ on T cells in mice infected with *S. choleraesuis* after a 48-h culture with PPD in the presence of normal adherent cells. Nonadherent PEC (2 × $10^6$ cells) from mice on day 6 after an intraperitoneal infection with *S. choleraesuis* were incubated with PPD at 30 μg/ml in the presence of syngeneic normal adherent cells (2 × $10^4$ cells) for 48 h. Cells were stained with PE-conjugated anti-Thy-1.2 mAb and biotin-anti-TCR γ/δ mAb followed by FITC-streptavidin. Analysis gate was set on Thy-1.2⁺ cells, and expression of TCR γ/δ heterodimer was displayed as a single histogram. Percentages were calculated by subtraction of the percentage of γ/δ T cells cultured without PPD from the percentage of γ/δ T cells cultured with PPD. Closed profile indicated Thy-1.2⁺ cells cultured without PPD. Open profile indicated Thy-1.2⁺ cells cultured with PPD.

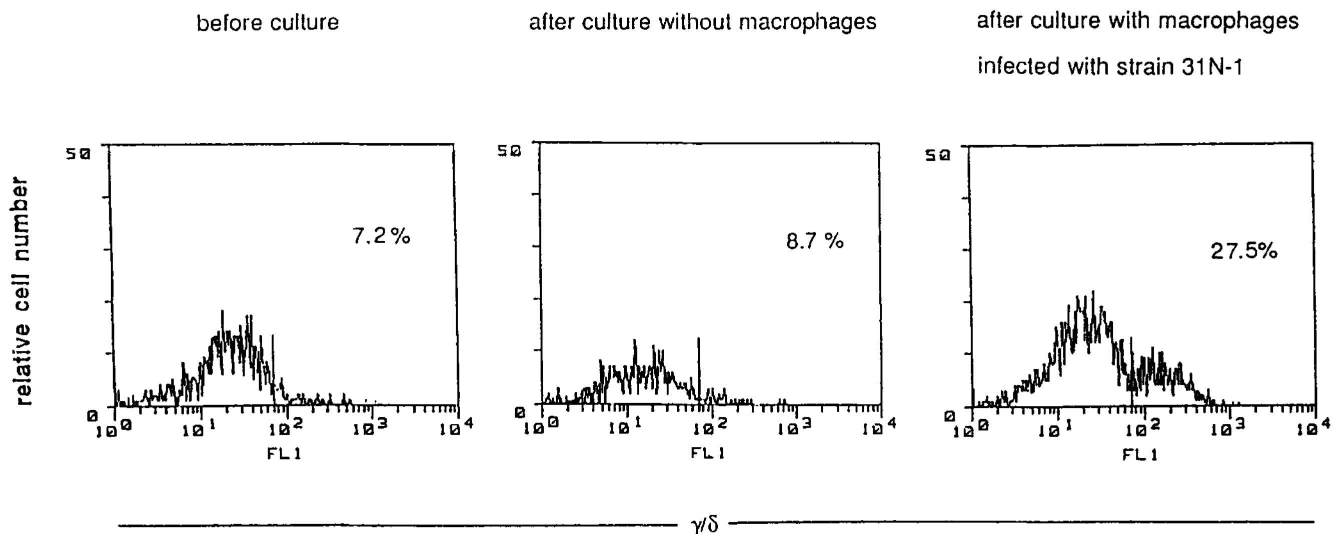


**Figure 5.** Expression of TCR $\gamma/\delta$ on T cells in normal mice after a 48-h culture with macrophages from mice infected with *S. choleraesuis* strain 31N-1. Nonadherent PEC ($2 \times 10^6$ cells) from normal mice were cultured with syngeneic *S. choleraesuis* strain 31N-1-infected macrophages ($2 \times 10^6$ cells) for 48 h. Cells were stained with PE-conjugated anti-Thy-1.2 mAb and biotin-anti-TCR $\gamma/\delta$ mAb followed by FITC-streptavidin. Analysis gate was set on Thy-1.2$^+$ cells, and expression of TCR $\gamma/\delta$ heterodimer was displayed as a single histogram.

