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Salmonella typhimurium aroA, *htrA*, and *aroD htrA* Mutants Cause Progressive Infections in Athymic (*nu*/*nu*) BALB/c Mice

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Athymic (*nu*/*nu*) BALB/c mice and their euthymic (*nu*/+) littermates were inoculated intravenously with live attenuated vaccine strains of *Salmonella typhimurium*. All strains caused progressive infections in the athymic mice but not in their euthymic littermates. Athymic mice given strain SL3261, an *aroA* derivative of SL1344, in doses between log 4.7 and 5.7 CFU were all severely ill and were killed by weeks 4 to 5. Athymic mice given log 4.7 CFU of a derivative of *S. typhimurium* C5 carrying a mutation in *htrA*, encoding a stress protein, were ill and were killed by week 7 in one experiment but survived to week 13 in another. Athymic mice given log 4.6 CFU of a C5 *aroD htrA* double mutant were ill and were killed at week 7. Athymic mice given SL3261 had high bacterial counts in the reticuloendothelial system at 4 weeks. Athymic mice given SL3261 or C5 *htrA* made immunoglobulin G3 (IgG3) (and to a lesser extent IgM) antibody to lipopolysaccharide (LPS), whereas euthymic mice made IgM, IgG1, IgG2a, IgG2b, and IgG3 anti-LPS antibodies. The results indicate that both *aroA* and *htrA* strains will produce slow, progressively lethal infections in athymic mice, that the *htrA* strain is more attenuated than the *aroA* strain as measured by time to death in this model, and that IgG3 anti-LPS antibody alone cannot suppress the progress of infections by very attenuated strains in athymic mice.

The new generation of live salmonella vaccines is proving effective in mice, cattle, sheep, and chickens; human trials are showing them to be safe and immunogenic (9, 10). Strains with lesions in genes of the aromatic pathway are believed to be attenuated due to their requirement for *p*-aminobenzoic acid (PABA), which is lacking in mammalian tissues (21). We have reported that salmonellae harboring lesions in *htrA*, a stress protein gene (12), are attenuated and are good vaccines in mice (2). Aromatic-dependent mutants of *Salmonella typhi* are immunogenic in humans (25), and a new candidate typhoid vaccine is an *aroC aroD htrA S. typhi* mutant which is nonreactogenic and immunogenic in volunteers following a single oral dose (25a).

Attenuated salmonella vaccine strains are not invasive in animals with moderate immune suppression, especially in the short term (11, 21, 22, 26). However, Hess et al. (6) have recently reported that an *aroA S. typhimurium* is invasive in gene-targeted mice deficient in $CD4^+$ TCR$\alpha\beta$ cells and gamma interferon (IFN-γ) receptor. In the present study, we compared the invasiveness of *aroA*, *htrA*, and a double *aroD htrA* mutant of *S. typhimurium* in athymic *nu*/*nu* mice in which the infection was allowed to progress for several weeks. Intravenous injection of organisms in doses that were well tolerated by euthymic mice caused slowly progressive infections in athymic *nu*/*nu* mice which were eventually lethal. Athymic mice succumbed to both *aro* and *htrA* salmonellae, although the latter organisms allowed more prolonged survival. Athymic mice which survived 6 weeks after inoculation with *aroA* salmonellae made a T-cell-independent antibody response to the salmonella lipopolysaccharide (LPS) but were unable to control the infection, stressing the need for T cells for containing the spread of even these attenuated strains.

Female athymic *nu*/*nu* BALB/c mice (innately susceptible to salmonellae [Ity^s] [8]) and their age-matched euthymic *nu*/+ littermates were purchased from Harlan Olac Ltd., Blackthorn, United Kingdom, and used when 12 weeks old. *S. typhimurium* SL3261 is an *aroA* derivative of the mouse virulent SL1344 (6). C5046 is a derivative of the mouse virulent *S. typhimurium* C5 harboring a Tn*phoA* insertion in *htrA* (12) and is referred to hereafter as C5 *htrA*. CU38 is a derivative of *S. typhimurium* C5 with a Tn*10*-generated lesion in *aroD* (17) which received the same *htrA*::Tn*phoA* lesion as C5046 by P22 transduction; the strain is referred to as C5 *aroD htrA* (1). Organisms for inoculation were grown as overnight static cultures in Luria-Bertani (LB) broth (Oxoid, Basingstoke, United Kingdom) at 37°C, and aliquots were snap frozen and stored in liquid nitrogen. For intravenous inoculation, vials of organisms were rapidly thawed and diluted in phosphate-buffered saline. Mice were inoculated with 0.2 ml of bacterial suspension in a lateral tail vein, and the inoculating dose was checked by pour plates in LB agar (8). Animals appearing clearly ill were killed. Enumeration of bacteria in liver and spleen was performed as previously described (8). Results are expressed as geometric mean counts per whole organ. Mice were bled from the tail, and sera from groups of four to five mice were pooled and stored at −20°C. Antibodies to LPS were measured by enzyme-linked immunosorbent assay (ELISA) as previously described (5).