$\gamma/\delta$ T cells within the tissues during salmonellosis. The $\gamma/\delta$ T cells were significantly increased in the inflamed sites after an intraperitoneal infection with an avirulent strain of *S. choleraesuis*, which is a derivative cured of the 50-kb virulence plasmid, while such an increase in a number of $\gamma/\delta$ T cells was not evident in any organ of mice infected with a virulent strain carrying the virulence plasmid. The $\gamma/\delta$ T cells appearing during salmonellosis with an avirulent strain, which preferentially used V$\gamma$1/V$\delta$6, are specialized to recognize mycobacterial antigens and are presumably homologous to the mycobacterial antigens on the infected macrophages. The adherent peritoneal cells from mice infected with an avirulent strain expressed a high level of endogenous hsp homologous to the mycobacterial 65-kD hsp, while those from mice infected with a virulent strain expressed a smaller amount of the hsp. It would, thus, appear that the appearance of $\gamma/\delta$ T cells during salmonellosis is associated with expression of the endogenous hsp in the adherent cells infected with *Salmonella*. We speculate that macrophages phagocytozing an avirulent strain of *S. choleraesuis* may easily kill the bacteria via their respiratory burst and consequently synthesize endogenous hsp to protect themselves from the toxic molecules such as reactive oxygen metabolites. On the other hand, a virulent strain might survive in the host macrophages for a long period without giving stress to them and induce indirectly only a few endogenous hsp in the macrophage, which in turn fail in stimulating the $\gamma/\delta$ T cells in the host after infection.

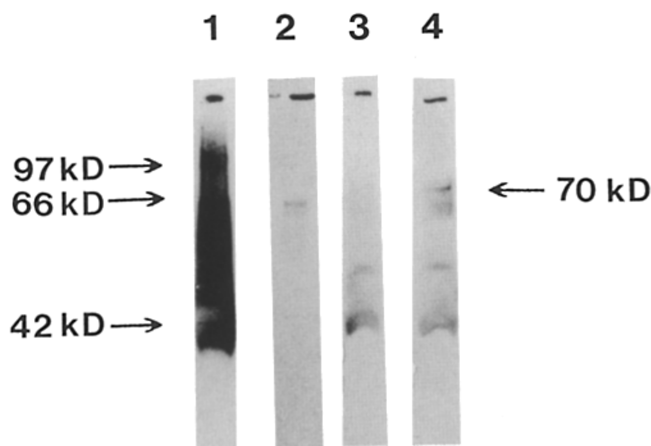


**Figure 6.** Identification of 65-kD hsp crossreactive proteins in PEC from mice injected with *S. choleraesuis* 6 d previously. Cell lysates were run under reducing conditions in an SDS-PAGE system, electroblotted, and stained with mAb IA 10. Lane *1*, PPD (*M. tuberculosis*); lane *2*, r-65-kD hsp (BCG); lane *3*, PEC from mice infected with strain RF-1; lane *4*, PEC from mice infected with strain 31N-1.

It has been reported that $\gamma/\delta$ T cells are increased in number before $\alpha/\beta$ T cells in the inflamed sites at the early phase of infection with *L. monocytogenes* (10), *M. tuberculosis* (8), and *M. bovis* (9). These observations raised the possibility that the early appearing $\gamma/\delta$ T cells during infection with facultative intracellular microorganisms may serve as a first defense against microbial pathogens. *Salmonella sp* are also facultative intracellular pathogens, and cellular immunity is regarded as one of the most important defense mechanisms, although humoral immunity is also involved in the protection against *Salmonella sp* (44–46). Our data presented here indicate that the $\gamma/\delta$ T cells appear in the peritoneal cavity and liver at an early stage after intraperitoneal infection with an avirulent strain of *S. choleraesuis*. Using anti-TCR $\alpha/\beta$ mAb– or anti-TCR $\gamma/\delta$ mAb–treated animals, we have re-

cently obtained direct evidence for a protective role of $\gamma/\delta$ T cells at an early stage after infection with *L. monocytogenes* in both mice (32) and rats (47). As in the case of *Listeria*, the early appearing $\gamma/\delta$ T cells during salmonellosis may also serve as a first defense against salmonella infection.