Survival of mice infected with attenuated salmonellae. Table 1 shows the survival of mice following intravenous inoculation with various doses of salmonellae. In four separate experiments, groups of 4 to 10 mice were given log 5.7, 5.0, 4.7, and 5.0 CFU of the *aroA* strain, SL3261. Euthymic mice showed no ill effects as late as 13 weeks postinfection (experiment 4). By contrast, athymic mice all became ill or died, the earliest at week 3 with the highest dose and the latest by week 5 to 6 at

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TABLE 1. Times to death of *nu*/*nu* and *nu*/+ BALB/c mice injected with attenuated salmonellae

Expt	Strain	Log_{10} dose	Genotype	No.	Outcome	Log viable counts	
						Liver (*n*)	Spleen (*n*)
1	SL3261	5.7	*nu*/*nu*	10	Dead by wk 3 to 5	nd[a]	nd
			nu/+	10	Healthy and killed by wk 5	nd	nd
2	SL3261	5.0	*nu*/*nu*	10	Sick and killed by wk 4	7.92 ± 0.39 (7)	7.52 ± 0.47 (7)
			nu/+	10	Healthy and killed by wk 4	2.16 ± 0.99 (10)	2.23 ± 0.89 (10)
3	SL3261	4.73	*nu*/*nu*	4	Sick and killed by wk 5	nd	nd
			nu/+	4	Healthy and killed by wk 7	nd	nd
	C5 *htrA*	4.70	*nu*/*nu*	4	Sick and killed by wk 7	nd	nd
			nu/+	4	Healthy and killed by wk 7	nd	nd
4	SL3261	5.0	*nu*/*nu*	5	Sick and killed wk 5 to 6	6.69 ± 0.99 (5)	7.14 ± 0.42 (5)
			nu/+	5	Healthy and killed by wk 13	nd	nd
	C5 *htrA*	4.7	*nu*/*nu*	4	Sick and killed by wk 13	2.25 ± 0.38 (3)	2.23 ± 0.23 (3)
			nu/+	4	Healthy and killed by wk 13	nd	nd
	None[b]	n/a[c]	*nu*/*nu*	4	Healthy and killed by wk 13	nd	nd

[a] nd, not determined.
[b] Uninoculated sentinel athymic mice.
[c] n/a, not applicable.

lower doses. No athymic mice survived infection with SL3261 at the doses employed.

The C5 *htrA* mutant also killed athymic but not euthymic mice, the latter showing no ill effects. However, when mice were given similar doses (log 4.7 CFU) in two separate experiments, athymic mice all became ill in experiment 3 whereas they appeared healthy and survived for 13 weeks in experiment 4, suggesting that the chosen dose was at or near the 50% lethal dose for this particular strain in athymic mice.

The C5 *aroD htrA* double mutant given at log 4.64 CFU killed athymic mice by week 7 (experiment 3).

Experiment 4 also included four uninoculated sentinel athymic mice, which were apparently healthy when killed at week 13.

Bacterial numbers in internal organs. Table 1 shows that in athymic mice, the *aroA* SL3261 strain reached high numbers in both livers and spleens (approximately 10^7) 4 to 6 weeks after inoculation (experiments 2 and 4). By contrast, counts in euthymic mice (which appeared healthy) were under 10^3 organisms in both liver and spleen.

In experiment 4, salmonellae recovered from the organs of mice immunized with SL3261 which showed high counts were tested for auxotrophy; no revertants were found.

Antibody responses to LPS. In experiment 2, anti-LPS antibodies were detected as early as week 2 in euthymic and athymic mice (results not shown); euthymic mice made immunoglobulin M (IgM), IgG2a, IgG2b, and IgG3 anti-LPS antibodies, whereas athymic mice, as expected, only produced IgG3 and low levels of IgM. At week 4, all athymic mice were showing signs of severe infection and were sacrificed immediately after bleeding; viable counts demonstrated that they were carrying high numbers of bacteria in livers and spleens (Table 1). Euthymic mice appeared healthy and had high levels of IgM, IgA, and all IgG subclasses specific to LPS; in contrast, no antibodies to LPS were found in sera from athymic mice (not shown).

In experiment 4, mice were immunized with the lower dose of log 5 CFU of SL3261; athymic mice survived to weeks 5 to 6 (Table 1), but many mice appeared severely ill and were killed after collection of sera. Figure 1a shows that at 6 weeks euthymic mice made a good antibody response to LPS of IgM and the IgG classes comprising IgG1, IgG2a, IgG2b, and IgG3. The athymic mice produced IgM and IgG anti-LPS antibodies, with the bulk of the IgG response being almost exclusively IgG3. The level of anti-LPS IgG3 in athymic mice was high but was lower than in euthymic animals. No IgG1 was detected, and the levels of IgG2a and IgG2b were only slightly higher than in nonimmunized control mice at the highest concentration of serum used.

Thus, athymic mice which died 4 weeks after inoculation with the *aroA* mutant showed little anti-LPS antibody, whereas mice which survived to weeks 5 to 6 made significant anti-LPS responses, especially IgG3.

In experiment 4, all mice appeared healthy and survived immunization with log 4.7 CFU of the *htrA* mutant. The antibody response in euthymic mice to the C5 *htrA* mutant at 13 weeks after inoculation was qualitatively similar to the response to the *aroA* mutant but was less intense (Fig. 1b). Euthymic mice showed little IgM, the response being mainly IgG; the IgG3 response was lower than that seen with SL3261. The antibody response in athymic mice was indistinguishable from that of euthymic mice in terms of IgM and IgG3; as expected, no other IgG subclasses were detected.

The present results show that live salmonella vaccines attenuated by lesions either in genes of the aromatic pathway or in *htrA* could cause slowly progressive but eventually lethal infections in athymic mice carrying the *nu*/*nu* defect on a BALB/c background, with high CFU counts in internal organs (*aroA* mutant). Time to death was longer in mice injected with strains harboring a lesion in *htrA* than in mice injected with an *aroA* mutant. Athymic mice which survived infection with *aroA* salmonellae to weeks 5 to 6 appeared severely ill despite showing high levels of IgG3 anti-LPS antibodies.

These results suggest that, in this experimental model, T cells are essential for the control of salmonella vaccine strains attenuated by lesions in either genes of the aromatic pathway or *htrA*, with IgG3 anti-LPS antibody alone being insufficient. It is known that T cells are essential in the control of infections by wild-type salmonellae in animals (3, 14, 15, 18–20); proliferative and cytotoxic T-cell responses have been described in peripheral blood leukocytes from humans immunized with aromatic-dependent mutants of *S. typhi* (24, 25). In a recent study of gene-targeted immunodeficient mice, Hess et al. (6) found that an *aroA S. typhimurium* was invasive and caused lethal infections in mice deficient in $CD4^+$ TCR-αβ T cells and IFN-γ receptor, whereas mice devoid of conventional $CD8^+$ T cells or TCR-γδ T cells were not unduly affected.