Several investigators have reported that a significant fraction of $\gamma/\delta$ T cells can respond to mycobacterial antigens (8–10, 14–18). Some of these $\gamma/\delta$ T cells can be stimulated with the mycobacterial 65-kD hsp (9, 14–18). Limited receptor repertoire in the mycobacterial antigen–reactive $\gamma/\delta$ T cells is described in both mice (41) and humans (48). Happ et al. (41) have shown that PPD-reactive $\gamma/\delta$ T cell hybridomas derived from murine newborn thymus preferentially used V$\gamma$1/V$\delta$6 gene segments. We have recently reported that the early appearing $\gamma/\delta$ T cells during murine listeriosis preferentially used V$\gamma$1/V$\delta$6 gene segments and showed a specificity for the mycobacterial 65-kD hsp (32). The PPD-reactive $\gamma/\delta$ T cells during Bacillus Calmette Guérin (BCG) infection were also shown to preferentially use V$\gamma$1/2/V$\delta$6 (9). Consistent with these findings, the early appearing $\gamma/\delta$ T cells during salmonellosis used V$\gamma$1/2/V$\delta$6 and proliferated in response to PPD. We also show here that the $\gamma/\delta$ T cells in PEC from normal mice preferentially used V$\gamma$1/2/V$\delta$6 and significantly proliferated in response to the adherent PEC infected with a virulent strain of *S. choleraesuis*. Koga et al. (43) have reported that a 70-kD homologue of the mycobacterial 65-kD hsp, present in the murine macrophages subject to stimulation with IFN-$\gamma$, is recognized by T cells raised against the mycobacterial 65-kD hsp in mice, suggesting that this molecule may serve as a target for protective immune responses. Our present study revealed that molecules recognized by antimycobacterial 65-kD hsp mAbs were detected in the lysate of macrophages infected with an avirulent strain of *S. choleraesuis* but not in those infected with a virulent strain. Havlir et al. (49) have reported that monocytes infected with live salmonella in vitro are effective inducers of $\gamma/\delta$ T cells. Cron et al. (50) have reported that ~70% of the $\gamma/\delta$ T cells in LN of normal mice are CD44 (Pgp-1$^+$), which has been proposed to be a marker for memory T cells. Taken together, it appears that normal $\gamma/\delta$ T cells, which may be previously activated by stimulation with phylogenically highly conserved antigens such as 65-kD hsp, may expand in response to the endogenous hsp in macrophages infected with an avirulent strain of *S. choleraesuis* more rapidly than naive $\alpha/\beta$ T cells, resulting in preceding $\alpha/\beta$ T cells in appearance during salmonellosis. A virulent strain of *S. choleraesuis* may fail to induce the $\gamma/\delta$ T cells in the infected host because of lack of ability to induce endogenous hsp in the infected macrophages. So far, it is not known why a virulent strain of *S. choleraesuis* can not induce the endogenous hsp in the macrophages. The ingestion of microorganisms by macrophages is accompanied by a respiratory burst, which activates reduced nicotinamide-adenine dinucleotide phosphate oxidase with increased consumption of oxygen, and generates superoxide anion and hydrogen peroxide (51). The oxygenic metaborates are known to be inducers of endogenous hsp in autologous stressed cells (52). The virulent strain may fail to trigger the cell respiratory burst, resulting in induction of only a few endogenous hsp in the infected macrophages.

Buchmeier and Heffron (53) have recently shown that a virulent strain of *S. typhimurium* inhibits macrophage phagosome-lysosome fusion. Phagosome-lysosome fusion is known to be an important step for processing of exogenous antigens (54). In addition, there has been evidence for the involvement of endogenous hsp in the processing and presentation of exogenous antigens to T cells (55). Therefore, it is alternatively possible that a virulent strain of *S. choleraesuis* may inhibit the process of antigen processing and presentation of the bacterial hsp to the $\gamma/\delta$ T cells via inhibition of phagosome-lysosome fusion and inhibition of expression of endogenous hsp in the APC. Our preliminary results revealed that $\gamma/\delta$ T cells in normal mice show blastogenesis in response to PPD in the presence of the peritoneal APC from mice infected with an avirulent strain but not in the presence of those from mice infected with a virulent strain, which may support this possibility. Detailed analysis of the relationship between the bacterial virulence and its ability to induce the 65-kD hsp–reactive $\gamma/\delta$ T cells in the infected host may not only help elucidate the role of the $\gamma/\delta$ T cells during salmonellosis but also could clarify the function of virulence factors of the bacteria within the host phagocytes.

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