The susceptibility of T-cell-deficient mice to infection with

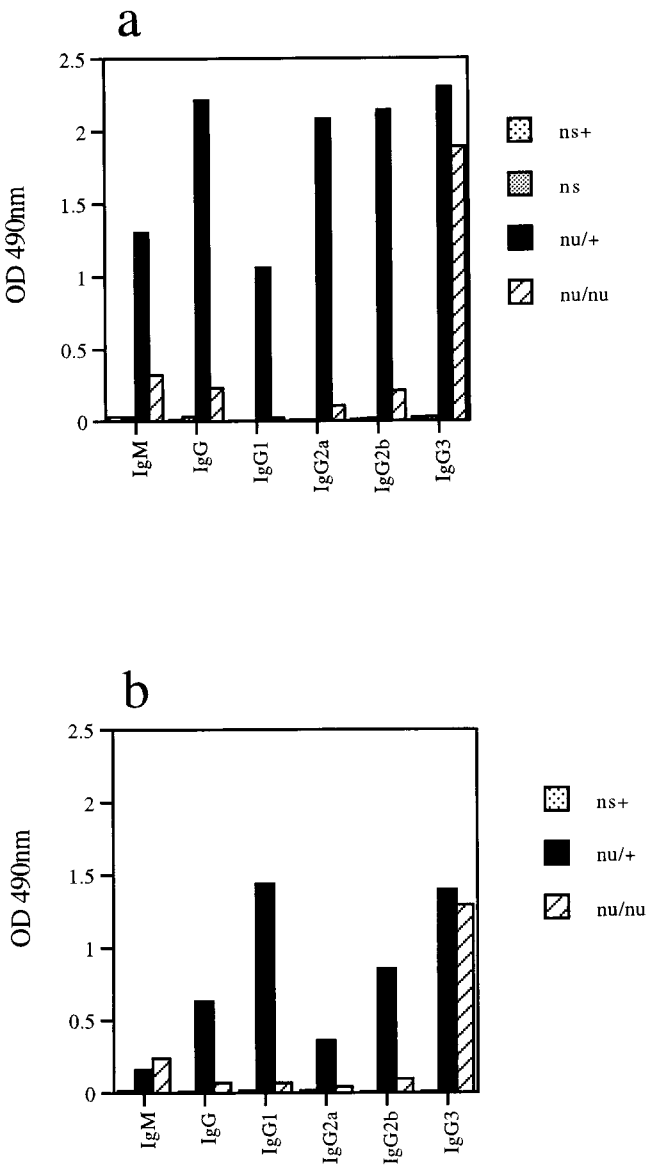

FIG. 1. (a) Antibody response to LPS in *nu*/*nu* and *nu*/+ mice immunized with 10^5 CFU of *S. typhimurium* SL3261. Measurements of IgM, IgG, IgG1, IgG2a, IgG2b, and IgG3 were made by ELISA in pools of serum samples diluted 1/100 (*nu*/*nu*, $n = 5$) (*nu*/+, $n = 5$) 6 weeks after inoculation and were obtained just before death. Pools of sera from five nonimmunized *nu*/+ mice (ns+) and three nonimmunized *nu*/*nu* mice (ns) are included as normal controls. (b) Antibody response to LPS in *nu*/*nu* and *nu*/+ mice immunized with 5×10^4 CFU of *S. typhimurium* C5 *htrA*. Measurements of IgM, IgG, IgG1, IgG2a, IgG2b, and IgG3 were made by ELISA in pools of serum samples diluted 1/100 (*nu*/*nu*, $n = 4$) (*nu*/+, $n = 4$) 13 weeks after inoculation. A pool of sera from five nonimmunized *nu*/+ mice (ns+) is included as a normal control.

aromatic-dependent or *htrA* mutants of salmonellae was unexpected. Whereas both antibody and T cells are essential for resistance to virulent salmonellae (15), we showed that *aroA* mutants were not invasive in mice carrying the *xid* sex-linked agammaglobulinemia (low serum IgM and IgG3) (11). Further, we showed that *aroA* salmonellae were not invasive in mice given a sublethal dose of radiation, which made them highly susceptible to wild-type salmonellae of moderate virulence (11). However, sublethal irradiation caused a transitory delay in clearance of the *aroA* salmonellae from the tissues, after which they were cleared, presumably indicating the recovery of the immune system (11). There are also reports of noninvasiveness of aromatic-dependent salmonellae in animals with other immune defects such as Lps^d or *scid* mice or in mice with defense mechanisms impaired by administration of microparticulate silica or cyclophosphamide (21) or by administration of anti-tumor necrosis factor alpha antiserum (26); the latter procedure exacerbates the early stages of infection with virulent salmonella (13, 14). Taken collectively, these results were consistent with the current view that aromatic-dependent salmonellae owe their attenuation to their requirement for PABA, which is unavailable in mammalian tissues (reviewed in reference 21). It is therefore surprising to find that aromatic-dependent salmonellae can cause lethal infections in athymic mice.

One difference with experiments reported earlier on the noninvasiveness of aromatic-dependent salmonellae is that the results presented in this report (and those of Hess et al. [6]) were obtained with innately susceptible mice with a persistent T-cell deficiency and monitored over many weeks. It is known that PABA in food will affect the level of parasitemia in mice infected with *Plasmodium* spp. (16). The pelleted diet fed to mice used in the experiments described here was reported by the manufacturer (Special Diets Services, Wiltham, United Kingdom) to contain 5 mg of PABA per kg as naturally occurring; no supplementary PABA was added. It remains to be seen whether a PABA-free diet would affect the course of an infection with aromatic-dependent salmonellae in this experimental model.

However, dietary factors cannot explain the invasiveness of the *htrA* mutants. We have reported that *S. typhimurium htrA* mutants are more susceptible to oxidative stress than the wild type, which could contribute to their reduced virulence (12). They are efficient vaccines (2) and are not invasive in mice with impaired resistance to infection due to the *xid* defect, sublethal irradiation, or administration of anti-tumor necrosis factor alpha antiserum (22). The present results indicate that, despite their marked attenuation in normal mice, *htrA* salmonellae can nevertheless cause slow, progressive infections in animals devoid of functional T cells. The longer time to death of athymic mice infected with *htrA* rather than *aroA* salmonellae suggests that the *htrA* lesion causes a greater attenuation than aromatic dependency in this experimental model; the time to death of mice infected with the mutant carrying both the *aroD* and *htrA* lesions was also longer than that for the *aroA* mutant.

We have shown that both antibody and T cells are necessary for protection against virulent salmonellae in normal mice (15); it is, however, surprising to find that lack of T cells should predispose to a lethal infection with very attenuated salmonellae such as *aro* or *htrA* mutants, especially in the face of the clear antibody response mounted by the athymic mice. This result underlines the need for T cells in the control and clearance of salmonella infections in mice.

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